Strongly integrated host–parasite system: the transcriptomic analysis

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ABSTRACT: The mollusc–trematode system exemplifies a parasitic relationship with a very long evolutionary history and has significantly altered the functions of both organisms. The influence of trematode parasites on molluscs is complex and multifaceted: it may include immune suppression, alterations in energy metabolism, gigantism, parasitic castration, shell deformation, changes in behavior and motility, etc. Nevertheless, the molecular basis of host-parasite interactions between snails and flukes is still underinvestigated, and thorough studies of trematode-caused changes in the host at the molecular level are of high demand in parasitology. In this study, we analyzed changes in gene expression in tissues of *Littorina saxatilis* snails infected with *Microphallus piriformes* trematodes. Our comparative transcriptomic analysis of infected vs healthy molluscs revealed significant changes in expression of 160 genes associated with biomolecule catabolism, morphogenesis and tissue remodeling, immune system function, etc. The observed changes in expression of genes associated with lipid and protein metabolism are likely to be associated with parasitic exploitation of the host's energy resources, while tissue remodeling processes may be attributed to homeostasis maintenance in the infected host. Additionally, we performed a targeted search and identified homologues of a number of immune-related genes: toll-like receptors, peptidoglycan recognition proteins, etc. In the context of immune response-related genes, there was a noticeable decrease in the expression of genes associated with the production of reactive oxygen species and reactive nitrogen species in the infected snails, specifically the dual oxidase and nitric oxide synthase genes. Our data suggests a fundamental modification of the host's metabolism induced by the parasite and add to the previous studies on the interaction between the *Littorina* molluscs and the *Microphallus* trematodes.
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KEY WORDS: host-parasite interactions, parasitism effects, host resources exploitation, invertebrate immunity, *Microphallus*, *Littorina*.

Глубоко интегрированная система паразит–хозяин: транскриптомный анализ

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РЕЗЮМЕ: Система трематоды–моллюски представляет собой паразитарную ассоциацию с длительной коэволюционной историей и значительными взаимными функциональными воздействиями. Влияние трематод на организм моллюсков сложно и многогранно: оно может включать подавление иммунитета, изменение энергетического обмена, гигантизм, паразитарную кастрацию, деформацию раковины, изменение поведения и подвижности хозяина и т.д. Тем не менее, молекулярные основы паразито-хозяинных взаимодействий между моллюсками и трематодами по-прежнему недостаточно исследованы. В данной работе мы проанализировали изменения экспрессии генов в тканях улиток *Littorina saxatilis*, заражённых трематодами *Microphallus piriformes*. Проведенный нами сравнительный транскриптомный анализ инфицированных и здоровых моллюсков выявил значительные изменения в экспрессии 160 генов, среди которых можно отметить гены, связанные с катаболизмом биомолекул, морфогенезом и ремоделированием тканей, функционированием иммунной системы и др. По-видимому, обнаруженные изменения липидного и белкового обмена связаны с эксплуатацией паразитом энергетических ресурсов хозяина. Процессы ремоделирования тканей, вероятно, отражают попытки поддержания гомеостаза в организме инфицированного моллюска. Также мы провели целенаправленный поиск и выявили гомологи ряда генов, ассоциированных с иммунной защитой: Toll-подобных рецепторов, пептидогликан-распознающих белков и др. Среди генов, участвующих в иммунном ответе, отмечено заметное снижение экспрессии генов, ответственных за продукцию АФК (активных форм кислорода) и АФА (активных форм азота) у заражённых моллюсков: гены двойной оксидазы и NO-синтазы. Наши результаты указывают на существенные модификации метаболизма хозяина, вызванные паразитом, и дополняют предыдущие исследования взаимодействий моллюсков *Littorina* с трематодами рода *Microphallus*.
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КЛЮЧЕВЫЕ СЛОВА: паразито-хозяинные взаимодействия, эффекты паразитизма, эксплуатация ресурсов хозяина, иммунитет беспозвоночных, *Microphallus*, *Littorina*.

Introduction

Parasitic organisms are a taxonomically diverse and ubiquitously distributed group that inhabit other living organisms and exploit their metabolic capacities (Bush *et al*., 2001; Combes, 2001; Poulin, 2007; Von Brand, 2013). Parasites alter their host's biochemical and physiological functions, often changing their morphology, behavior, and reproductive capability (e.g. Moore, 2002; Granovitch *et al*., 2009; Thomas *et al*., 2010; Lafferty, Shaw, 2013; Granovitch, 2016). Such changes can cause whole-community shifts in matter and energy cycling patterns, highlighting the necessity of integrating parasites into the ecosystem-scale framework (Mouritsen, Poulin, 2002; Lafferty *et al*., 2008, 2012; Rynkiewicz *et al*., 2015). Nevertheless, these high-level consequences start from the molecular level of host-parasite interactions, which are highly variable both in mechanisms and in the depth of parasite incorporation into the metabolism and physiology of its host. Strongly integrated host-parasite systems are highly informative models, clarifying how the biological function of one organism can be efficiently exploited by another. As such, detailed studies of host-parasite interaction mechanisms in such systems are of high demand.

Trematodes (Platyhelminthes: Digenea) and their gastropod hosts form a finely tuned system replete with complex mutual influences with a long evolutionary history (Galaktionov, Dobrovolsky, 2003). Trematodes are common parasites in the intertidal zone, exploiting the snails living there as intermediate hosts (Mouritsen, Poulin, 2002; Galaktionov, Dobrovolskij, 2003; Granovitch, Mikhailova, 2004; Galaktionov *et al*., 2019). One of the high-density species present on the North Atlantic rocky shores is *Littorina saxatilis*. These invertebrates are low-mobile micrograzers which consume bacterial biofilms, as well as microalgae (Reid, 1996). In the northeast part of their ranges, these snails are often parasitized by highly prevalent trematode species of the 'pygmaeus' group of the Microphallidae family; particularly, *Microphallus piriformes* Galaktionov, 1983 and *M. pygmaeus* Levinsen, 1881 (Granovitch *et al*., 2000, 2004).

In this study, we used the *Microphallus piriformes* and *Littorina saxatilis* Olivi, 1792 as the most common host-parasite system on the Barents and the White Sea coasts to characterize the trematode-caused changes in host tissues. We applied the transcriptomic methodology, which is a highly informative tool to describe host-parasite interplay (rev. in Swann *et al*., 2015), including gastropod–trematode systems (e.g. Adema *et al*., 2020; Gorbushin, Borisova, 2015; Schultz *et al*., 2020, etc.). Transcriptomic analysis showed that in infected molluscs (at the fully-formed stage) processes of polysaccharide and lipid catabolism and protein degradation are upregulated. The acquired data also indicates the upregulation of tissue remodeling processes. Also, we identified through targeted BLAST of the *L. saxatilis* transcriptome the homologues of the TLR (toll-like receptors), PGRP (peptidoglycan recognition proteins), and GNBP (Gram-negative bacteria-binding proteins) receptors, as well as transcripts of DuOX (dual oxidase) and NOS (nitric oxide synthase); the expression level of the two latter enzymes was lower in infected snails, which may be an important factor in the course of the infection in snails.

Materials and methods

Sample collection

Specimens of *L. saxatilis* were collected from the wild populations at the following sites: Levin Navolok (Chupa Inlet, Kandalaksha Bay, White Sea; 66°18′04″ N, 33°27′27″ E), surroundings of Dalniye Zelentsy (East Murman, Barents Sea; 69°6′22″ N, 36°3'35" E) and the estuary of Kulonga River (Kola Bay, Barents Sea; 69°4′34″ N, 33°7′25″ E). Specimens were transported to the laboratory, where they were exposed to 3 weeks of acclimation in the tank under standard aquarium conditions to minimize region-specific physiological heterogeneity. Namely, the molluscs were kept as groups of 10–15 individuals in aquarial cells (10x10x30 cm) mechanically separated from each other with partially permeable mesh (1x1 mm) within the common tank with small pebbles on the cell bottom. Water temperature was 10 °C; water salinity was 33‰; water in the tank was kept running and aerated, and 6-h tidal cycles were artificially generated with a pump; small fragments of dried algae were used as a food source. Then, molluscs were dissected and checked for a parasitic infection. Parasites were identified to the species level based on the morphological features of the daughter sporocysts and metacercariae (Galaktionov, 1980, 1983) using uncovered microscopic slides (as described in Galaktionov, 1980; Repkin *et al*., 2020). Head and foot of every mollusc (not affected by parasites if they are

presented) of healthy[1] and infected molluscs were rinsed in distilled water, then fixed individually in TRIzol (TRIzol, Merck) and frozen at –80 °C. Eight healthy and six infected molluscs were included in the analysis (3 healthy and 3 infected snails from Levin Navolok; 3 healthy and 3 infected snails from Kola Bay; 2 healthy snails from Dalniye Zelentsy).

Library preparation and sequencing

The tissues were mechanically homogenized, and total RNA was isolated with a customized standard TRIzol extraction protocol. Briefly: (1) Mixture chloroform/isoamyl alcohol (24:1) was added to homogenate in TRIzol in a ratio of 1:5. (2) The supernatants after centrifugation were transferred to clean tubes (nuclease-free, Eppendorf), the co-precipitants: sodium acetate (to a final concentration of 0.1 M), Satellite Red (Evrogen, 1 µl) and isopropanol in a ratio 1:1 were added. (3) After being cooled at –20 °C for 30 min, the samples were centrifuged at 4 °C, 15000 g, 15 min. (4) After rinsing with 75% ethanol, the precipitated RNA was reconstituted in nuclease-free water (Evrogen) and stored at –80 °C until the next steps. The quality of RNA was evaluated by measurements of 260/280 nm (values varied in a range 1.92–1.99) and 260/230 nm (values varied in a range 1.87–2.01) absorbance ratios using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). cDNA synthesis (for poly(A) RNA fraction) was performed using the Mint cDNA synthesis kit (Evrogen) following the manufacturer's recommendations.

Library preparation and sequencing were performed with the Evrogen commercial service provider (Moscow, Russia); the instrument was a NovaSeq 6000 system (Illumina); 150-bp paired-end (PE) sequencing was used.

The raw sequencing data have been deposited to the NCBI Sequence Read Archive under BioProject ID PRJNA1048994.

Data processing and transcriptome assembly

Raw read quality was assessed with FastQC (v.0.11.5, S. Andrews, 2010). Subsequent correction of sequencing errors was performed using karect (v 1.0; Allam *et al*., 2015) with the following parameters -celltype=diploid and -matchtype=hamming. Adapters and low quality nucleotides were removed with Trimmomatic (v0.39, Bolger *et al*., 2014) in the paired-end mode with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:2:TRUE, SLIDINGWINDOW:4:20, MAXINFO:50:0.8, MINLEN:25. Additionally, barcodes were removed with cutadapt (v2.8, Martin, 2011) in the paired-end mode with parameters -q 20,20 and -m 25. Decontamination of reads was performed with kraken2 (v2.1.2, Wood *et al*., 2019) against its standard database including Eubacteria, Archaea, Viruses and *H. sapiens*, and against the NCBI RefSeq Eubacteria (downloaded 28.11.2021).

Reference transcriptome was assembled from reads from all 14 samples using Trinity (v2.12.0, Grabherr *et al*., 2011) with default parameters. Assembly quality control and filtration of well-assembled transcripts were performed with TransRate (v1.0.1, Smith-Unna *et al*., 2016). Then, transcripts were clustered with CD-HIT-EST (v4.7, Fu *et al*., 2012) with the threshold value of sequence identity 95%. Quality of obtained assembly was assessed with BUSCO v4.1.3 (Simão *et al*., 2015). For the BUSCO database, metazoan-odb10 was used with the e-value cutoff of 1e-3.

The search for the open reading frames in the obtained transcripts was conducted in TransDecoder. LongOrfs (v5.5.0, Haas, https://github.com/TransDecoder/TransDecoder) with the minimal ORF size of 100 amino acids. The ORFs found were annotated according to the Uniprot database (downloaded 23.01.2022) with the blastp search (blast+, v2.12.0+, Camacho *et al*., 2009) and according to the Pfam-A database (Mistry *et al*., 2021) with HMMscan (v3.1b2, Eddy, HMMER). Transcripts with e-value < 1e-3 and identity percent ≥ 50 were included in further analysis. Then, TransDecoder.Predict was used to extract annotated transcripts.

The obtained transcriptomic assembly was in a targeted way searched for the presence of trematode transcripts, since despite separation of infected and uninfected host tissues, the possibility of contamination with parasitic stages can not be fully excluded due to artificial (incompleteness of host-parasite separation) or natural reasons (in rare cases some sporocysts can be brought into pedal sinus). For filtering out transcripts with high homology with parasites, we created a database from a number of the trematode full genomes available from NCBI (NCBI Assembly Accessions: GCA_000237925.2, GCA_000699445.2, GCA_000699445.3, GCA_000715545.1, GCA_003604175.2, GCA_003958945.1, GCA_004794785.1, GCA_008360955.1, GCA_008508345.1, GCA_013368495.1, GCA_013407085.1, GCA_014220965.1, GCA_014338405.1, GCA_018104335.1, GCA_025215515.1, GCA_900302435.1, GCA_900617995.1, GCA_900618395.1, GCA_900618425.1, GCA_900618515.1, GCA_907275115.1, GCF_000237925.1, GCF_000699445.2, GCF_000715545.1). Filtration was performed as two steps using the blastn tool (blast+, v2.12.0+, Camacho *et al*., 2009) for testing sequence homology. At the first stage, transcripts with the blastn result e-value ≤ 1e-5, identity percent ≥ 70 and bit score ≥ 100 were selected out for the next filtration step as potentially contaminative (number

[1] In the context of this article, the word "healthy" means hosts that are not infected with trematodes.

of filtered transcripts was 17 802). At the second stage, we searched for homologues of these selected transcripts in published *L. saxatilis* genome (Westram *et al*., 2018; the unmasked version from Dr. Marina Panova) using the blastn algorithm with the thresholds e-value ≤ 0.001 and bit score ≥ 150. The transcripts demonstrating high homology with the *Littorina* genome were returned back to the decontaminated transcriptome for further analysis; the final number of excluded transcripts was 10 381.

Differential expression, annotation and ontology analyses

Statistical analysis was carried out using the R language (v4.1.3, R Core Team, 2022) and Bioconductor project (Gentleman *et al*., 2004; https://www.bioconductor.org/).

Quantification (TPM) was performed using the Salmon tool (v1.5.2, Patro *et al*., 2017) with --validateMappings flag. Tximport library was used to summarise transcript expression to gene expression. Then, genes with median expression values above 1 at least in one group PERM of molluscs (either healthy or infected) were selected. Significance of the infection influence on transcriptome was estimated using permANOVA (100 000 permutations, Anderson, 2001) implemented in the vegan package (v2.6, Oksanen *et al*., 2013).

DESeq2 package (v1.34.0, Love *et al*., 2014) was used to identify differentially expressed genes (DEGs); the differential expression analysis was performed with the log fold change shrinkage "apeglm" parameter (Zhu *et al*., 2019). Selection of genes with significant difference in expression level was done based on the adjusted p-value (FDR correction) cutoff of 0.05 and the 4-fold difference in expression levels (abs(logFoldChange) ≥ 2).

For the between-sample comparison, logarithmic regularization was performed (rlog function from DESeq2). Normalised data were used for the principal component analysis (PCA). To assess the effect of potential outliers on the ordination, a sensitivity analysis was performed (Supplement 6).

Sequences of differentially expressed genes were annotated in the EggNOG mapper (Cantalapiedra *et al*., 2021, Huerta-Cepas *et al*., 2019). Further gene ontology (GO) term analysis was carried out with the topGO package (v2.46.0, Alexa, Rahnenfuhrer, 2021) in the Biological Processes mode. The Kolmogorov-Smirnov test was used for evaluating statistical significance. Processes with a significance value < 0.05 and significant genes in the process ≥ 10 were selected.

Using the EggNOG mapper we also obtained metabolic pathway codes from the KEGG database (Kanehisa, 2000) and visualised them using the KEGGREST package (v1.34.0, Tenenbaum, 2016).

The immune system is the most important interface for interactions between host and parasite. Changes in its functioning following infection are to be expected and especially interesting in the context of host-parasite interactions (van der Knaap, Loker, 1990; Mone *et al*., 2011; Gorbushin, 2016, 2019). As such, we performed a targeted analysis of changes in the expression of key genes associated with immune response in invertebrates. The search for genes associated with immunity was performed using blastn (blast+, v2.12.0+, Camacho *et al*., 2009) with the e-value cutoff of 1e-3. We used the following reference sequences: Toll-like receptor *L. littorea* (GenBank: MT683613.1), MyD88 *L. littorea* (GenBank: MT683556.1), peptidoglycan recognizing protein (PGRP) *L. littorea* (GenBank: OK030845.1), gram-negative bacteria binding protein (GNBP) *Anopheles quadriannulatus* (GenBank: FJ653840.1), NO-synthase *Aplysia californica* (GenBank: AF288780.1), dual oxidase (DuOX) *Marsupenaeus japonicus* (GenBank: AB744213.1), thioester containing protein (TEP) *Biomphalaria glabrata* (GenBank: FJ480411.1). Since we were interested in detecting any changes in the expression of immune system-associated genes, there was no fold change limit in this analysis; statistical testing of significance of these changes was performed in a pairwise comparison using the Mann–Whitney U test in R (wilcox.test standard function) to decrease the false negative rate (error type II).

All the figures were visualized using the ggplot2 (Wickham, 2016) package.

Results and discussion

Characteristics of the libraries and the transcriptome assembly

The size of individual libraries varied from 10.5 to 23.5 million raw reads prior to filtration, and from 9.1 to 20.9 million reads following filtration (Supplement 1). Assembly quality statistics are presented in Supplement 5. The final transcriptome after all filtration steps includes 38 361 contigs (25 029 Trinity genes). The BUSCO search against the metazoan-odb10 database resulted in 879 (92.1%) of the complete orthologs and 58 (6%) of the missing ones (Fig. 1). This assembly was later on used for quantification and differential expression analysis.

Transcriptomic comparison and differentially expressed genes

The Salmon tool quantified abundances of the transcripts were merged to the gene levels with the tximport R library. During the denoising procedure, genes with the median expression level ≥ 1 in at least one group were selected as significantly expressed; the final expression

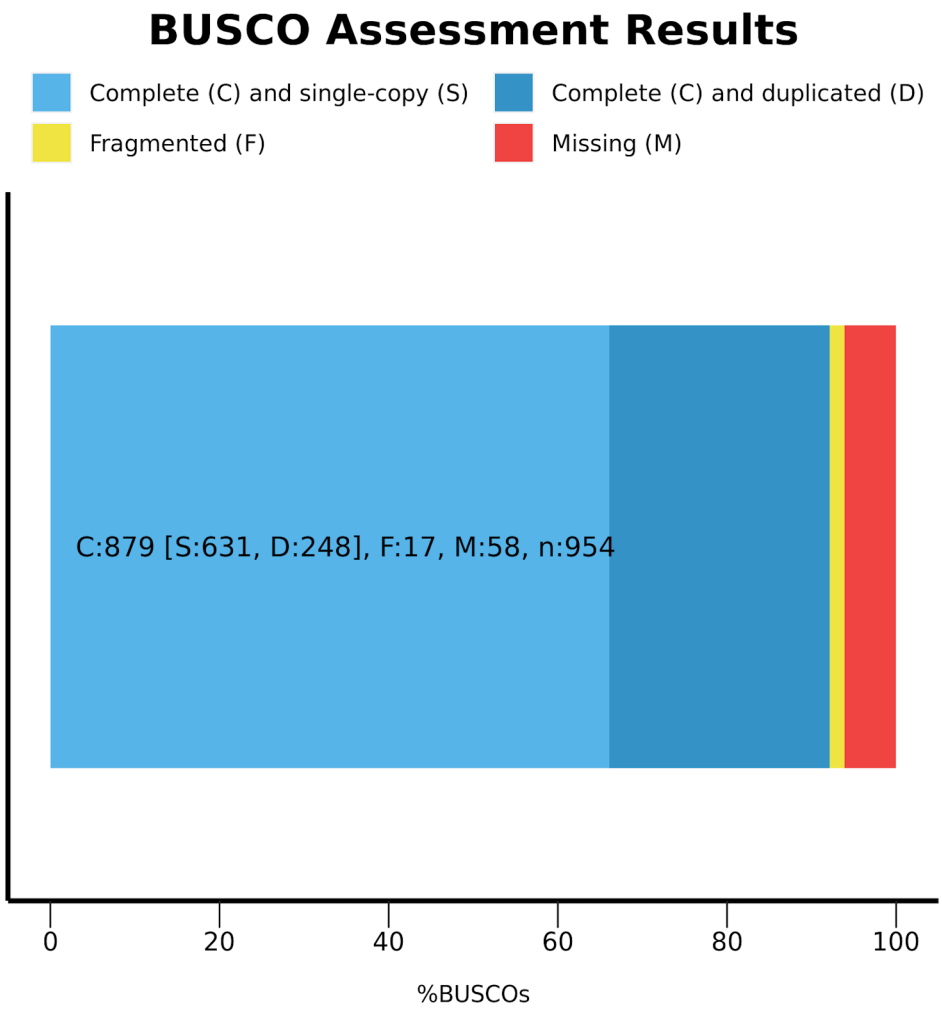

Fig. 1. Bar chart demonstrating the completeness of the decontaminated transcriptome of *L. saxatilis* based on the BUSCO analysis against the Metazoa database.
Рис. 1. Диаграмма, демонстрирующая полноту деконтаминированного транскриптома *L. saxatilis*, основанная на анализе BUSCO по базе данных Metazoa.

dataset included 13 588 genes. No significant geographical variability of transcriptomes was revealed. This is evidenced by the results of both principal component analysis (Fig. 2B) and the perMANOVA test (100 000 permutations, p-value 0.52). At the same time, comparative analysis demonstrated the significance of trematode-induced changes in the transcriptomic profiles of molluscs (perMANOVA $p < 0.01$, 100 000 permutations; Fig. 2B). This finding is supported by previously published data: alterations in host functioning were demonstrated in the *Littorina–Microphallus* parasitic system at diverse levels: biochemical, physiological, morphological and behavioural (Mikhailova *et al*., 1988; Granovitch, 1992, 2000, 2016; Galaktionov, 1993; Panova *et al*., 1999; McCarthy *et al*., 2000; Kaliberdina, Granovitch, 2003; Arakelova *et al*., 2003, 2004; Granovitch *et al*., 2009; Repkin *et al*., 2024).

In total, changes in expression were observed for 160 genes (Fig. 2A); among them, 58 genes with log-fold change $\geq \pm 2$, of which 48 were upregulated and 10 downregulated in the infected

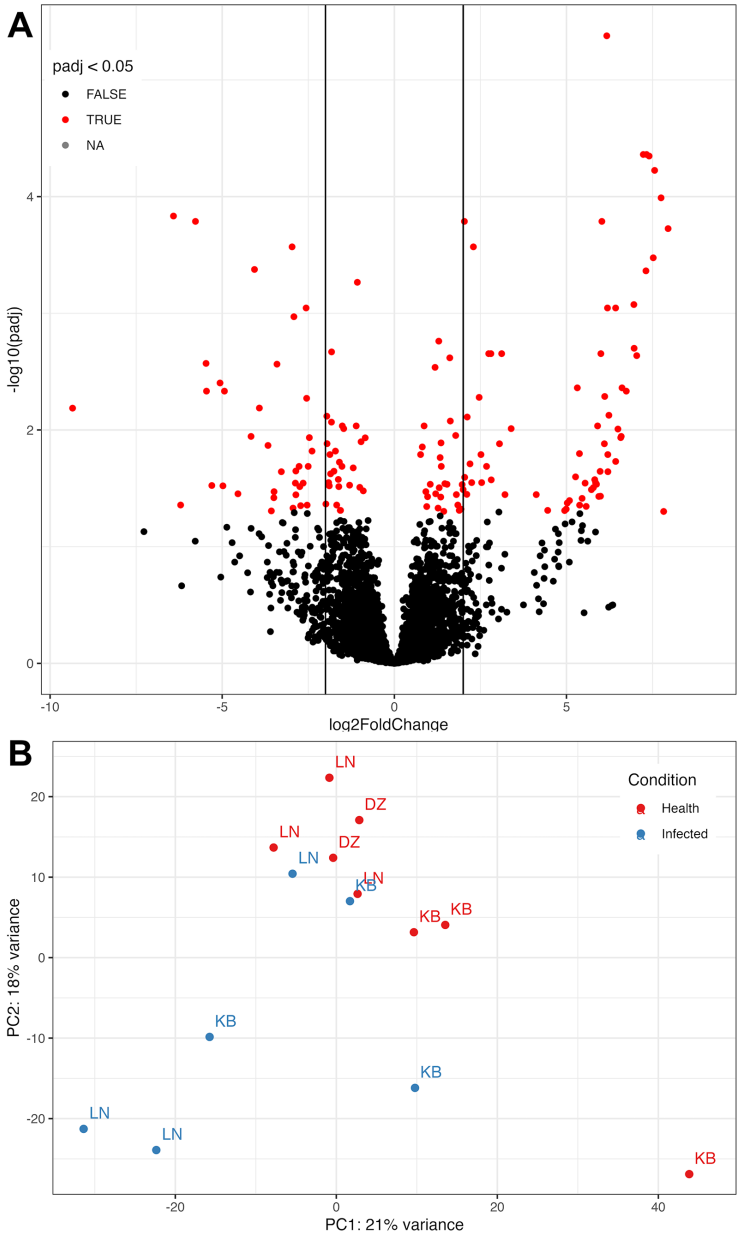

Fig. 2. A — volcano-plot demonstrating the significance of expression changes (Y axis) and logFC of expression between groups (infected against healthy individuals; X axis). Red points — significant DEGs (differentially-expressed genes), padj — adjusted p-value; B — PCA plot shows two diverging groups supported with perMANOVA (p-value = 0.0045, 100000 permutations). LN — Levin Navolok, White Sea; KB — Kola Bay, Barents Sea; DZ — Dalniye Zelentsy, Barents Sea.

Рис. 2. A — диаграмма, демонстрирующая значимость изменения экспрессии (ось OY, красные точки — гены со значимым уровнем отличий в уровне экспрессии) и логарифм кратности изменения экспрессии (logFC) между заражёнными и незаражёнными моллюсками (ось OX); B — визуализация расхождения двух групп моллюсков, подтвержденная статистическим анализом perMANOVA (p-value = 0.0045, 100000 пермутаций). LN — Левин наволок, Белое море; KB — Кольский залив, Баренцево море; DZ — Дальние Зеленцы, Баренцево море.

individuals compared to the uninfected ones (Supplement 2). The upregulated genes included triacylglycerol lipase, glucose transporter, regulators of proteasome-dependent protein degradation, autophagy, cell cycle and apoptosis, component of the shell matrix (perlwapin homolog). This may reflect the active utilization of the host's resources, encompassing both the breakdown of storage molecules (e.g. hydrolysis of triacylglycerols and glycogen), as well as the degradation of cellular structures and associated proteins. Our recent study (Repkin *et al*., 2024) demonstrated the active transport of glucose recruited from the host's storage to the parasite. An increase in sodium-dependent glucose transporter 1 (TRINITY_DN31468_c0_g2, logFC = 2.11) and trafficking regulator of GLUT4 1 (controlling the exposure of glucose transporter GLUT4 on cell surface, TRINITY_DN14618_c0_g1, logFC = 2.78) gene expression is consistent with this data. The fast drop in glycogen (and lipid) content accompanied by active catabolization of proteins in snails following a trematode invasion is well known (Arakelova *et al*., 2003, 2004, 2007; Tunholi *et al*., 2011, 2013; Repkin *et al*., 2024) and is congruent with our observations. The downregulated group included components of the extracellular matrix (collagen, hemicentin) (Supplement 2). Changes in the expression of the shell matrix protein (perlwapin homolog, TRINITY_DN6036_c0_g1, logFC = 2.45) might have an effect on the process of shell growth and shell shape. Deformation of the shell in trematode infected *Littorina* snails is well-described (Panova *et al*., 1999; McCarthy *et al*., 2004). Overexpression of the shell matrix may be a contributing factor to this abnormality.

Analysis of gene ontology

In addition, using the EggNOG web-service, we annotated the functional groups of differentially-expressed genes (DEGs) based on gene ontology (GO) group. Further GO statistical analysis revealed the significant association of 13 upregulated genes with 2 GO processes: positive regulation of cellular processes and biological processes. In the case of the downregulated genes, no significant associations with GO biological processes were found.

The GO processes associated with the upregulated genes are compatible with the trend of active metabolization of the host resources, inferred from the analysis of the DEGs transcriptomic annotation results.

Metabolic pathways analysis in KEGG

Involvement of DEGs in metabolic pathways (MPs) was assessed via the KEGG pathway analysis. The most enriched MPs group was associated with protein degradation (map04974: Protein digestion and absorption). In particular, among the upregulated members of this group were enzymes annotated as carboxypeptidases A1 (CPA1; contigs TRINITY_DN18353_c0_g1 and TRINITY_DN8522_c0_g1) (logFC = 7.56), though in our case these enzymes should be considered as non-digestive (not related to intestinal digestion), because the hepatopancreas, the main producer of the digestive enzymes, was not among the analyzed organs (Supplement 3). As such, these CPA1s are likely to be involved in non-digestive protein degradation and are associated with such MPs as 'Necroptosis' (Fig. 3). Our recent study on the metabolic changes in the *Littorina* snails after the *Microphallus* trematode infection (Repkin *et al*., 2024) implies active engagement of proteins (and later amino acids) as an alternative to glucose energy source in infected snails (while glucose is actively transported to the parasite). Accordingly, the aromatic-amino-acid aminotransferase (TRINITY_DN7418_c0_g1, logFC=5.45), an enzyme with predominant role in the metabolism and transfer of amino acids (Ge *et al*., 2023), was upregulated in the infected group. The increased catabolic utilization of proteins to meet energy requirements has also been observed in *Biomphalaria glabrata* snails infected with the *Echinostoma paraensei* trematode (Tunholi *et al*., 2011), which aligns with our current findings.

It should also be noted that several additional enzymes associated with protein digestion were identified as DEGs, e.g. chymotrypsin-like serine proteinases (TRINITY_DN1270_c0_g1, logFC=5.84 and TRINITY_DN8979_c0_g1, logFc=6.23), chymotrypsin-like elastase family member 2B (TRINITY_DN5105_c0_g1, logFC=6.91) and procathepsin (TRINITY_DN267_c0_g1, logFC = 2.45) The role of cathepsin in resistance of *B. glabrata* snails to *Schistosoma mansoni* trematodes has been previously documented (Myers *et al*., 2008). Similarly, in the study we noted an increase in the cathepsin expression levels in infected individuals, which

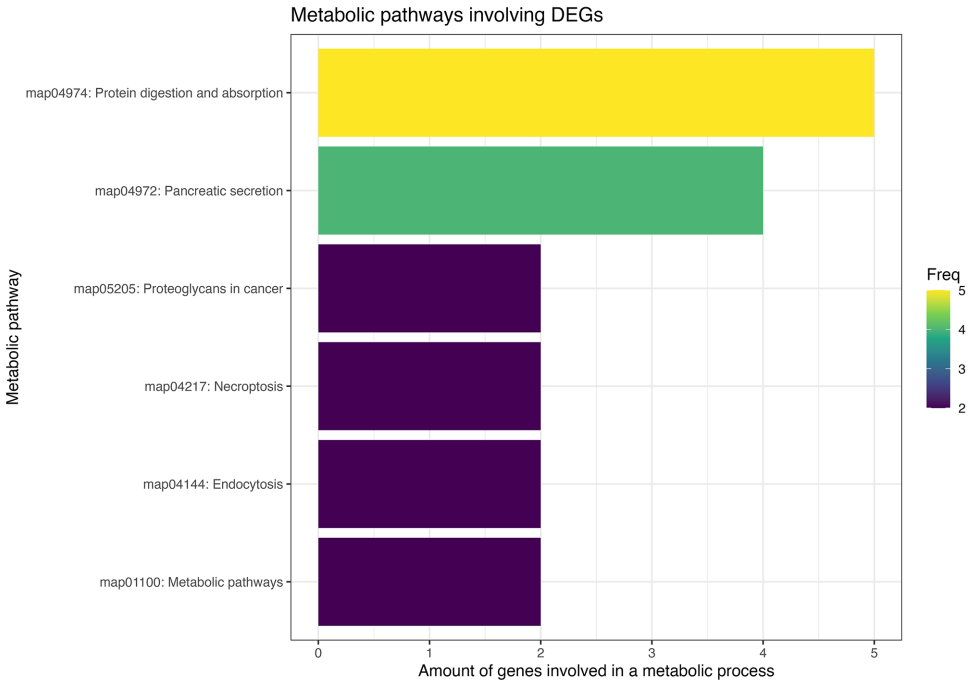

Fig. 3. KEGG metabolic pathways associated with DEGs.
Рис. 3. Метаболические пути KEGG, ассоциированные с дифференциально экспрессирующимися генами.

may be associated with the molluscs' defensive mechanisms against trematodes.

Another significant category of identified MPs is 'Pancreatic secretion' (Fig. 3, Supplement 3), which, besides CPA1s, includes an enzyme annotated as pancreatic triacylglycerol lipase (PTGL, logFC = 2.7). This enzyme is responsible for the hydrolysis of triacylglycerols which are stored by *Littorina* snails in both the hepatopancreas and the pedal muscle (Arakelova, 2008). The previous study noted a reduction in the neutral lipid content in trematode-infected *L. saxatilis* (Arakelova *et al*., 2004). The up-regulation of the PTGL-homologue shown in our study may indicate both intensification of lipolysis and a corresponding increase in the overall parasite-induced utilization of energy reserve in host tissues.

Targeted search for immune effectors/ regulator genes

The immune system of multicellular organisms encompasses interactions with other living entities, maintaining continuous metabolic contact. Gastropods possess an innate immune system based on pattern recognition (rev. in Loker, 2010; Al-Khalaifah, 2022). The primary classes of the pattern recognizing receptors in invertebrates include toll-like receptors (TLR), peptidoglycan recognition proteins (PGRP) and Gram-negative bacteria-binding proteins (GNBP). In our present study, we identified 18, 2 and 2 homologues of TLRs, PGRPs and GNBPs, respectively, in the foot and head transcriptome of *L. saxatilis* (Supplement 7). However, analysis using both DESeq2 and the Mann–Whitney U-test revealed no significant changes in expression of these genes following trematode infection (Supplement 4).

Key components of innate immunity in many invertebrates are enzymes involved in producing active oxygen and nitrogen species (ROS and RNS, respectively), specifically the DuOX (dual oxidase) and NOS (NO-synthase). These enzymes are involved in the gut immunity of *Drosophila* (rev. in Kim, Lee, 2014) and the antiplasmodial response of the *Anopheles* mosquito (rev. in Kakani *et al*., 2016). Both DuOX and NOS activity have been reported in molluscs (Castillo *et al*., 2015; Donaghy *et al*.,

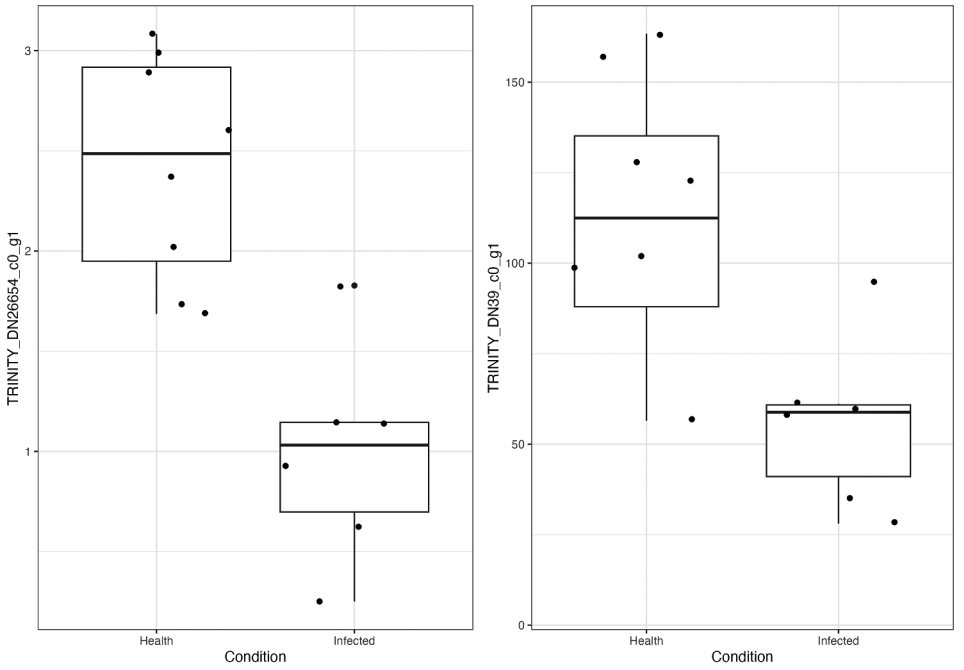

Fig. 4. Box plots demonstrating significant differences in expression levels of NO-synthase (left, Mann–Whitney U test p-value 0.001) and dual oxidase genes (right, Mann–Whitney U test p-value < 0.05). Legend: "health" — healthy molluscs; "infected" — molluscs infected with trematodes.
Рис. 4. Значимые различия в уровнях экспрессии генов NO-синтазы (слева, тест Манна–Уитни, p-value 0.001) и двойной оксидазы (справа, тест Манна–Уитни, p-value < 0.05). Обозначения: "health" — моллюски, не заражённые трематодами; "infected" — моллюски, заражённые трематодами.

2015), including gastropods (rev. in Sokolova, 2009; Al-Khalaifah, 2022). However, their specific roles in defensive response are still poorly understood. In our study, we identified the DuOX and NOS homologues in the *L. saxatilis* somatic transcriptome (three and one homologues were found, respectively; Supplement 7) and quantified their expression in infected and uninfected snails. Notably, one of the three LsDuOX (TRINITY_DN39_c0_g1) and LsNOS exhibited significantly lower expression levels in infected snails compared to uninfected ones, as determined by the Mann–Whitney U-test (logFC = –0.4 and –0.16, respectively, Fig. 4). However, their fold changes were insufficient to surpass the DESeq threshold for differential expression detection, resulting in their exclusion from the DEGs list. These findings suggest a potential role for ROS and RNS in the antiparasitic response; their reduced expression in the infected host may be a consequence of the parasite inhibition, or alternatively, snails with inherently lower ROS and RNS production may be more sensitive to parasitic invasion. Further research is needed to clarify these mechanisms.

Conclusion

In this study, we compared the transcriptomes of the trematode-infected vs healthy *Littorina saxatilis* snails, revealing changes in expression of genes associated with energy metabolism, digestive and vascular system remodeling, and immune suppression in infected specimens. The findings, when integrated with metabolomic data (Repkin *et al*., 2024) suggest that infected hosts may shift to alternative energy sources, particularly proteins, in response to carbohydrate allocation towards the parasite. Given the high population densities of *L. saxatilis* in the intertidal zone and the prevalence of *Microphallus* trematode infection, these processes are likely to have

broader implications for nutrient cycling within the community. Thus, the *Littorina–Microphallus* parasitic relationship serves as an informative model for ecological parasitology. Additionally, the observed changes in morphogenesis and organ/tissue restructuring may be associated with phenomena such as parasitic castration, replacement of the hepatopancreas by the parasite infrapopulation, shell deformation and other trematode-induced morphological modifications of the host. We also identified signs of immunodeficiency related to the lowered expression of genes responsible for the production of ROS and RNS. This immunological alteration may be an important factor in facilitating the successful development of *Microphallus* trematodes in the *Littorina* snails, highlighting the need for further investigation into the roles of ROS and RNS in mollusc–trematode interactions.

Electronic Supplements.

The following materials are available online.

Supplement 1. Sequencing results.

Supplement 2. Annotation of differentially expressed genes (DEGs) in comparison of trematode-infected and uninfected molluscs.

Supplement 3. Differentially expressed genes associated with particular metabolic pathways.

Supplement 4. Genes associated with immunity response.

Supplement 5. Transcriptome assembly statistics.

Supplement 6. PCA sensitivity analysis.

Supplement 7. Sequence identifiers (NCBI) of immune gene homologues found in this research.

Compliance with ethical standards

Conflict of interests

The authors declare that they have no competing interests.

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