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Original Article

Gonadal transcriptomic analysis of the mud crab *Scylla olivacea* infected with rhizocephalan parasite *Sacculina beauforti*

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ABSTRACT

Infection by the rhizocephalan parasite *Sacculina beauforti* can have detrimental effects on mud crab *Scylla olivacea*. However, the molecular changes that occur during rhizocephalan infection are poorly understood. Due to the disruption in the reproductive system after infection, the gonadal transcriptomic profiles of non-infected and infected *Scylla olivacea* were compared. A total of 686 and 843 unigenes were differentially expressed between non-infected and infected males, and females, respectively. The number of DEGs increased after infection. By comparing shared DEGs of non-infected and infected individuals, potential immune- and reproduction-related of host, and immune- and metabolism-related genes of parasite are highlighted. The only shared KEGG pathway between non-infected and infected individuals was the ribosome pathway. In summary, findings in this study provide new insights into the host-parasite relationship of rhizocephalan parasites and their crustacean hosts.

1. Introduction

Rhizocephalans (Crustacea: Maxillopoda: Cirripedia) are parasitic barnacles whose hosts are mainly decapods [23]. Unlike other crustaceans, rhizocephalans are morphologically modified, lacking segmentation and appendages as adults. As with barnacles, rhizocephalans exhibit free-living nauplius and cyprid larva stages. After infection, the female parasite consists of a root-like structure called the interna, which spreads within the body of the host to absorb nutrients, and the externa, an external reproductive structure [24,74]. The externa is often visible under the abdomen of the host and connects with the interna via a narrow stalk [23]. Rhizocephalans, especially of the family Sacculinidae, are known to induce morphological, physiological and behavioural changes on their hosts [23]. Some of these changes include feminization [32,71], anecdysis [21,45], gonadal atrophy and castration [57,69]. Also, hosts – including males – would exhibit typical behaviours only found in berried females such as tending to the externa as if it were their own eggs [64]. Sacculinids have been reported to infect various crab species worldwide, some of which are commercially important species, including *Portunus pelagicus* [77], *Portunus sanguinolentus* and *Charybdis feriatus* [13].

Host immune response towards the invading rhizocephalan parasites is generally marked by the melanization of invading rootlets [33] and is often observed during two phases of infection, initial invasion stage (prior to the solid establishment of interna) and post-externa stage (injury to or loss of the externa) [62]. Melanization is a typical immune response of arthropod, including crustaceans where foreign bodies are encapsulated by melanin [6,37]. Some species of rhizocephalans such as *Sacculina carcini*, however, manage to bypass the host immune detection and allowing itself to be recognized as a part of the host instead of as foreign bodies even during initial invasion stage. This is evident by the absence of melanized bodies in the thoracic ganglia of its usual host the European green crab *Carcinus maenas,* despite numerous exposures [33]. The molecular mechanism regulating the cellular defense response of host, however, is still unclear.

Mud crab *Scylla* spp. are economically important in the Indo-west Pacific region, with high market values [15,72]. Only two species of sacculinids are known to infect *Scylla* spp., *Loxothylacus ihlei* in Australia and *Sacculina beauforti* in Malaysia [8,29]. After the revision of genus *Scylla* into four morphologically and genetically distinct species, i.e. *Scylla olivacea*, *S. tranquebarica*, *S. paramamosain* and *S. serrata* [28], Waiho et al. [71] recently reported the occurrence of *Sacculina beauforti*

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infecting *Scylla olivacea* population in Sabah, Malaysia. Some of the noticeable adverse effects on the external morphology of the infected crabs include reduced gonopods (in males) or pleopods (in females), dented abdomens, female-like abdomen in males and higher body weight relative to carapace width [16,71].

With the advent of technology, next generation sequencing is now widely available and has become a valuable tool for studying biological changes at a molecular level, especially in non-model organisms where full genomes are unavailable for comparison [73,82]. To date, very little is known about the molecular mechanism occurring in the host upon sacculinid infection. A more in-depth understanding of this process could aid in the development of prevention methods for sacculinid infection on mud crabs and on other crustacean species. Here, we conducted the first transcriptome analysis to examine the genes that were differentially expressed in the gonads of *Scylla olivacea* after being infected with *Sacculina beauforti*, with a special focus on genes involved in reproduction, immune response and growth.

2. Method

2.1. Specimen collection

Mud crab species identification was based on the taxonomic classification keys provided by Keenan et al. [28] whereas *Sacculina beauforti* infection status of *Scylla olivacea* was based on previous descriptions [16,71]. In short, infected individuals bearing externa of more than 25 mm in diameter from Marudu Bay, Sabah (6°44′N117°1′E) were used in this study. To ensure that *Sacculina beauforti* was absent in non-infected individuals, we obtained *Scylla olivacea* samples from a known population (Kuala Sepetang, Perak, 4°45′N100°37′E) free of rhizocephalan infection [71]. Healthy non-infected individuals with no external morphological changes, no missing appendages and no external parasites were selected. Due to the distance between the two sampling locations, their sampling periods were a week apart (Marudu Bay on 21–23 September 2017; Kuala Sepetang on 28–30 September 2017). Healthy non-infected mature females (NF) (n = 3; mean carapace width, CW = 102.26 ± 2.39 mm), healthy non-infected mature males (NM) (n = 3; mean carapace width, CW = 104.91 ± 1.49 mm), infected males (IM) (n = 3; mean CW = 104.21 ± 1.53 mm; mean externa diameter = 27.27 ± 2.14 mm) and infected females (IF) (n = 3; mean CW = 99.19 ± 2.19 mm; mean externa diameter = 26.61 ± 1.03 mm) were collected live from the wild using standard crab pots (pots were deployed during low tide and retrieved during subsequent high tide). In the field, after selection, specimens were immediately anaesthetized in ice for 5 mins. Approximately 300 mg of gonadal tissues were extracted, stored in 10-fold volume of RNA Keeper Tissue Stabilizer (Nanjing Vazyme Biotech Co., Ltd., China) and maintained on ice to ensure RNA quality. Samples were immediately transported back to the lab and preserved in −80 °C following manufacturer's protocol until RNA extraction. All animal experimental procedures were approved and conducted in strict accordance with the recommendations in the *Guide for the Care and use of Laboratory Animals* outlined by the Institutional Animal Care and Use Ethics Committee of Shantou University, comply with the ARRIVE guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. As interna rootlets of rhizocephalan parasites are known to intricately invade all parts of the crab gonads [7,51], the gonadal transcriptomic results of infected crabs in this study were of *Scylla olivacea* and *Sacculina beauforti*.

2.2. Total RNA extraction, library construction and sequencing

Total RNAs of each tissue sample was extracted using TRIzol reagent (Invitrogen) following manufacturer's protocol. The quantity and quality of the extracted RNAs were checked using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA), respectively. RNA libraries were constructed using TruSeq RNA sample preparation kit (Illumina, USA) according to the manufacturer's instructions. In brief, Poly(A) mRNA was isolated and purified from the total RNA with the aid of magnetic oligo-dT beads after denaturation. The isolated mRNAs were then fragmented into short fragments of about 200 bp and reverse-transcribed into cDNAs (first-strand) using reverse transcriptase and random primers. Subsequently, double-stranded cDNA was synthesized using DNA polymerase I and RNase H. This served as the template for the construction of paired-end libraries using Genomic Sample Prep Kit (Illumina, USA). QIAquick PCR extraction kit (Qiagen, USA) was used for the purification of cDNA fragments prior to adaptor ligation. After adaptor ligation, unsuitable fragments and adaptors were filtered out using AMPure XP beads (Beckman Coulter, China). Suitable fragments were amplified using polymerase chain reaction (PCR) for final cDNA library preparation. Lastly, the cDNA library was sequenced using the Illumina HiSeq X Ten SBS instrument by Genergy Biotechnology Co. Ltd. (Shanghai, China).

Table 1
Functional annotation of assembled unigenes.

Public database	Number of unigenes	Percentage (%)
Swiss-Prot	35,266	7.1
Nt	21,247	4.3
Nr	58,776	11.8
KOG	19,470	3.9
KEGG	8767	1.8
Interpro	28,404	5.7
GO	33,924	6.8
At least 1 database	72,522	14.5
All databases	2355	0.5
Total unigenes	499,084	

2.3. Pre-processing, de novo assembly and functional annotation

Raw reads were subjected to adaptor clipping and trimming to filter out low-quality reads using Trim Galore software version 0.4.1 (Babraham Bioinformatics, Cambridge, UK, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Reads with the mean phred score of ≤30 were filtered out to ensure sequence quality. The read qualities before and after pre-processing were assessed using FastQC v0.11.5. All subsequent analyses were conducted using clean reads. Clean reads were then assembled into contigs using Trinity software v2.3.2 [20] based on *k-mer* value of 25. All libraries were assembled separately. Contigs with less than 200 bases were excluded from further analysis after assembly. Unigenes were formed when contigs could not be extended on either end. To select unigenes, firstly, TransDecoder v.5.0.2 (http://transdecoder.github.io) was used to translate transcripts into proteins. Next, transcript with the longest translated protein is selected as unigene. If all transcripts of the gene cannot be translated, then the longest one was selected as unigene. Benchmarking Universal Single-Copy Orthologs (BUSCO) v2.0 [61] was used to evaluate the completeness of the transcriptome assembly. Functional annotation of the assembled unigenes were conducted using Blastx or Blastn (*E*-value $< 10^{-5}$) against public databases (Annotation date: November 2017) including Nr/Nt (NCBI non-redundant protein/nucleotide sequences, http://www.ncbi.nlm.nih.gov/) (NCBI [48]), Swiss-Prot (a manually annotated and reviewed protein sequence database, http://www.ebi.ac.uk/uniprot) [67], InterPro (a protein sequence analysis and classification database, https://www.ebi.ac.uk/interpro/) [43], KOG/COG (Clusters of Orthologous Groups of proteins, https://www.ncbi.nlm.nih.gov/COG/) [65], KEGG (Kyoto Encyclopedia of Genes and Genomes database, http://www.genome.jp/kegg/) [26] and GO (Gene Ontology, http://www.geneontology.org/)

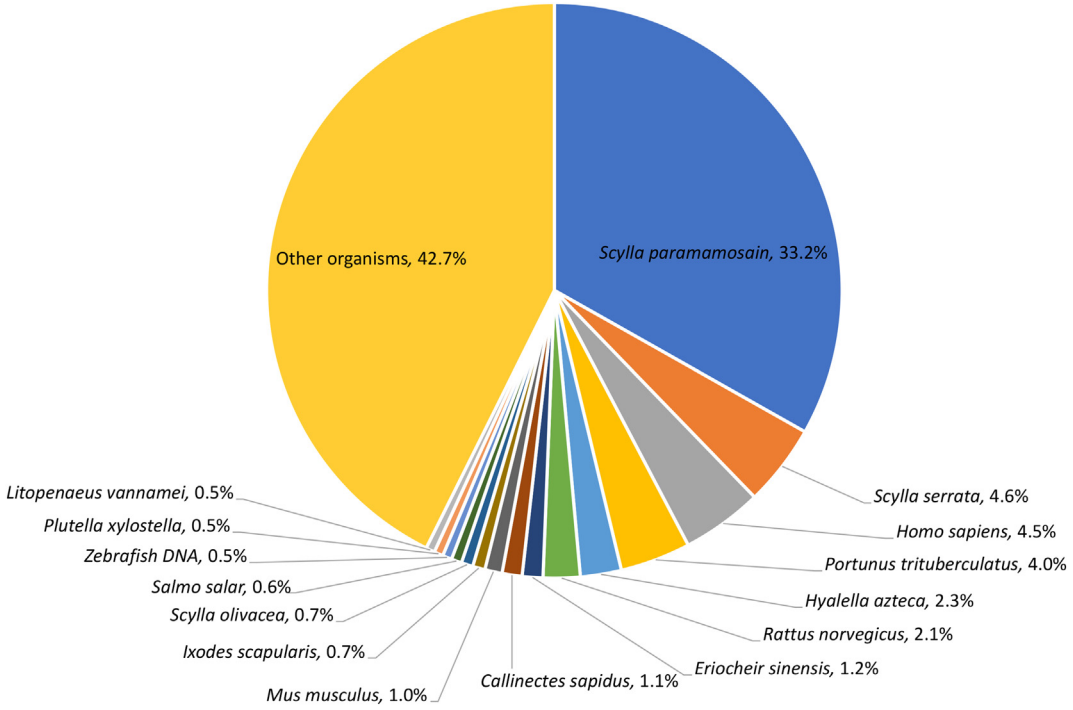

Fig. 1. Species distribution of the top 15 species with the highest number of unigenes matches against Nr database.

[3,66]. Further classification of unigenes was mainly based on GO terms analyzed using Blast2GO software [12,18] based on default parameters (*E*-value < 1^{-6}, annotation cut-off > 55 and a GO weight > 5).

2.4. Differential expression analysis

The abundance of each transcript was determined by mapping clean reads onto the assembled transcriptome using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) [35]. Abundance calculation for each library was done using RSEM (RNA-Seq by Expectation-Maximization, https://github.com/deweylab/RSEM) [36]. Differentially expressed genes (DEGs) among treatments were calculated based on unnormalized counts using *DESeq2* [40]. Fragments Per Kilobase of exon model per Million mapped reads (FPKM) was used to normalize gene expression. The BH procedure was applied to account for false discovery by producing adjusted *P* (P_{adj}) values [5]. Since FPKM values might be 0, all FPKM values were added with 1 when calculating fold change, i.e. $\log_2$(FPKM + 1). DEGs were identified when the P_{adj} < 0.05 and the absolute value of $\log_2$ fold change (log2FC) > 1. Unigenes were considered as specifically expressed if one of the treatment groups exhibit zero expression in all three samples and the difference observed between treatment groups were significant; they were regarded as differentially expressed if the expression of two treatment groups was statistically significant. Multidimensional scaling (MDS) analysis was performed on normalized and log2 transformed gene expression data sets using edgeR [56]. Replicates of each treatment were grouped together (Supplementary Fig. S1). DEGs were subsequently subjected to function and signaling pathway enrichment analysis using GO and KEGG database. The enriched pathways were analyzed using the method of hypergeometric test and considered significant when P_{adj} < 0.05 and ≥ 5 affiliated genes were included. Venn diagram between comparison groups was constructed using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) [49]. To understand the changes caused by *Sacculina beauforti* infection on the reproductive organs of its host, we compared the DEGs of 'NM versus IM' and 'NF versus IF' groups. Based on the shared DEGs of these two groups, immune-related genes that might be of interest were manually selected.

2.5. Quantitative real-time PCR (qPCR) validation

Total RNA from gonadal tissues of infected and non-infected specimens were extracted as described above. RNA quality and quantity were checked on a 1% agarose gel and Nanodrop 2000 spectrophotometer. cDNA was synthesized from 1 μg DNase-treated RNA using GoScript Reverse Transcription System (Promega, USA). qPCR was conducted using Talent qPCR Premix (SYBR Green) kit (Tiangen Biotech, China) according to manufacturer's protocol. Six DEGs were selected for the validation of RNA-Seq results, namely glutathione S-transferase 1 (*GST1*), C-type allatostatin, ovary development-related protein (*ODRP*), crustacean hyperglycemic hormones (*CHH*), crustin 4 (*PJC4*) and estrogen sulfotransferase (*SULT1E1*). 18S rRNA served as the reference gene in which the expression levels of selected genes were normalized against. Comparative Ct ($2^{-\Delta\Delta Ct}$) value method was used to calculate gene expression levels [38]. Primer premier 6.0 software was used for primer design (Supplementary Table S1). Correlation between qPCR and RNA-seq results were tested using Pearson correlation coefficient conducted in IBM SPSS Statistics version 25. Statistical significance level was set at *P* < .05.

3. Results

3.1. Transcriptome sequencing and read assembly

In total, 77 billion raw bases and 514 million raw reads were generated from 12 cDNA libraries (i.e. IF, IM, NF, NM, each with three biological replicates). After removing adapter, low-quality sequences and sequences of < 200 bp, all libraries retained more than 97% of the raw reads (now termed as clean reads) (Supplementary Table S2). Clean reads were then assembled into 585,459 contigs with an average length of 624.91 bp (range = 224–18,152 bp). Subsequently, 499,084 unigenes were detected, with a total length of 261,367,256 bp, an average length of 523.69 bp and a N50 assembled length of 550 bp. The length distribution of all transcripts and unigenes is depicted in Supplementary Fig. S2. More than half (67.2% of the transcripts and 72.7% of the unigenes) of the assembled transcripts/unigenes were in the range of

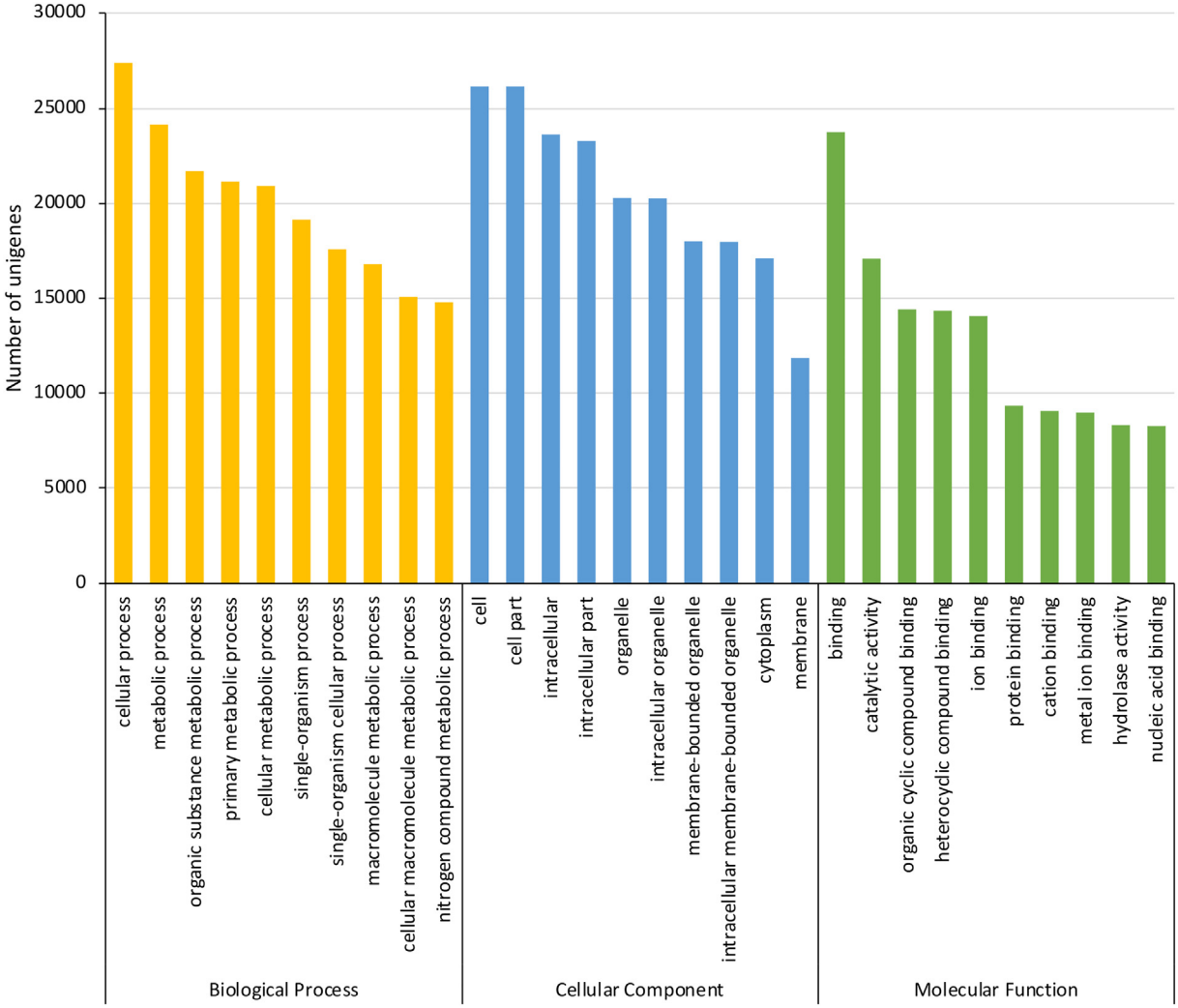

Fig. 2. Top 10 Gene Ontology (GO) classification of assembled unigenes of *Scylla olivacea* for biological process, cellular component and molecular function.

200–500 bp. Approximately 97.6% and 97.1% of complete BUSCO genes were matched when compared to the Metazoan database and Arthropoda database, respectively. Based on these two databases, only 0.6% (Metazoan database) and 0.4% (Arthropoda database) are missing. The remaining percentages represent partial BUSCO gene match.

3.2. Functional annotation of unigenes

Out of the 499,084 unigenes, 72,522 unigenes (14.5%) were annotated in at least one of the databases whereas only 2355 unigenes (0.5%) were annotated in all selected databases. Detailed functional annotation of assembled unigenes is shown in Table 1. According to the annotation based on Nr database, the assembled unigenes matched with the known sequences of 1266 species. Among the top 15 species with the highest number of unigenes matches, more than half (53.3%) were crustaceans, of which 6 were brachyuran crabs, including three *Scylla* species (Fig. 1). The top five species with the greatest number of hits were *S. paramamosain* (33.2%), *S. serrata* (4.6%), *Homo sapiens* (4.5%), *Portunus trituberculatus* (4.0%) and *Hyalella azteca* (2.3%).

A total of 33,924 unigenes were GO-categorized into one of the three GO domains, i.e. biological process (90.3% of unigenes in 11,076 GO terms), cellular component (88.0% of unigenes in 1314 GO terms) and molecular function (87.3% of unigenes in 3438 GO terms) (Fig. 2). Each unigene could be categorized into more than one GO term. Out of the 11,076 GO terms in the biological process, the top three GO terms were “cellular process”, “metabolic process” and “organic substance metabolic process”. “Cell”, “cell part” and “intracellular” were the three highest GO terms in the cellular component domain. In the molecular function domain, most of the unigenes were involved in “binding”, “catalytic activity” and “organic cyclic compound binding”.

Based on the KEGG analysis, most unigenes (n = 3607) were assigned to “metabolism” pathway class, followed by “signal transduction” (n = 2566) and “infectious disease bacterial” (n = 1396) (Fig. 3). Additionally, some unigenes were mapped to several growth and reproduction-related pathways, including MAPK signaling pathway (n = 306), PI3K-Akt signaling pathway (n = 301) and Wnt signaling pathway (n = 225).

3.3. Validation of RNA-Seq data using qPCR

Six genes were selected to validate the results of RNA-Seq. Based on Fig. 4, the results of RNA-Seq and qPCR were largely consistent between each other. Similar FPKM and relative expression patterns were observed for genes such as *GST1, C-type allostatin, ODRP* and *CHH*. Two genes, *PJC4* and *SULT1E1*, exhibited opposite patterns of expression between RNA-seq and qPCR results in the relationship of ‘NM versus IM’ and ‘NF versus IF’, respectively. Significant correlations were observed between qPCR and RNA-seq results for *C-type allostatin* (P < .001), *ODRP* (P = .004), *CHH* (P = .02) and *PJC4* (P = .047).

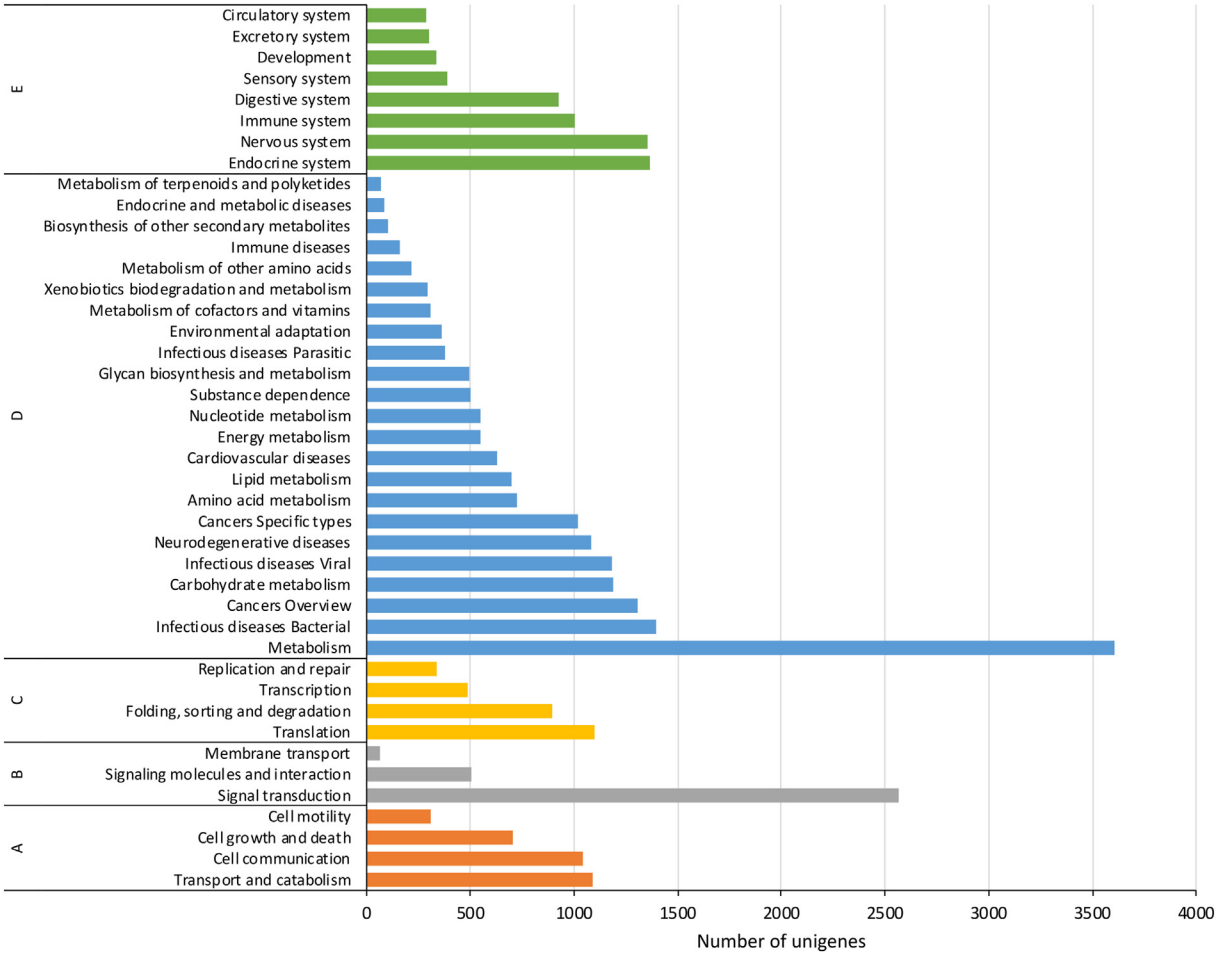

Fig. 3. Functional annotation of *Scylla olivacea* transcriptome assembly by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. All pathway classes were grouped into five main categories, namely cellular processing (A), environmental information processing (B), genetic information processing (C), metabolism (D) and organismal system (E).

3.4. Differentially expressed genes

A total of 686 and 843 DEGs were differentially expressed between 'NM versus IM' and 'NF versus IF', respectively, of which about half (393 and 436 DEGs) were annotated with gene names (Supplementary Table S3). The numbers of DEGs between sexes regardless of infection status – 'NM versus NF' and 'IM versus IF' – were more than three-fold compared to the number of DEGs between 'NM versus IM' and 'NF versus IF'. Of the 2794 DEGs in 'NM versus NF', only 572 were annotated whereas 927 DEGs were annotated among the 4996 DEGs of 'IM versus IF'. The number of DEGs that were shared between groups are illustrated in Fig. 5. Only 2 DEGs (0.02%) were shared among all four compared groups and only 4 (0.04%) DEGs were shared among compared groups with infected crabs – 'NM versus IM', 'NF versus IF', 'IM versus IF'. Interestingly, when comparisons were made between non-infected and infected crabs ('NM versus IM' and 'NF versus IF'), *Sacculina beauforti*'s infection resulted in an increase in up-regulated genes and down-regulated genes (Fig. 6).

3.5. Immune-related genes differentially expressed after infection

All 239 shared unigenes between 'NM versus IM' and 'NF versus IF' groups showed significant up-regulation in infected individuals, except for 2 and 3 unigenes in 'NF versus IF' and 'NM versus IM', respectively (Supplementary Table S4). Among these shared unigenes, some known host immune and reproduction-related genes were found. These included genes such as *crustin Pm5, 60 kDa heat shock protein (HSP), stress-70 protein* (*HSPA9*), *HSP90, cold shock protein 1, heat shock cognate 71 kDa protein, activator of 90 kDA HSP, urokinase-type plasminogen activator, serine protease 41, apolipophorins, innexin inx1, innexin inx2, lysozyme* and *97 kDA HSP*. Some shared immune-related genes such as *serine protease inhibitor precursor, fructose-biphosphate aldolase, phosphoglycerate mutase 2, enolase, acyl-CoA-binding protein, phosphoenolpyruvate carboxykinase, myophilin, calreticulin, cathepsin L* and *cathepsin F* are postulated to be of parasitic origin (*Sacculina beauforti*) and play key roles in facilitating parasite's metabolism and immune response.

Two reproduction-related genes, i.e. *ODRP* and *trypsin-1* were significantly down-regulated in females (log2FC = −3.34) and males (log2FC = −6.14), respectively.

3.6. Ribosome pathway

The sole significant KEGG pathway in 'NF versus IF' – ribosome pathway – was found to be significant as well in 'NM versus IM' (Supplementary Table S5). All 36 DEGs in the ribosome pathway of 'NF versus IF' were present in that of 'NM versus IM' (total DEGs = 47). Most predicted genes in this pathway were specifically- (only expressed in one group) or differentially-up-regulated in infected individuals, except for *40S ribosomal protein S5* and *40S ribosomal protein S19* (Table 2). The potential involvement of ribosome pathway following *Sacculina beauforti*'s infection was also supported by the strong association of ribosome-related GO terms in both 'NF versus IF' and 'NM versus IM' groups, such as structural constituent of ribosome

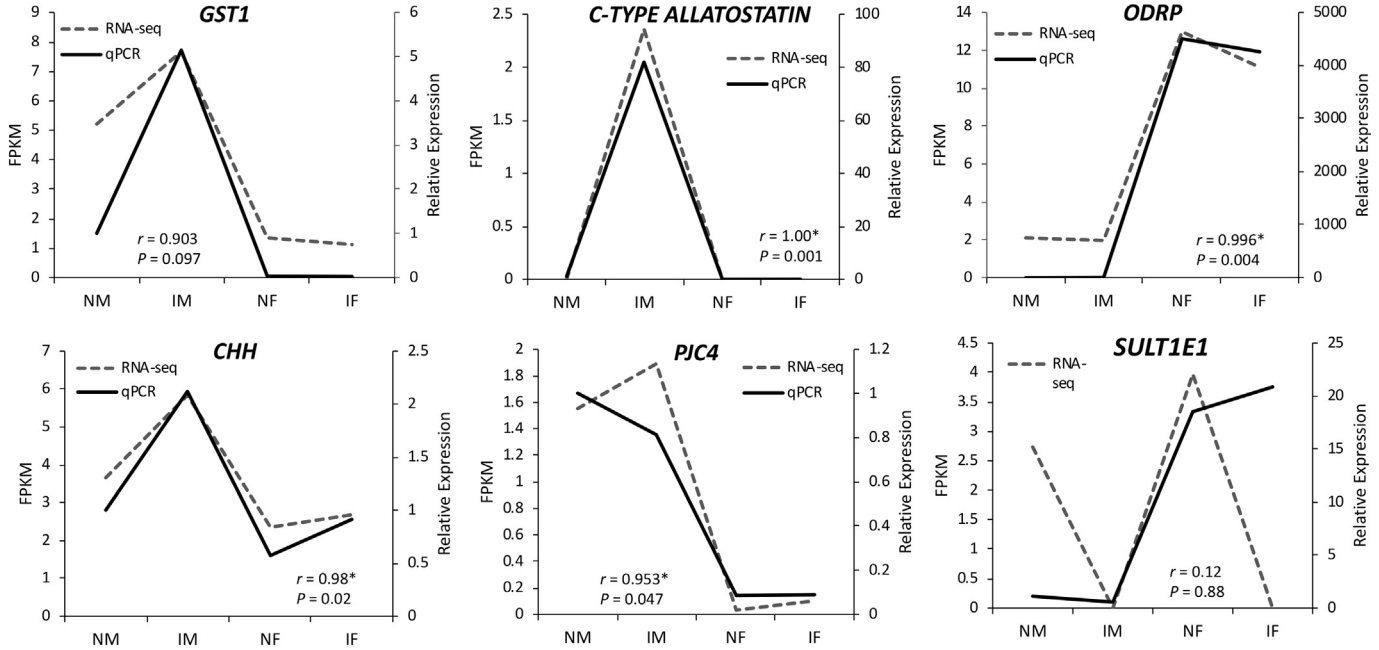

Fig. 4. Validation of RNA-seq and qPCR data in infected males (IM), infected females (IF), non-infected mature males (NM) and non-infected mature females (NF) using selected genes, i.e. sex determination protein fruitless (*FRU*), glutathione S-transferase 1 (*GST1*), C-type allatostatin, ovary development-related protein (*ODRP*), crustacean hyperglycemic hormones (*CHH*), heat shock factor protein 4 (*HSF4*), vitellogenin (*VIT*), crustin 4 (*PJC4*) and estrogen sulfotransferase (*SULT1E1*).

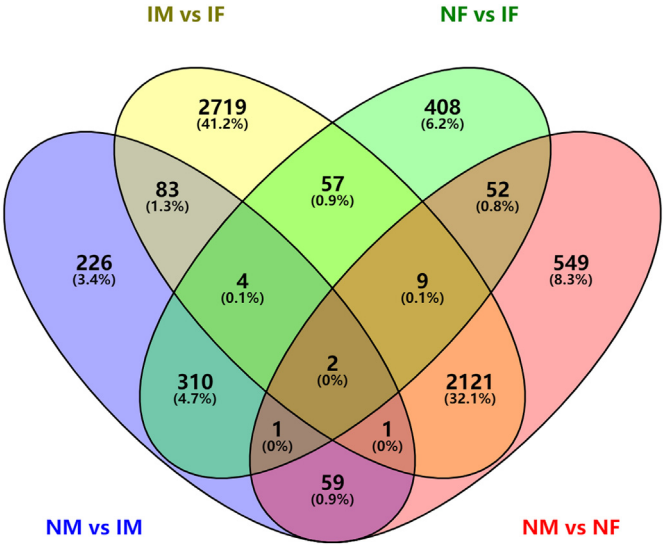

Fig. 5. Venn diagram of differentially expressed genes (DEGs) for all four comparison groups, i.e. 'non-infected mature males (NM) versus (vs) non-infected mature females (NF)', 'NM vs infected males (IM)', 'NF vs infected females (IF)', and 'IM vs IF'.

(GO:0003735), ribosome (GO:0005840), ribonucleoprotein complex (GO:0030529) and ribosomal subunit (GO:0044391) (Fig. 7).

4. Discussion

Sacculina beauforti can cause serious changes in its host, including sterilization and alterations of the host's primary and secondary sexual characters [71]. Therefore, transcriptomic characterization and comparative analysis of non-infected and infected individuals (males and females) were conducted to shed some light on the molecular interactions of this unique pathosystem. This study serves as the first transcriptome study conducted on rhizocephalan-infected crustaceans; we examined the molecular changes related to immune function of host upon infection. Although this study is limited by the lack of technical replicate, the results of this study, and the conclusions drawn from them, are still sound as demonstrated by the narrowing down of DEGs among groups, i.e. from the 499,084 unigenes obtained, only approximately 0.31% (1529 unigenes) were differentially expressed between non-infected and infected crabs of both sexes, and of that, only 239 DEGs were shared between sexes. The *DESeq2* tool used to estimate DEGs based on shrinkage estimation for dispersions and fold changes [40] in this study is known to exhibit minimal ($\leq 5\%$) false positive rate (FPR) regardless of fold-change threshold or numbers of biological replicate used [60]. The high quality of this de novo transcriptome assembly is reflected by the recovery of high percentage ($> 97\%$) of complete BUSCO genes and comparable to that of other crustaceans analyzed using the arthropod lineage, e.g. that of Caribbean spiny lobster *Panulirus argus* was 91.7%, the clawed lobster *Homarus americanus* was 91.5%, the red swamp crayfish *Procambarus clarkii* was 92.8% and the blue crab *Callinectes sapidus* was 96.2% [31].

4.1. Down-regulation of genes after infection

Total number of DEGs with down-regulated expression between infected and non-infected mature individuals increased after *Sacculina beauforti* infection. This might indicate that gonadal inhibition and promotion of feminization caused by *Sacculina beauforti* infection could be largely regulated by gene silencing.

4.2. Differentially expressed genes involved in immune response and reproduction of host

Rootlets of rhizocephalans are known to infiltrate host organs [7,51]. Thus, it would be appropriate to regard the gonadal samples of infected individuals as tissues of mixed origins (the gonads of *Scylla olivacea* and rootlets of *Sacculina beauforti*) and the resulting DEGs might be that of either host or parasite, or both. The availability of whole genome of both host and parasite in near future would be useful to facilitate the identification of the origin of expressed genes in mixed

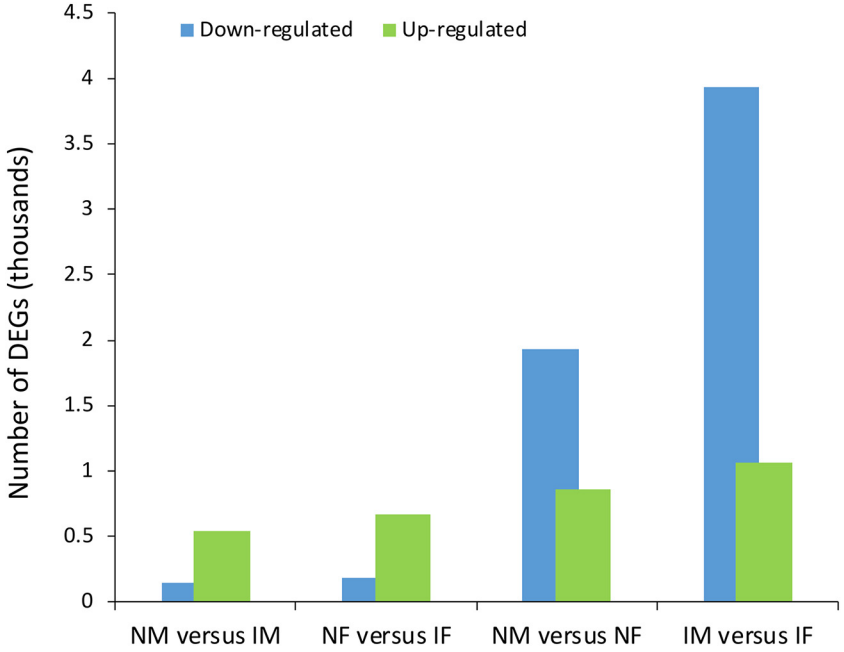

Fig. 6. Abundance of differentially expressed genes (DEGs) between infected and non-infected mature individuals.

samples.

Crustin is a known antimicrobial peptide that plays an essential role in immunological defense against various pathogens (e.g. bacteria, viruses and fungi), especially in organisms lacking an adaptive immune system such as crustaceans [63,81]. Crustin of mud crabs *S. paramamosain* [75] and *S. tranquebarica* [1] showed strong antimicrobial activity. However, nothing is known about the involvement of crustin in the defense against sacculinid infection. In our study, *crustin pm5* was highly up-regulated in infected individuals. Future research on crustin and its relationship with rhizocephalan infection might reinforce the role of crustin in crab's innate immunity response.

Among the shared DEGs between 'NM vs IM' and 'NF vs IF' groups were heat shock proteins (HSP), an important protein family that are involved protein homeostasis and stress protection [52,80]. For example, stress70 protein (also known as HSP70) regulates fundamental cellular processes, including signal transduction and innate immunity [34] and it is induced and used by many RNA viruses for replication purposes [9,34,47]. Similar to the function of HSP70, HSP90 is a chaperone protein that aids in protein folding and stabilization during stress. It is involved in various cellular processes such as development and immune response towards external stress factors such as hyperthermia, radiation, heavy metals and infection [59]. It was reported that western painted turtle *Chrysemys picta bellii* infected with *Citrobacter* sp. showed tissue-specific up-regulation of both HSP70 and HSP90 [54]. In our study, various HSP including *Stress 70 protein* and *HSP90* were up-regulated in males and females that were infected with *Sacculina beauforti*. Due to their cellular function especially during stress, the up-regulation of these HSP in infected individuals might be an innate immune response by the host.

Serine proteases catalyze intra- and extracellular hydrolytic reactions and are involved in various biological processes of eukaryotes, including homeostasis, inflammation and immune mechanisms [27]. In arthropods, serine protease cascades regulate immune response by inducing melanization – the production of melanin to envelop microbial invaders or to envelop wounds [11]. The process of melanization is also reported in some crab species infected with rhizocephalan parasites, especially in non-host species [17]. The significantly up-regulated *serine protease 41* gene in both infected males and females found in our study could be the host's internal immune response in attempt to activate the serine protease cascades to fight off the invading rootlets of *Sacculina beauforti*.

Innexins are transmembrane proteins in invertebrates. On top of their intercellular communication role in various physiological processes, innexins are also critical in mediating host immune response [19]. mRNAs of innexins (Sp-inx2) were found to be up-regulated in hepatopancreas tissue, gill and hemocyte of mud crab *Scylla paramamosain* after *Vibrio* (*Vibrio alginolyticus*, *V. parahaemolyticus*) challenge [76]. The similar up-regulation of *innexin inx1* and *inx2* in the gonadal tissues of infected individuals highlights the potential involvement of innexin in *Scylla olivacea*'s immune response towards the *Sacculina beauforti*'s infection.

By comparing DEGs between non-infected and infected individuals, two reproduction-related genes – *ODRP* in females and *trypsin-1* in males – were found to be down-regulated after infection. Trypsin is a pancreatic enzyme crucial for food digestion in animals, including crustaceans [58]. In addition, their production in testis and their role in the spermatogenesis pathway are evident in teleost fish *Anguilla japonica* [44] and nematode *Ascaris suum* [83]. Up-regulation of *trypsin* gene in testis of the Chinese mitten crab *Eriocheir sinensis* was reported by He et al. [22]. Thus, the significant down-regulation of these two genes suggests that by negatively regulating the gene expression of ODRP and trypsin-1 in *Scylla olivacea*, *Sacculina beauforti* could potentially inhibit and reverse the gonadal development of its host.

4.3. Differentially expressed genes involved in immune response and metabolism of parasite

Due to their highly conserved nature, HSP are also present and play important role in the pathogenic mechanism of parasite infection, especially during invasion stage [25]. Heat shock pathway is postulated to be involved in the invasion of parasitic schistosome larvae to its human host. More importantly, HSP70 is implicated as the key switch in the morphological transformation of cercaria into schistosomulum [25]. HSP90 is also proven to be essential for the development and invasion of parasitic protozoa [63]. As the structure and function of HSP70 and HSP90 proteins are highly conserved, the up-regulated *HSP70* and *HSP90* found in our study might be associated with the invasion, formation and growth of *Sacculina beauforti* interna's rootlets in the gonads of *Scylla olivacea*. In addition to host's immune response, serine proteases are also implicated in mediating host invasion by

Table 2
The predicted genes in ribosome pathway and their expression patterns in non-infected females (NF) versus (vs) infected females (IF), and non-infected males (NM) vs infected males (IM).

Gene name	NF vs IF	NM vs IM	$P_{adj\ (NF\ vs\ IF)}$	$P_{adj\ (NM\ vs\ IM)}$
40S ribosomal protein S10	+	+	0.009	0.003
40S ribosomal protein S13	+	+	0.007	0.001
40S ribosomal protein S14a	+	+	0.021	0.004
40S ribosomal protein S15	+	+	0.023	0.003
40S ribosomal protein S18	+	+	0.012	0.007
40S ribosomal protein S19	+*	−*	$6.61E^{-18}$	$3.29E^{-06}$
40S ribosomal protein S23	+	+	0.011	0.001
40S ribosomal protein S25	+	+	0.033	0.009
40S ribosomal protein S26	+	+	0.031	0.002
40S ribosomal protein S3	+	+	0.011	0.001
40S ribosomal protein S4	+	+	0.003	0.003
40S ribosomal protein S5	−*	−	$1.83E^{-6}$	$2.98E^{-07}$
40S ribosomal protein S5a	+*	+	0.007	$4.79E^{-4}$
40S ribosomal protein S8	+	+	0.005	0.001
40S ribosomal protein S9	+	+	0.001	0.001
60S acidic ribosomal protein P0	+	+	$1.64E^{-4}$	$7.47E^{-05}$
60S ribosomal protein L10a	+	+	0.008	0.001
60S ribosomal protein L11	+	+	0.013	0.003
60S ribosomal protein L12	+	+	0.012	0.008
60S ribosomal protein L13	+	+	0.022	0.005
60S ribosomal protein L17	+	+	0.016	0.002
60S ribosomal protein L18	+	+	0.009	0.002
60S ribosomal protein L18a	+	+	0.014	0.023
60S ribosomal protein L19	+	+	0.009	0.003
60S ribosomal protein L21-A	+	+	0.025	0.002
60S ribosomal protein L23	+	+	0.013	0.001
60S ribosomal protein L23a	+	+	0.006	0.001
60S ribosomal protein L24	+	+	0.030	0.030
60S ribosomal protein L31	+*	+	0.039	0.002
60S ribosomal protein L35a	+	+	0.031	0.005
60S ribosomal protein L36	+	+	0.017	0.006
60S ribosomal protein L4-A	+	+	0.010	0.003
60S ribosomal protein L5	+	+	0.006	0.004
60S ribosomal protein L6	+	+	0.014	0.008
60S ribosomal protein L7a	+	+	0.001	$1.33E^{-4}$
60S ribosomal protein L9	+	+	0.002	0.003
40S ribosomal protein S11	n.d.	+	n.d.	0.004
40S ribosomal protein S16	n.d.	+	n.d.	0.005
40S ribosomal protein S20	n.d.	+	n.d.	0.012
40S ribosomal protein S7	n.d.	+*	n.d.	$1.92E^{-06}$
60S acidic ribosomal protein P2	n.d.	+	n.d.	0.007
60S ribosomal protein L13a	n.d.	+	n.d.	0.024
60S ribosomal protein L14-B	n.d.	+	n.d.	0.004
60S ribosomal protein L27	n.d.	+	n.d.	0.026
60S ribosomal protein L3	n.d.	+	n.d.	0.028
60S ribosomal protein L30	n.d.	+	n.d.	0.021
Probable 60S ribosomal protein L37-A	n.d.	+	n.d.	n.d.

Note: +: specifically-up-regulated in infected individuals; +*: differentially-up-regulated in infected individuals; −: specifically-down-regulated in infected individuals; −*: differentially-down-regulated in infected individuals; n.d.: no data.

parasitic nematodes [68] and protozoans [78]. Therefore, *serine protease* up-regulated in our study might be from parasitic (*Sacculina beauforti*) origin as well. Further, serine protease inhibitors – the inactivator of serine proteases – are the key proteins that facilitate invasion and growth of parasite within its host system by counteracting and suppressing host proteases, as evident in parasitic protozoans [10,53] and trematodes [55]. Based on its conserved function in other parasites, we speculate that the up-regulated *serine protease inhibitor precursor* in infected individuals potentially involves in the synthesis of serine protease inhibitor protein by the rootlets of *Sacculina beauforti* to overcome the proteolytic activity of host's serine protease.

Glycolysis and gluconeogenesis supply ATP and synthesis glycoconjugates. These processes are essential for the survival of many parasites [70]. Genes in these two processes, however, have been shown to contribute towards parasite's invasiveness and virulence as well [4,79]. In our study, several glycolysis- and gluconeogenesis-related genes (i.e. *fructose-biphosphate aldolase, phosphoglycerate mutase 2, enolase and phosphoenolpyruvate carboxykinase*) were up-regulated in the gonadal tissues of infected individuals. Thus, it is postulated that the high expression of these genes by the rootlets of *Sacculina beauforti* might serve two functions; (1) facilitate nutrient absorption from host tissues via glycolysis and gluconeogenesis, and (2) involve in parasite general functions, including host cell adhesion, plasminogen binding and invasion.

Cysteine proteases are well characterized in parasitic helminths as the key components in host-parasite interactions, including nutrition metabolism, host tissue invasion and host immune evasion [42]. Among them, cathepsins are the most studied parasite proteases. Cathepsin F in trematode *Clonorchis sinensis* is involved in nutrient acquisition based on their intestinal localization and their ability to hydrolyze host (human) proteins [46]. In addition to nutrient absorption, cathepsin L is postulated to be associated with host invasion as juvenile trematodes *Fasciola hepatica* exhibited reduced ability to penetrate the wall of host's intestine after knockdown of cathepsin L [41]. Based on the high expression profiles of *cathepsin L* and *cathepsin F* in infected individuals, we postulate that the function of cathepsin L and cathepsin F proteins might be conserved across phylum and they contribute to nutrient uptake and host invasion by the rootlets of *Sacculina beauforti*, although more extensive molecular and cell biological studies are needed to validate this claim.

4.4. Ribosome pathway

Ribosomes are considered as the most basic components of living organisms and are responsible for protein synthesis [2]. In eukaryotes, ribosomes are made up of a large 60S subunit with 46 ribosomal proteins and a small 40S subunit with 33 ribosomal proteins [30]. In unicellular parasite *Plasmodium* spp. that are well-adapted to living in two different hosts (an insect vector and a vertebrate intermediate host), oscillations in ribosomal protein gene transcription (based on RNA-seq) and their synthesis are observed across life cycle [14,39]. The significant up-regulation of ribosomal protein genes (60S and 40S) in the ribosome pathway indicates the increase in active ribosomes after infection. Ribosome profiling (Ribo-seq) is recommended to further investigate mRNA regions that are associated with assembled ribosomes and subsequently identify the translated proteins [50].

5. Conclusions

This study describes the first gonadal transcriptome of a crustacean infected with a rhizocephalan and identified various DEGs involved in immune response and reproduction/metabolism of host and parasite after infection. In addition, KEGG pathway analysis showed that genes in ribosome pathway were significantly up-regulated, indicating the increase in active ribosomes after infection. As the first transcriptomic study using rhizocephalan-infected hosts, our results potentially pave ways for understanding the molecular mechanism of rhizocephalan infection on its crustacean host and their host-parasite relationship.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2020.05.007.

Availability of data and materials

The datasets generated during the current study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with the accession number SRP155128 (BioProject Accession No. PRJNA482745).

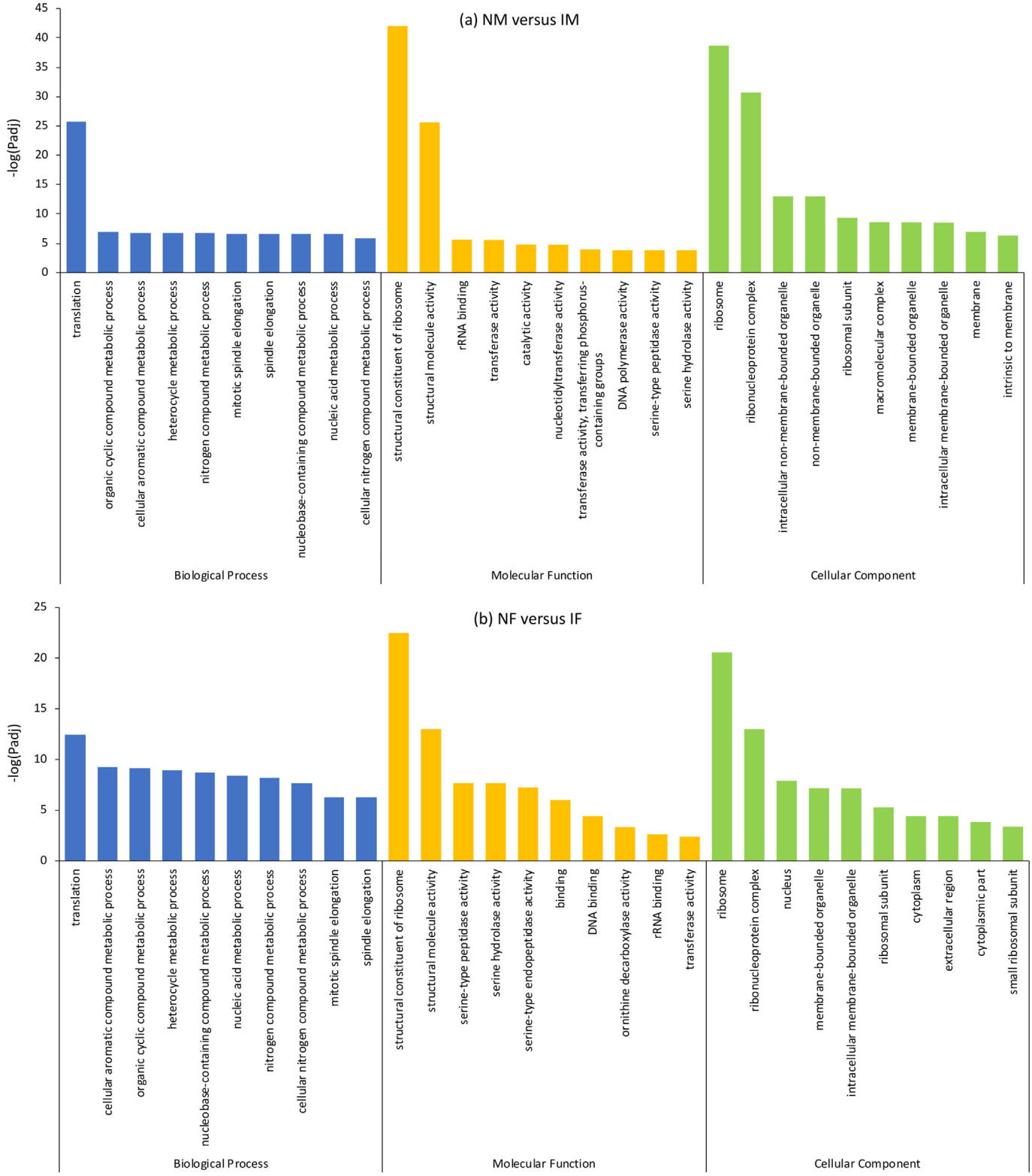

Fig. 7. Top 10 GO terms of each GO category (Biological Process, Molecular Function and Cellular Component) of (a) non-infected males (NM) versus infected males (IM) and (b) non-infected females (NF) versus infected females (IF).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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