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Decoding the gene expression response of *Cerastoderma edule* to chronic trematode infection: A comparison among host tissues

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ABSTRACT

Parasites can induce gene expression changes in their hosts, either benefiting the parasite or the host. In particular, trematodes are not only one of the most ubiquitous groups of aquatic parasites, they also have huge impacts on individual hosts with significant ecological and economic repercussions. The trematode *Bucephalus minimus* infects *Cerastoderma edule* (the edible cockle), a socioeconomically and ecologically important bivalve, as its first intermediate host. This parasite is one of the most harmful parasites infecting cockles, affecting their ability to reproduce, grow, and survive, thereby indirectly impacting ecosystem functioning. Despite the well-documented ecological effects of *B. minimus*, its impacts at a molecular level remain poorly understood. This study aimed to investigate the molecular mechanisms underlying *B. minimus* infection in cockles by analysing tissue-specific and systemic responses to long-term parasitic infection. It compared gene expression profiles in two critical tissues of naturally infected and non-infected cockles: the digestive gland, the primary target of infection, and the haemolymph, the backbone of the bivalve immune system. Results revealed extensive tissue-specific changes in gene expression. In the haemolymph, infected cockles showed significant downregulation of pathways related to cell division, cytoskeletal organization, and DNA repair, suggesting potentially parasite-induced reduction of immune responses and host cellular functions. Contrary to expectations, immune pathways did not show significantly increased expression, likely reflecting the chronic nature of infection and energy reallocation by the host. In the digestive gland, genes associated with gametogenesis, metabolism and immune function were downregulated, with no significant upregulation observed, except in some genes related to scavenger receptor activity and inflammation, suggesting localized immune responses. Shared responses among tissues included alterations in zinc ion transport and neurotransmitter biosynthesis, suggesting management of infection-induced stress. These findings highlight how *B. minimus* may manipulate host biology to suppress immunity and disrupt critical cellular processes, providing valuable insights into chronic trematode infections and host-parasite dynamics.

1. Introduction

Parasites are a highly diverse group of organisms with profound effects on their hosts and the ecosystems they inhabit (Poulin and Morand, 2000). However, the severity and nature of these impacts depend on the specific parasite-host interactions and environmental conditions (Wolinska and King, 2009). Parasite prevalence and abundance have undergone significant changes in recent years, largely driven by shifts in environmental factors such as climate change, pollution, habitat degradation, and the resulting changes in host-parasite dynamics (Harvell et al., 2002; Jones et al., 2008; Mastick et al., 2024; Poulin, 2006). These disturbances can elevate the susceptibility of ecosystems to parasite outbreaks, raising not only ecological concerns but also public health risks (Horwitz and Wilcox, 2005).

Trematodes are one of the most ubiquitous groups of aquatic parasites. These parasites are widely distributed in coastal systems

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(Lauckner, 1983; Mouritsen and Poulin, 2002). They have complex life cycles involving multiple hosts, alternating between asexual multiplication and sexual reproductive phases (Bartoli and Gibson, 2007). Due to this complexity, trematodes influence almost every trophic level of food webs, acting as regulators of host population dynamics and ecosystem processes (McLaughlin et al., 2020; Sures et al., 2017). Despite often being considered to have low pathogenicity, their impact on host health and population dynamics can be significant, depending on the species, parasite load, and, most importantly, the developmental stage of the parasite and its interaction with the host's vital organs, in many cases causing severe harm or even mortality to their hosts (Thieltges, 2006).

Studies have shown that trematode infections can trigger an intense immune reaction inducing gene expression changes in their host, potentially favouring either parasite establishment or host defence (Guler et al., 2015; Moreira et al., 2018; Schultz et al., 2020). In most cases, activation of the immune system is observed when a host encounters a trematode or other pathogens (Moreira et al., 2018; Schultz et al., 2020). Additionally, research has highlighted instances of acquired resistance, whereby organisms reinfected by previously encountered pathogens show a more effective and stronger immune response compared to that seen during the initial exposure, even in invertebrates (Lie et al., 1983; Montagnani et al., 2024; Novoa et al., 2019). However, most studies have focused on vertebrates or freshwater invertebrates, with considerably less attention given to marine invertebrates. Moreover, due to the significant diversity in immune systems across animal species (Buckley and Rast, 2015) and the focus on a limited number of model organisms, there remains a need to further investigate the molecular mechanisms underlying these host-parasite interactions.

The potential rise in trematode infection prevalence, driven by climate change scenarios that may increase pathogen outbreaks (Byers, 2021; Marcogliese, 2008), poses a growing threat to coastal ecosystems and their associated species. Molluscs, in particular, which serve as the preferred intermediate hosts for trematodes (Esch et al., 2001; Wright, 1959), are especially vulnerable to parasitic invasions. Bivalves are an important aquatic mollusc group for coastal communities, providing numerous ecosystem services. On one hand, bivalves play an important ecological role in the ecosystem functioning as links between primary producers and higher trophic levels, key contributors to nutrient cycling, and habitat facilitators for other species due to their bioturbation activity (Carss et al., 2020; Morgan et al., 2013). On the other hand, these organisms are of great socio-economic importance, representing approximately 15 % of global marine production for human consumption (FAO, 2024; Wijsman et al., 2019). The European edible cockle, *Cerastoderma edule*, a bivalve species widely distributed along the northeast Atlantic coast (Honkoop et al., 2008; Krakau, 2008), is among the most commercially targeted bivalves in Europe, mainly in Portugal and Spain (Oliveira et al., 2013). Cockles are host to several trematode species (de Montaudouin et al., 2021; de Montaudouin et al., 2009). These parasites have effects on individual fitness in terms of growth (Curtis et al., 2000), reproduction (SchulteOehlmann et al., 1997), and overall physiological performance (Magalhães et al., 2019). One of the most pathogenic trematodes infecting *C. edule* is *Bucephalus minimus* (family Bucephalidae) (de Montaudouin et al., 2021). This parasite uses cockles as its first intermediate host, where it develops as sporocysts via asexual multiplication and severely invades host tissues, starting with the digestive gland (Magalhães et al., 2015; Pina et al., 2009). In its complex life cycle, *B. minimus* also infects *Pomatoschistus microps* (the common Goby) as the second intermediate host and *Dicentrarchus labrax* (the European seabass) as the definitive host (Pina et al., 2009). During the early stages of infection, *B. minimus* disrupts the digestive gland of cockles, leading to reduced energy reserves, impaired immune responses, and altered reproductive capacity (Carballal et al., 2001; Dubois et al., 2009), leading to the total castration of infected individuals (Magalhães et al., 2015). However, the mechanisms underpinning infection by *B. minimus* sporocysts, and the immune response triggered in cockles (including the impacts at a molecular level in terms of gene expression) remain largely unexplored.

In contrast, other trematode species such as *Gymnophallus minutus* can reach very high prevalence in cockle populations (Correia et al., 2020) while exhibiting low pathogenicity (de Montaudouin et al., 2021). This species typically settles in the hinge and mantle margin and does not cause major physiological disruption (Fermer et al., 2011; de Montaudouin et al., 2009). Nevertheless, it can reach high abundances, with some cockles parasitised by hundreds to thousands of individuals, which may lead to more harmful effects (de Montaudouin et al., 2021; Goater, 1993).

To address this knowledge gap and gain a deeper understanding of the true effects of this infection, transcriptomic techniques (RNA-seq) were employed to examine gene expression differences between chronically infected and non-infected cockles. This study aims to elucidate the molecular mechanisms underlying the infection of cockles by *B. minimus* sporocysts. By examining tissue-specific responses in naturally infected and non-infected individuals, the primary objective of this study is to uncover how chronic trematode infection of cockles modulates the gene expression in two critical tissues: the digestive gland, which is the primary target of parasitic infection, and the haemolymph, the backbone of the bivalves' immune system. We hypothesised that infected cockles would exhibit a higher number of upregulated genes associated with immune responses, reflecting a sustained defensive reaction to the parasite. It is also expected that the haemolymph would show stronger expression of immune-related genes, due to its systemic immunological role, whereas the digestive gland would display altered expression in genes involved in tissue damage repair and stress, potentially suggesting necrosis.

2. Material and methods

2.1. Sample collection and processing

A hundred adult cockles (*Cerastoderma edule*) were hand-collected by digging in a 10 m^2 area in the Ramallosa Lagoon, Baiona, Spain (42° 06′ 59.5″N; 8° 49′ 19.6″W), in April 2022. Cockles were transported to the lab within 1 h under cool controlled temperature conditions for immediate processing and analysis.

Upon arrival, dissections were immediately initiated, and from each cockle, haemolymph and digestive gland tissues were collected for RNA extraction. First, haemolymph was extracted by carefully inserting a 23G syringe into the anterior adductor muscle of the cockle. The quality of the collected haemolymph was immediately verified using a light microscope to avoid contamination with other cell tissue or even with bacteria, then, samples passing the first quality control were centrifuged at 1000 rpm for 10 min at 4 °C and the resultant pellet was snap-frozen in liquid nitrogen and stored at −80 °C. Following haemolymph extraction, cockles were dissected to collect the digestive gland and assess infection by *B. minimus*. A piece of digestive gland was extracted and preserved in RNAlater and stored at −20 °C until RNA extraction. Cockle infection was assessed by gently compressing the remaining soft tissues between two glass slides and examining them under a stereomicroscope. Trematode infection (including *B. minimus* or other more prevalent species such as *G. minutus*) was morphologically identified following the identification key provided by de Montaudouin et al. (2009). Parasite abundance (number of individuals per cockle) and prevalence (percentage of infected cockles) were determined following Bush et al. (1997). For *B. minimus*, abundance could not be estimated due to the diffuse nature of trematode sporocysts. Thus, for this species, only prevalence was assessed, with individuals classified as either infected or non-infected. The intensity of other parasitic infections in cockles used for transcriptomic analysis was compared between *B. minimus*-infected and non-infected cockles using a two-sample *t*-test performed in R statistical software v4.4.1, after verifying the assumptions of normality and homogeneity of variances.

Finally, to determine the sex and gonadal stage of cockles, and the presence of other infections, tissues were preserved in Davidson's solution and subsequently processed for histological sectioning and stained with hematoxylin and eosin following the procedure described in Mahony et al. (2021).

2.2. Sample sequencing and editing

RNA was extracted from both tissues from 5 infected cockles (2 males, 2 females, and 1 indeterminate sex) and 5 non-infected cockles (3 males and 2 females) using the NZY Total RNA Isolation Kit from NZYTech (Lisbon, Portugal), according to the manufacturer's instructions. RNA concentration and purity were measured using a DeNovix DA-11 spectrophotometer and Qubit RNA-HS (high sensitivity) Assay (Invitrogen). RNA integrity was assessed using a 2 % agarose gel stained with GelGreen nucleic acid stain (NZYTech, Lisbon, Portugal) by evaluating the presence of distinct 28S and 18S rRNA bands and the absence of smearing.

Samples were sent to Novogene Company Limited (UK) for sequencing using an Illumina mRNA-seq platform (i.e., Illumina NovaSeq 6000). The sequencing platform generated 150-base pair paired-end reads, with an insert size of 350 base pairs, yielding between 20 and 35 million reads per sample (with an average of 30 million), corresponding to approximately 6 Gb of raw data per sample. After quality filtering and trimming, an average of 13 million reads per sample was retained for downstream analysis.

The quality of the raw RNA-seq reads was assessed using FastQC (Andrews, 2010). Trimmomatic v0.39 was then used to remove adapters, trim low-quality bases, and filter out reads using the default settings: an average Phred score cutoff of 33, a 4 bp sliding window with a quality threshold of 15, and a minimum post-trimming read length of 30 base pairs (Bolger et al., 2014). Finally, trimmed sequences were mapped against the available *C. edule* genome (GenBank Accession Number: GCA 947846245.1) using Kallisto v0.46.1, a pseudo-alignment-based tool (Bray et al., 2016), with default parameters and 100 bootstrap samples to generate transcript abundance estimates and transcripts per million values for each transcript.

2.3. Gene differential expression analysis and Ontology term enrichment analysis

Gene differential expression and Gene Ontology (GO) enrichment analyses were performed using R Statistical software v4.4.1. Normalised transcripts per million values were imported using '*tximport*' package (Soneson et al., 2016) for downstream analysis.

Differential expression analysis was conducted separately for haemolymph and digestive gland tissues using the '*DESeq2*' package (Love et al., 2014) to identify genes that were significantly differentially expressed between infected and non-infected cockles. The Wald test was applied to evaluate the differential expression for each gene, and the corresponding *p*-values were adjusted using the Benjamini-Hochberg method to control the false discovery rate (FDR). Genes were considered significantly differentially expressed if a threshold of adjusted *p*-value of 0.05 was met, along with a minimum fold change of 2. Data visualization, including the Principal Component Analysis (PCA) and volcano plots, was performed using the '*ggplot2*' package (Wickham et al., 2016). PCA was used to explore global gene expression patterns and assess variation among samples, while volcano plots helped to visualise the magnitude of differential expression and the significance of each gene.

The significantly upregulated and downregulated genes identified from each of the two analysed tissues (haemolymph and digestive gland) were individually subjected to GO enrichment analysis using the '*clusterProfiler*' package (Yu et al., 2012). Annotation data was provided using a custom database (org.Cedule.eg.db) created with the '*AnnotationForge*' package (Carlson and Pagès, 2024). A Bonferroni-corrected *p*-value threshold of 0.05 was applied for identifying enriched pathways. The top 20 most significantly enriched pathways (i.e., those with the lowest *p*-values) and their respective clusters (grouped using Ward.D clustering method) were visualised in a treeplot. However, since no significantly enriched pathways were detected at this threshold in the haemolymph upregulated genes, the same analysis was repeated using a relaxed *p*-value cutoff of 0.20. This relaxed threshold was adopted for exploratory purposes to identify potentially relevant trends in gene expression that may have been missed under stricter significance criteria, especially considering the low sample size and the biological variability inherent to field-collected samples. The enriched functional groups were visualized using a hierarchical clustering tree diagram generated by the '*enrichplot*' package (Yu and Gao, 2024).

2.4. Comparison of tissue-specific genes

To identify genes shared between haemolymph and the digestive gland, a comparison of differentially expressed genes (genes with adjusted *p*-value $\leq$ 0.05 and fold change $\geq$ 2) was conducted using a Venn diagram generated with the '*ggvenn*' library (Yan, 2023). Functional enrichment analyses were then performed as previously described on the shared genes, focusing on four specific categories: genes activated in both tissues, genes upregulated in haemolymph but downregulated in the digestive gland, genes downregulated in haemolymph but upregulated in the digestive gland, and genes repressed in both tissues.

3. Results

3.1. Prevalence and infection levels of parasites in cockles

Out of the 100 collected cockles, 18 % were infected with *Bucephalus minimus*. In addition to *B. minimus*, other parasite species were also identified, namely the trematode species *Gymnophallus minutus* with a prevalence of 100 %, 32 cockles infected with *Himasthla* spp., and two cockles infected with *Monorchis parvus*, another trematode parasite that uses cockles as its first host. Furthermore, one cockle was found to be infected by the copepod *Herrmannella rostrata*.

For the present transcriptomic study, in addition to the 5 non-infected control cockles, only cockles infected by *B. minimus* and *G. minutus* (100 % prevalence) were used. It must be noted that *G. minutus* uses cockles as its second intermediate host, where its metacercariae stage is much less invasive and damaging than the sporocyst stage of *B. minimus*. No significant difference in *G. minutus* infection levels was observed between cockles infected by *B. minimus* (mean *G. minutus* intensity: 31.4 ± 16.9 metacercariae/cockle) and uninfected cockles (mean *G. minutus* intensity: 26.8 ± 11.0 metacercariae/cockle) ($t = -0.5089$, df = 8, $p = 0.625$).

3.2. Gene expression profiles in haemolymph

The differential gene expression analysis of haemolymph between *B. minimus* infected and non-infected cockles revealed 312 upregulated and 896 downregulated genes (see Supplementary Material). These differentially expressed genes were visualized in a volcano plot (Fig. 1A). The differences between infected and non-infected cockles were also visualized in a heatmap (Fig. 1B). Principal component analysis (PCA) showed a marked distinction in patterns of differential gene expression between infected and non-infected individuals, with 53 % of the variance explained by dimension 1. However, one of the non-infected cockles clustered with samples from the infected group (Fig. 1C).

Non-infected cockles showed a significant enrichment of Gene Ontology (GO) terms associated with meiotic cell cycle and cytoskeleton organization. These included Microtubule cytoskeleton organization (GO:0000226), Chromosome segregation (GO:0007059), and Meiotic

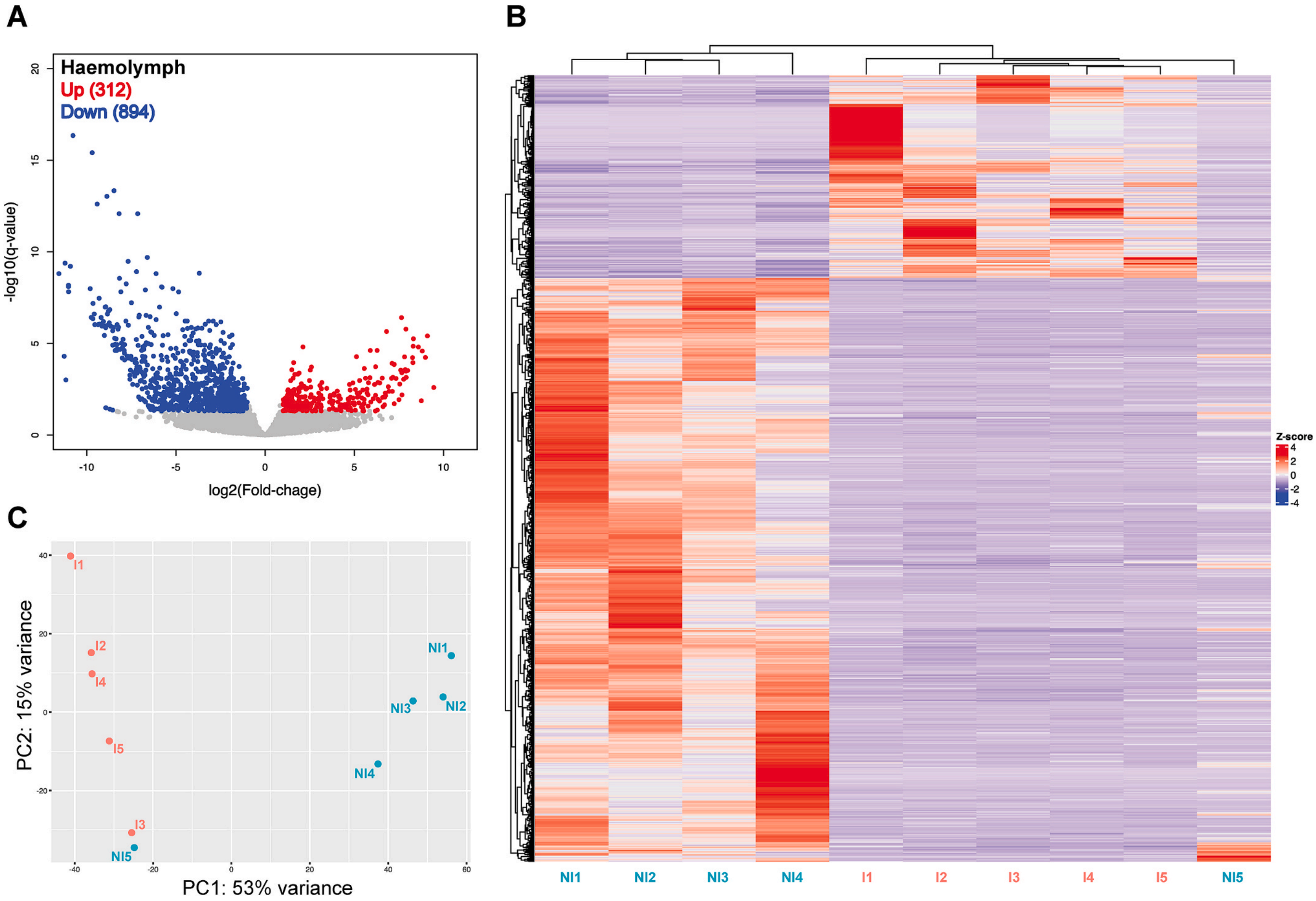

Fig. 1. Differential gene expression analysis in the haemolymph of infected (I) and non-infected (NI) *Cerastoderma edule*. A. Volcano plot displaying differentially expressed genes. Red represents upregulated genes, and blue downregulated genes. B. Heatmap of the differentially expressed genes illustrating the degree of up- or down- regulation for each gene. C. Principal component analysis (PCA) illustrating the different patterns of gene expression among the samples. Infected samples are shown in orange and non-infected samples in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell cycle process (GO:1903046). Additionally, GO terms associated with DNA-templated processes were enriched, such as Chromosome organization (GO:0051276), DNA damage response (GO:0006974), and DNA replication (GO:0006261). Other enriched categories included those associated with the actomyosin contractile ring apparatus, specifically Actomyosin contractile ring assembly (GO:0000915) and organization (GO:0044837), as well as pathways involved in ncRNA and rRNA metabolic processes, such as rRNA processing (GO:0006364), ncRNA processing (GO:0034470), ribosome biogenesis (GO:0042254), and the maturation of the ribosomal small subunit (SSU)-rRNA (GO:0030490 and GO:0042274) (Fig. 2A).

Conversely, infected cockles did not show any significantly enriched functional pathways at a 95 % confidence interval (adjusted p-value $\leq$ 0.05). However, when the significance threshold was relaxed to a p-value of 0.20, several pathways emerged as significant, primarily related to immune responses. These included innate immune response (GO:0045087), positive regulation of immune system process (GO:0002684), and activation of innate immune response (GO:0002218). Other highlighted pathways were linked to extracellular anion transport and dopaminergic synaptic transmission, such as organic anion transport (GO:0015711) and regulation of synaptic transmission, dopaminergic (GO:0032225). Additionally, pathways associated with nucleobase metabolic processes (GO:0009112), nucleoside catabolism, and actin filament organization, including Actin Filament Bundle Organization (GO:0061572), Assembly (GO:0051017), and Actin Filament Organization (GO:0007015), also stood out. Finally, pathways related to cell differentiation and cell–cell adhesion were also identified, such as positive regulation of leukocyte cell–cell adhesion (GO:1903039), cell–cell adhesion (GO:0045785), and T cell activation (GO:0050870) (Fig. 2B).

3.3. Gene expression profiles in the digestive gland

Regarding digestive gland samples, only four infected and four non-infected individuals were successfully sequenced. A total of 358 genes were found to be upregulated in infected cockles, while 846 genes were downregulated (see Supplementary Material), as shown in the volcano plot (Fig. 3A). Once again, the differences in gene expression between infected and non-infected individuals are also highlighted in a heatmap (Fig. 3B). Principal Component Analysis (PCA) revealed a clear separation, with infected individuals on the right side of the x-axis and non-infected individuals on the left. Notably, one non-infected sample, which was also previously separated in the haemolymph analysis, clustered more closely with the infected individuals (Fig. 3C).

For non-infected cockles, GO enrichment analysis identified five main enriched pathways (i.e., downregulated in infected individuals). The first pathway was related to Microtubule Axoneme (Dynein Arm Extracellular Assembly), with GO terms such as Microtubule Cytoskeleton Organization (GO:0000226) and Axoneme Assembly (GO:0035082), Plasma Membrane Bounded Cell Projection Assembly (GO:0120031), Microtubule-Based Movement (GO:0003341), and Cilium Movement (GO:0007018). The second pathway involved Cilium-

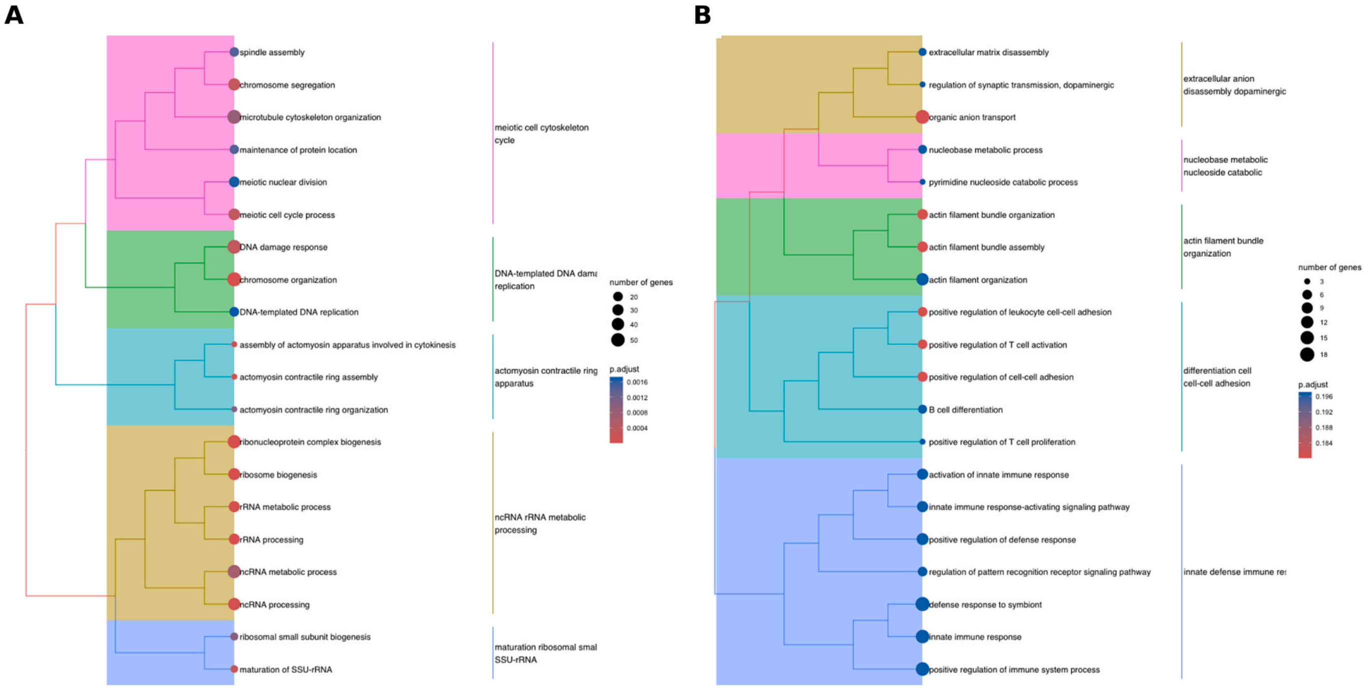

Fig. 2. Treeplot of the 20 most representative pathways identified by Gene Ontology analysis of haemolymph from infected *Cerastoderma edule*. A. Pathways showing significant downregulation. B. Pathways showing upregulation under a less strict *p*-value threshold.

Dependent Flagellated Sperm Mobility, characterised by terms such as Sperm Motility (GO:0097722), Cilium Movement Involved in Cell Motility (GO:0060294), and Cilium or Flagellum-Dependent Cell Motility (GO:0001539). The third pathway was associated with Spermatid Development and Differentiation, including GO terms such as Male Gamete Generation (GO:0048232) and Spermatid Differentiation (GO:0048515). Additional pathways included nuclear division processes (Metaphase/Anaphase Sister Mitotic Chromatid Separation and Homologous female chromosome pairing at), with GO terms such as Chromosome Segregation (GO:0007059), Organelle Fission (GO:0048285), Nuclear Division (GO:0000280), Mitotic Nuclear Division (GO:0140014), Meiotic Cell Cycle (GO:0051321), Meiotic Chromosome Segregation (GO:0045132), and Homologous Chromosome Pairing at Meiosis (GO:0007129) (Fig. 4).

For infected cockles, no pathways were found to be significantly enriched at a 95 % confidence interval (or any other *p*-value threshold). Among the 358 upregulated genes, approximately 38 % were annotated, primarily associated with scavenger receptor activity and C-type lectin (carbohydrate-recognition) functions. Despite these annotations, the number of transcripts involved in these processes was insufficient to reach statistical significance.

3.4. *Comparative analysis of gene expression profiles across tissues*

Nearly 95 % of differentially expressed genes were specific to a single tissue. Seven genes were upregulated in both the haemolymph and the digestive gland, while 84 were downregulated in both tissues. Six genes were upregulated in the haemolymph but downregulated in the digestive gland, whereas 29 genes showed the opposite pattern, being downregulated in the haemolymph and upregulated in the digestive gland (Fig. 5).

Genes downregulated in both tissues were primarily linked to cell division, microtubule cytoskeleton movement, and axoneme-cilium bundle formation (Fig. 6A). Conversely, genes upregulated in both the haemolymph and the digestive gland were associated with the adult amine compound biosynthetic pathway and zinc ion transport pathways (Fig. 6B).

No statistically significant pathways were identified for genes upregulated in the haemolymph but downregulated in the digestive gland, or those showing the opposite pattern (upregulated in the digestive gland but downregulated in the haemolymph). However, genes upregulated in the haemolymph were linked to glucose metabolism, leptin signalling in muscle cells, pyramidal neuron development, and the glucuronate-xylulose 5-phosphate biosynthetic pathway. Genes upregulated in the digestive gland were associated with metabolic processes involving eicosanoids, unsaturated fatty acids, vasoconstriction responses, and inflammatory biosynthesis.

4. Discussion

Cockles are exposed to several parasites and pathogens due to their role as filter feeders (de Montaudouin et al., 2021; Longshaw and Malham, 2013). Among other parasites with lower infection levels, all cockles from Baiona were found to be infected by the trematode parasite *Gymnophallus minutus*. Although this trematode species is highly prevalent and abundant in cockles (de Montaudouin et al., 2010; Magalhães et al., 2018), its pathogenicity is generally considered low (de Montaudouin et al., 2021).

In this study, all analysed cockles were exclusively infected by *G. minutus* or co-infected with *Bucephalus minimus*. While *B. minimus* was the primary focus, *G. minutus* was consistently present in all individuals, but at relatively low abundance. Since no protocol for infection under controlled conditions has yet been developed, this study relied on naturally infected field samples, potentially exposed to various stressors and pathogens, different infection intensities, or dynamics. Importantly, the consistent presence of *G. minutus* across all samples suggests that any observed differences are more likely attributed to *B. minimus* infection, rather than the co-occurrence of *G. minutus*. Therefore, the focus of this study was on the gene expression profiles of cockles infected by *B. minimus*, a highly deleterious parasite that causes castration, suppression of immune defences, and even death of cockles (Dubois et al., 2009; Magalhães et al., 2015). However, despite analysing cockles from the same area (presumably exposed to similar environmental stressors), and showing relatively comparable infection levels, the PCA revealed

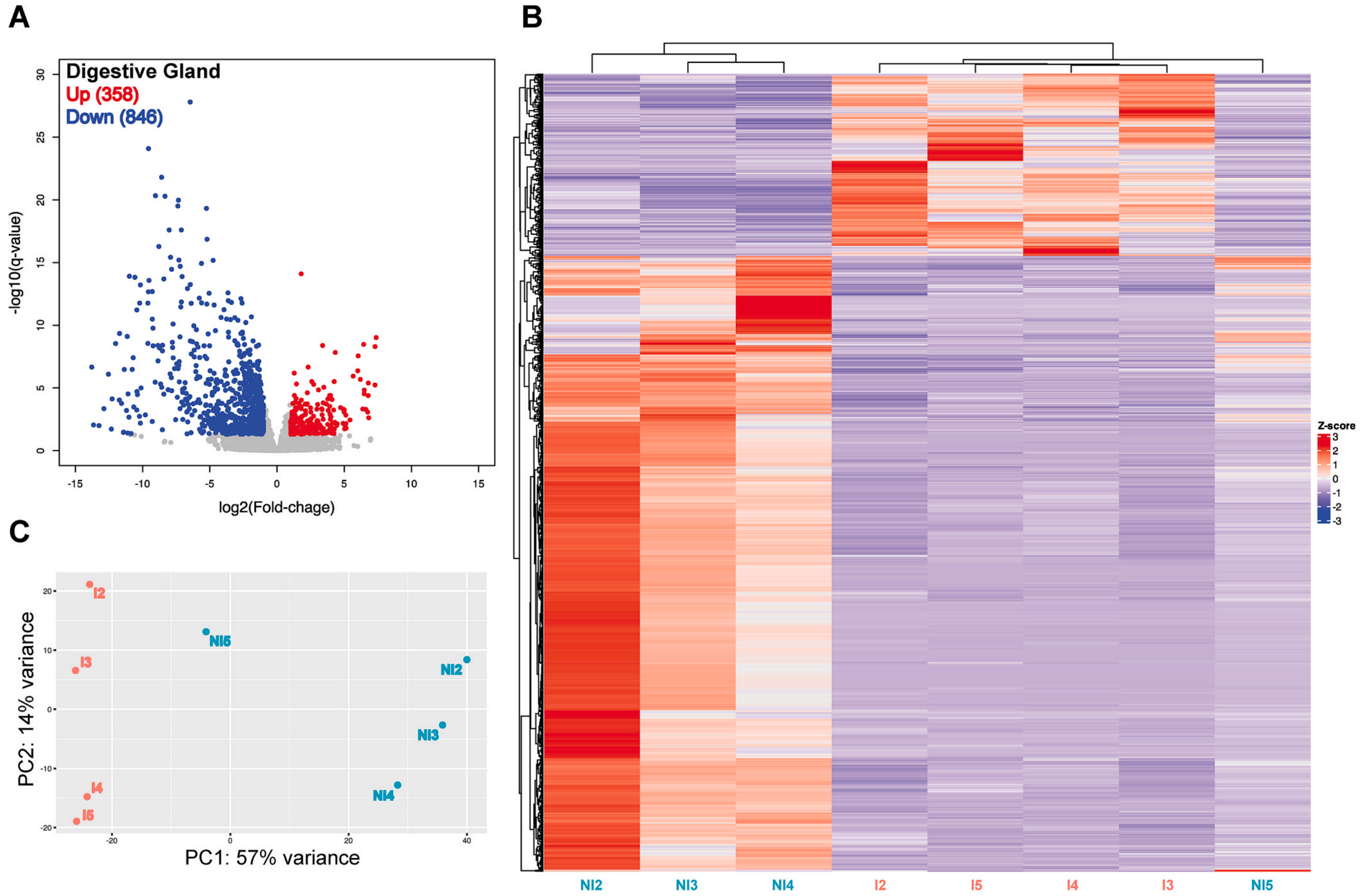

Fig. 3. Differential gene expression analysis in the digestive gland of infected (I) and non-infected (NI) *Cerastoderma edule*. A. Volcano plot displaying differentially expressed genes. Red represents upregulated genes, and blue downregulated genes. B. Heatmap of the differentially expressed genes illustrating the degree of up- or down- regulation for each gene. C. Principal component analysis (PCA) illustrating the different patterns of gene expression among the samples. Infected samples are shown in orange and non-infected samples in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

some inter-individual variation, including one control sample clustering close to the infected group. These patterns may reflect differences in infection progression and challenges of working with naturally infected samples.

This study specifically targeted two crucial tissues, i.e. the haemolymph, which plays a central role in immune function (Allam and Raftos, 2015), and the digestive gland, the primary site of infection (Magalhães et al., 2015). Contrary to our initial hypothesis that infection would trigger a higher number of upregulated immune and stress-related genes, particularly in the haemolymph, results showed that infected cockles displayed a higher number of downregulated genes in the haemolymph compared to non-infected individuals. This contrasts with findings from previous studies on bivalves and other organisms infected by trematodes (Schultz et al., 2020; Wijayawardena et al., 2016) or other parasitic infections (Fernández-Boo et al., 2024; Saco et al., 2020; Taube et al., 2023), which reported an upregulation of immune system related genes. It is important to note that those studies relied mostly on experimental infections, where samples were collected shortly after infection (i.e. within days or hours post-infection), while this study analysed naturally infected cockles collected from a wild population in the Ramallosa lagoon (Baiona, Spain), making it hard to determine when the infection first occurred. Furthermore, the lack of a higher number of upregulated genes, typically associated with immunity, could be explained by the prevalence of *G. minutus* in the non-infected cockles, which may activate some immune-related pathways in this group. Nonetheless, as *G. minutus* was present at relatively low abundance and is often considered to have low impact on its host (de Montaudouin et al., 2021), other factors are likely contributing to observed gene expression patterns.

Curiously, this study did find several upregulated immune related pathways and genes, but these did not reach statistical significance ($0.05 < p\text{-value} \leq 0.20$). For instance, genes related to C-type lectins were identified as over expressed in the haemolymph. These receptors are crucial for recognising pathogens and activating immune system responses (Brown et al., 2018; Hertel et al., 1994; Jia et al., 2016) and are frequently reported as upregulated in similar studies (Moreira et al., 2018). Additionally, pathways associated with dopaminergic signalling were identified. Dopamine is known to modulate immune functioning (de la Ballina et al., 2022; Liu et al., 2018), and alternations in dopaminergic pathways during infection could influence immune responses, inflammation, and even behaviour, potentially limiting the capacity of the parasite to spread (Curtis, 2014; de la Ballina et al., 2022; Matt and Gaskill, 2020).

Other pathways identified as differentially expressed in the haemolymph, but with a more relaxed cut-off ($0.05 < p\text{-value} \leq 0.20$), such as actin filament organisation, cell–cell adhesion, or innate immune defence response, suggest that the upregulated genes were involved in pathogen recognition and early stages of immune defence. Activation of the innate immune response is a crucial early step in identifying and combating parasitic infection (Canesi and Pruzzo, 2016; Gowda and Wu, 2018). This involves recognising the parasite through pattern recognition receptors (PRR), and the release of cytokines and chemokines to recruit immune cells (Venier et al., 2011).

However, the lack of statistically significantly altered immune

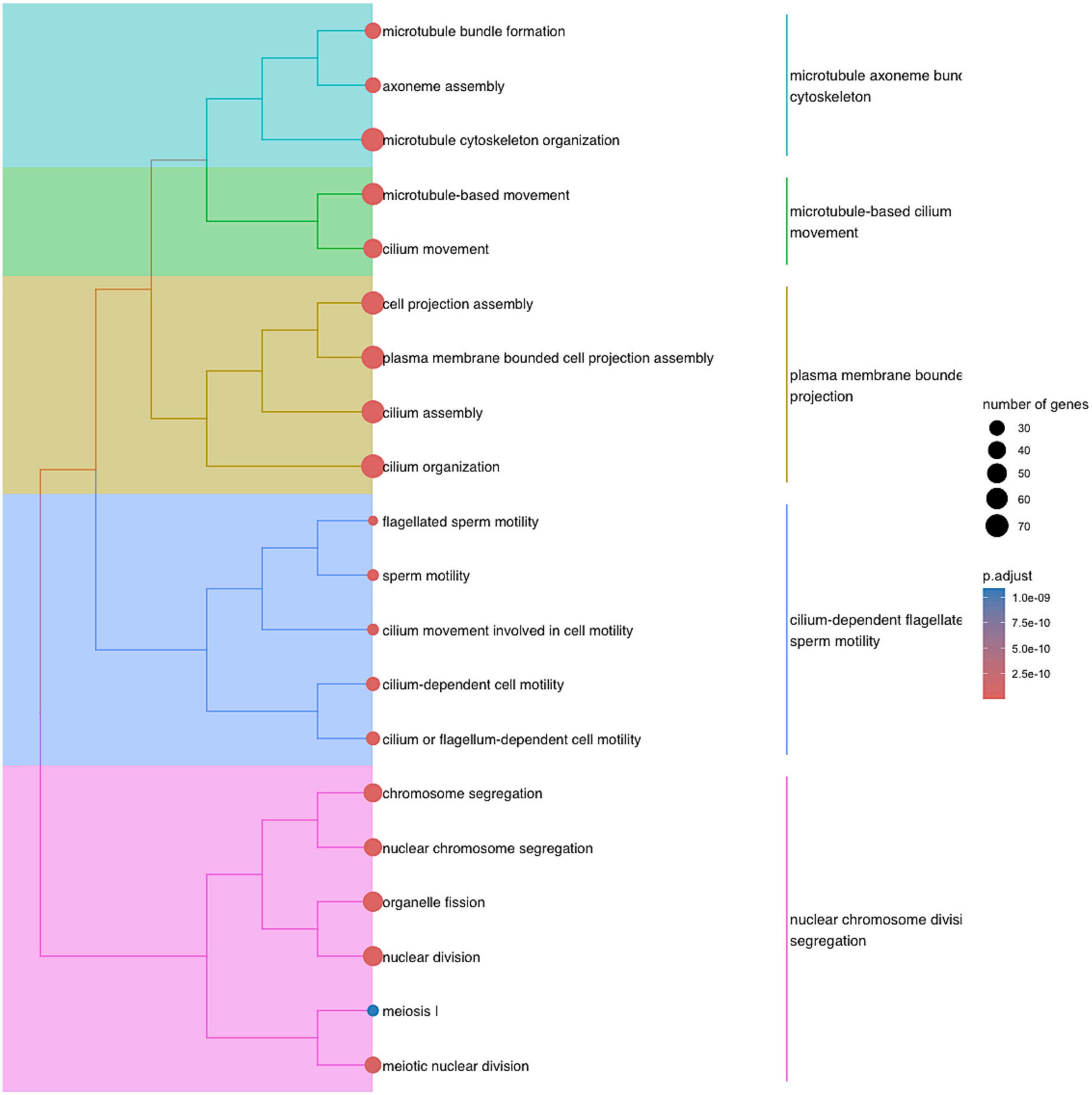

Fig. 4. Treeplot of the 20 most representative downregulated pathways identified by Gene Ontology analysis of digestive gland from infected *Cerastoderma edule*. No upregulated pathways were identified.

pathways in the haemolymph could suggest that the immune system, initially activated during early stages of infection, may have since been reduced by the parasite. Alternatively, it could indicate that infected cockles, having failed to clear the infection, are no longer investing resources in immune activation. Instead, cockles may be experiencing energy trade-offs, reallocating these resources to other physiological processes more beneficial for survival under chronic infection conditions. This hypothesis aligns with findings from other studies on immune signalling in bivalves, which report an initial peak in immune activity following infection. If the immune response is unsuccessful, the host reduces its investment in immune defences, shifting resources towards other strategies (Garcia, et al., 2022; Novoa et al., 2019; Rey-Campos et al., 2019). Another possibility is the development of immune tolerance to avoid chronic inflammation or tissue damage (Rey-Campos et al., 2019).

The repressed pathways in the haemolymph were mainly linked to cell division and the immune system, which may reflect a potential impact of the parasite on the host's cellular machinery. The cytoskeleton, mainly composed of microtubules, actin filaments, and intermediate filaments (Hohmann and Dehghani, 2019; Li et al., 2023), is vital for maintaining cell shape, transporting organelles, and aiding cells in completing immune processes like phagocytosis (Li et al., 2023; Mylvaganam et al., 2021). Some parasites can exploit these cytoskeletal functions to move and spread within the host (Cyrklaff et al., 2011; Frénal and Soldati-Favre, 2009), raising the hypothesis that similar mechanisms could theoretically benefit *B. minimus*. However, since *B. minimus* is an extracellular macroparasite, direct use of host's cytoskeletal network is unlikely. Instead, repression of these pathways could be a defence against the host's recognition and response mechanisms (Balbi et al., 2021).

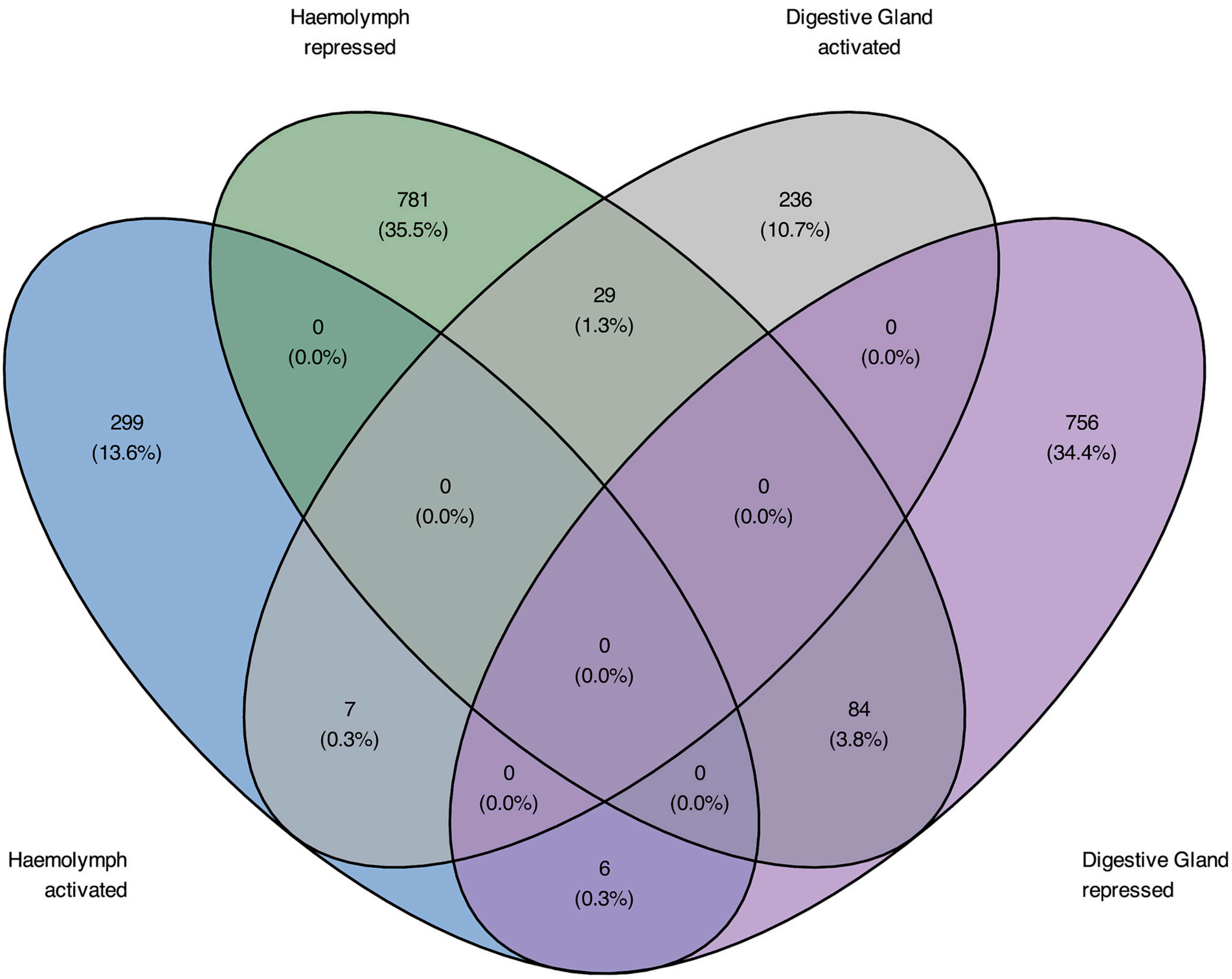

Fig. 5. Venn diagram comparing differentially expressed genes across tissues in *Cerastoderma edule*. The numbers indicate the total number of genes in each group.

Several downregulated pathways in the haemolymph may point to disruptions in host cell cycle processes. For example, the meiotic cell cycle and the actomyosin contractile ring provide the mechanical force needed for cell division during cytokinesis (Gerien and Wu, 2018; Hammarton, 2019), essential as previously discussed for immune cell recruitment. This could suggest a more permissive environment for *B. minimus* development. Besides, the repression of DNA damage replication pathways suggests that the parasite may inhibit the host's DNA repair mechanisms, potentially to prevent the activation of apoptosis or other defense strategies (Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Zhou et al., 2023). This hypothesis, while plausible, remains speculative, and proteomic studies could be helpful to validate whether these transcriptomic changes are reflected at the protein level, and to detect post-translational modifications or interactions that may further reveal how *B. minimus* influences host cell functions.

In addition to the possibility that the parasite manipulates host gene expression for its own benefit, an equally plausible explanation might be that the observed changes in gene expression are part of a complex adaptive response of the host to attenuate the effects of infection. Furthermore, it is also possible that the transcriptomic alterations observed are non-adaptive side effects of infection-induced tissue degradation or metabolic disruption, rather than active regulatory responses by the host. Distinguishing such effects from host strategies will require additional histological or proteomic validation to assess tissue integrity and functional outcomes.

Gene pathways related to RNA processing, including non-coding RNA (ncRNA), ribosomal RNA (rRNA) metabolic processing, and the maturation of the small subunit of ribosomal RNA (SSU-rRNA), were also found to be differentially expressed in cockles infected by *B. minimus*. Trematodes, such as the well-studied *Schistosoma mansoni*, have been shown to interfere with pathways involving ncRNA (Kim et al., 2020). There is evidence that ncRNAs play a role in regulating the expression of protein-coding genes (Hongkuan et al., 2021; Kim et al., 2020). Consequently, *B. minimus* may act similarly, with this disruption potentially impairing protein synthesis and ribosome function, thereby hindering the cockles' ability to produce proteins essential for immune responses. However, this hypothesis requires further investigation, as no data is currently available for *B. minimus*.

Regarding the digestive gland, there was once again a higher number of differentially expressed genes downregulated compared to upregulated genes in infected cockles, with the total number of differentially expressed genes similar to that found for the haemolymph. As previously discussed, haemolymph is the primary component of the immune system in bivalves (Allam and Raftos, 2015). It was expected that during early stages the infection would trigger activation of immune genes (Allam and Raftos, 2015; Rey-Campos et al., 2019; Schultz et al., 2020), which would then become less active as the infection progresses into a chronic stage. In contrast, the opposite was anticipated in the digestive gland. During the initial phase, this organ was expected not to show significant alterations (due to immune system response targeting the parasite via the haemolymph), but as the infection develops into a chronic infection, the digestive gland was expected to show an increase of gene activity

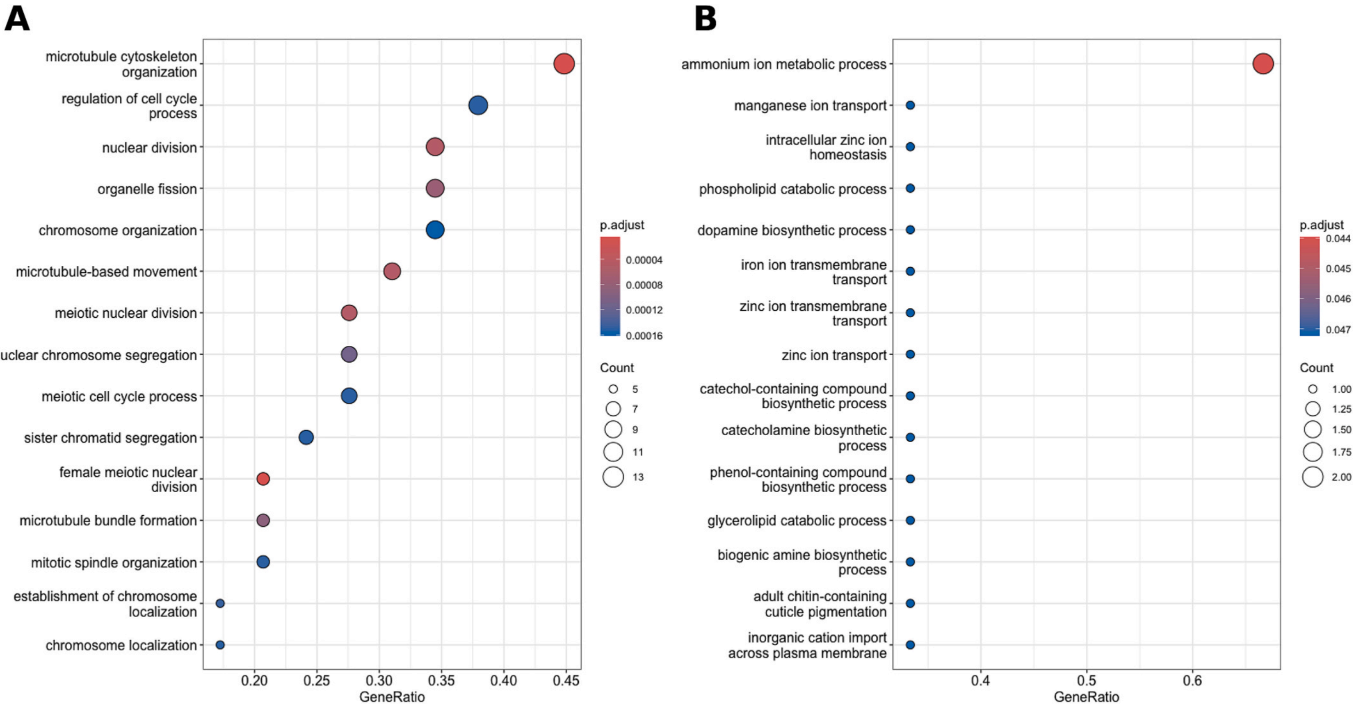

Fig. 6. Dotplot of the 20 most representative pathways identified by Gene Ontology analysis across tissues in *Cerastoderma edule*. A. Pathways with significant upregulated genes in both tissues. B. Pathways with significant downregulated genes in both tissues.

and consequently higher number of pathways upregulated. This was, however, not observed as the high number of downregulated genes in this study outnumbered that of upregulated genes. Moreover, there were no significantly upregulated pathways in the digestive gland of infected cockles.

Among the few upregulated genes in the digestive gland, several were associated with C-type lectins and scavenger receptors. These processes were previously discussed in the context of haemolymph as being linked to the immune response and could indicate a local immune response due to inflammation (Adema et al., 1997; Wang et al., 2016). Regardless, many of the differentially expressed genes found remain unannotated, making it difficult to infer their function. This knowledge gap highlights the need for further studies to uncover the molecular mechanisms underpinning how cockles cope with parasitic infection.

As documented in other studies, trematodes in their first parasitic life stages (i.e. sporocysts) are known to castrate their hosts (Coustau et al., 1993; Crews and Yoshino, 1989; Thieltges, 2006). This was consistent with the observed downregulation of several reproduction related pathways, such as Spermatid Development and Differentiation, Homologous Chromosome Pairing in Females, and Cilium-Dependent Flagellated Sperm Mobility. All these pathways are directly associated with reproductive processes. Reproductive systems are highly energy-consuming for organisms (Bayne, 1976; Berthelin et al., 2000). Meiosis is particularly demanding and essential for gamete formation. During infection, however, cockles may prioritise survival over reproduction, and repressing these functions may reflect a broader strategy to suppress reproductive functions and save resources for other functions. Additionally, as previously said, it is well established that trematodes may actively disrupt gametogenesis and gamete function in their hosts (Evensen et al., 2023; Valderrama et al., 2004).

Finally, the reduction of genes expressed in pathways related to the sister chromatid separation during mitosis may represent a defensive response of cockles to infection. Studies have shown previously that host cell cycle arrest is a common reaction to stress and infection (Allam and Raftos, 2015), as it helps to prevent the proliferation of potentially damaged cells. Besides, by stopping cell division, the host may limit the resources available to the parasite, which might otherwise exploit the host's cellular machinery for its own replication and development (Coakley et al., 2016; Schmid-Hempel, 2008).

In addition to tissue-specific differentially expressed genes, certain genes were differentially expressed in similar ways in both the haemolymph and the digestive gland. These shared genes likely represent systemic host responses to chronic infection by *B. minimus*. Genes involved in pathways such as cell division, microtubule cytoskeleton movement, and axoneme-cilium bundle formation were found to be downregulated in both tissues. As previously discussed, these genes are vital for cellular replication, intracellular transport, and ciliary function, all of which play critical roles in maintaining cellular homeostasis and immune responses (Li et al., 2023; Mylvaganam et al., 2021). The downregulation of these pathways could be due to a parasite-driven disruption of host cellular machinery, aligning with the broader strategy of *B. minimus* to evade immune detection and establish a favorable environment for its development.

Conversely, the upregulation of genes involved in zinc ion transport and the amine compound biosynthetic pathway in both tissues points to conserved host responses aimed at mitigating the infection and supporting essential physiological functions under stress. In the immune responses of several organisms, zinc functions as a structural component of key enzymes and transcription factors critical for pathogen recognition, inflammatory responses, and oxidative stress mitigation (Kambe et al., 2015). In bivalves, similar roles are expected (Kong et al., 2020). The upregulation of zinc ion transport genes may reflect cockles attempting to redistribute zinc to areas of highest need, such as sites of infection or tissue repair. This is consistent with the known role of zinc in enhancing the efficacy of immune cells, such as phagocytes and lymphocytes, which are crucial in responding to parasitic infections (Prasad, 2008; Shankar and Prasad, 1998). Moreover, zinc homeostasis is tightly regulated to prevent exploitation by pathogens, as some parasites sequester zinc to support their growth and metabolism (Ginsburg et al., 1986; Marvin et al., 2012). The observed upregulation could, therefore, represent a dual strategy by the host to bolster immune defenses and counteract potential parasitic zinc scavenging.

The amine compound biosynthetic pathway, on the other hand, is essential for producing key molecules such as neurotransmitters, hormones, and other signaling compounds (de Jong et al., 2011). These molecules play diverse roles in host responses to infection, including modulating stress responses, maintaining tissue integrity, and regulating immune signaling (Canesi et al., 2022; Li et al., 2005). For example, serotonin and dopamine, both products of this pathway, are known to influence immune cell function and stress adaptation, which may be critical in managing the systemic effects of *B. minimus* infection. Additionally, the activation of this pathway could represent an attempt by the host to mitigate the metabolic and structural disruptions caused by the parasite, ensuring continued support for essential physiological processes.

In conclusion, this study presents a detailed analysis of gene expression profiles in cockles (*C. edule*) exposed to a long-term infection by the trematode *B. minimus* across two tissues associated with different responses to the infection. This study identified a predominance of downregulated genes and functions in both haemolymph and the digestive gland. In the haemolymph, the Gene Ontology enrichment analysis suggests a potential reduction of the immune response over time due to a chronic infection. Moreover, the changes in host gene expression could result from parasite manipulation of the host's cellular machinery, namely the cytoskeletal dynamics and cell division processes, possibly to enhance its survival within the host. However, further experimental validation is needed to confirm such mechanisms. In the digestive gland, the observed downregulation of metabolic and immune related genes further highlights the parasite's impacts on host metabolism, potentially due to compromised cockle immune efficiency or reallocation of resources to more vital functions. Overall, this study highlights the molecular mechanisms driving host-parasite interactions, revealing the impacts of *B. minimus* on its first intermediate host *C. edule*. These findings represent an initial step toward understanding how trematodes adapt and interact with their hosts over time, highlighting the need for further research focusing on how infection intensity may modulate gene expression or proteomic responses. Future studies should also consider the use of controlled infections and complementary functional assays.

CRediT authorship contribution statement

Simão Correia: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andrés Blanco-Hortas:** Writing – review & editing, Software. **Daniel Garcia-Souto:** Writing – review & editing, Resources. **Robert Poulin:** Writing – review & editing, Supervision. **Manuel Vera:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Luísa Magalhães:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sergio Fernández-Boo:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization.

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Ethical standards

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2025.108451.

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