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The *Caligus rogercresseyi* miRNome: Discovery and transcriptome profiling during the sea lice ontogeny

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ABSTRACT

Small RNA sequencing in the copepod ectoparasite *Caligus rogercresseyi* was conducted to evidence putative roles of non-coding RNAs during the sea louse ontogeny. Here, differentially expressed miRNAs and mRNAs for each developmental stage were analyzed in parallel with bioinformatic gene target predictions. Based on sequence analysis, *C. rogercresseyi* miRNome comprises 673 conserved miRNAs, including precursors, 5′ and 3′ isomiRs. The conserved miRNAs include 40 families found in twelve different arthropods species. The results also showed that *C. rogercresseyi* miRNome exhibit stage-specific expression patterns, with miRNA-996-4 and miRNA-124 displaying sex-biased expression. Target prediction of these miRNAs identifies possible silencing mechanism of sex-related genes. Furthermore, bantam isomiRs were highly transcribed during the infective stage of copepodid and target prediction using differentially expressed genes in Atlantic salmon infested with sea lice, suggests a putative role of these miRNAs in the host-pathogen interaction. This is the first study reporting a miRNA repertoire in a marine copepod ectoparasite that affects the salmon aquaculture worldwide.

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1. Introduction

Sea lice are naturally occurring parasites for seawater salmon, but, compared to natural conditions, parasite infection and transmission are exacerbated under intensive fish farming. The sea louse *C. rogercresseyi* is the main copepod ectoparasite responsible for significant economic losses in the Chilean salmon farming industry (Kristoffersen et al., 2013). This parasite is known to cause surface damage to fish, which results in mucus breakdown that leads to open sores and lesions Further problems may arise in fish chronically stressed by the presence of sea lice (Bowers et al., 2000; Skilbrei et al., 2013), resulting putatively in immunosuppression and, consequently, to an increased susceptibility to secondary infections (Saksida et al., 2013). Moreover, sea lice infestations have been managed with antiparasitics such as organophosphates (Jones et al., 1992; Roth et al., 1996), pyrethroids (Sevatdal and Horsberg, 2003), hydrogen peroxide (Bravo et al., 2010), and avermectins (Duston and Cusack, 2002; Bravo et al., 2008). However, overexposure to these chemical agents tends to generate drug resistance in wild populations of parasites (ffrench-Constant et al., 2004). These concerns promote the development of novel strategies in sea lice control.

Advances in molecular biology, primarily based on next generation sequencing technologies, have tremendously changed our understanding on how the transcriptome is modulated and how the non-coding information is pivotal for key biological processes. One of these non-coding RNAs, denoted microRNAs (miRNAs), was first reported to regulate development timing in *Caenorhabditis elegans* (Lee et al., 1993). Since then, miRNA sequences have been reported in a wide variety of organisms, from animals to viruses, and these sequences are already publicly available through the miRBase database (Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2014). The main function of miRNAs is regulating gene expression at the post-transcriptional level by adding switch controls to complex cell signaling pathways associated with developmental timing. For instance, miRNAs have been shown to be essential in obtaining optimal expression levels of genes and in regulating the transcripts of target genes involved in the different stages and sites of ontogeny (Kim and Nam, 2006). However, although research has been conducted on miRNAs for twenty years, much is still unknown

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(Ambros, 2004), such as in regards to miRNA biogenesis and the dynamic interactions of miRNAs with target genes. Moreover, one of the major challenges facing future miRNA research will be relating the discovery of new miRNAs to specific biological processes.

The biogenesis of miRNAs in animals is a complex and multi-step process starting in the nucleus, passing through many post-transcriptional modifications, and ending in the cytoplasm. The canonical maturation pathway (Makarova and Kramerov, 2007; Zhang et al., 2007; Chawla and Sokol, 2011), which is similar to protein-coding genes, initiates at transcription (mostly by RNA polymerase II or III), thus generating a primary miRNA (pri-miRNA). The pri-miRNA is characterized by a hairpin RNA structure recognized by the nuclear RNAse III enzyme Drosha and its cofactor DGCR8 in vertebrates or Pasha in invertebrates. These proteins work within a complex of several proteins known as the microprocessor. The microprocessor cleaves the pri-miRNA to generate a shorter hairpin about 70 nt in length called pre-miRNA. Pre-miRNA, an intermediate stage, is exported from the nucleus to the cytoplasm *via* Exportin-5. In the cytoplasm, a second RNase III enzyme, Dicer, makes the pair of cuts that defines the other end of the miRNA, generating a siRNA-like duplex known as the miR/miR* duplex. This duplex is incorporated into the RNA Inducing Silencing Complex (RISC) in which the Argonaute-1 protein is the main component. The miRNA* passenger strand is then degraded, and the miRNA guide strand leads the RISC complex to the target mRNA. Assembly of the mature, single stranded miRNA (~22 nucleotides in length) from the duplex into the RISC completes miRNA biogenesis. Recently, our research group has reported for the first time the biogenesis pathway of miRNAs in the marine copepod ectoparasite *C. rogercresseyi* (Valenzuela-Miranda et al., 2015), evidencing that RNA silencing mechanisms can be found during the life cycle of this sea louse species.

The aim of this study was to conduct a comprehensive miRNome analysis in the sea lice *C. rogercresseyi* in order to explore novel control strategies based on gene silencing. In previous studies, we have shown how specific molecular gene pathways are primary modulated during the *C. rogercresseyi* development (Gallardo-Escarate et al., 2014a; Gallardo-Escarate et al., 2014b; Nunez-Acuna et al., 2014a; Valenzuela-Munoz and Gallardo-Escarate, 2014; Maldonado-Aguayo et al., 2015; Valenzuela-Munoz et al., 2015). However, the knowledge of *C. rogercresseyi* miRNA expression profiles and their target genes remain unknown. To gain an understanding of the role of miRNAs through the *C. rogercresseyi* ontogeny, we have conducted high-throughput small RNA sequencing and profiling of miRNAs at different life cycle stages. Here, we find putative roles of *C. rogercresseyi* miRNAs to evade host immune response and discuss further research in parasite control strategies through gene silencing control as possible avenues for future sea lice research.

2. Methods

2.1. Salmon lice culturing

Female specimens of *C. rogercresseyi* were collected from recently harvested fish at commercial farms located in the south of Chile. Individuals were transported to the laboratory on ice, and their egg strings were removed and placed in culture buckets supplied with seawater flow at 12 °C and with gentle aeration. Eggs were allowed to hatch and develop until the infectious copepodid stage and were used to inoculate a tank containing host fish according to Bravo (2010). Prior to the collection of sea lice, fish were anaesthetized and then were harvested for RNA extraction and cDNA library construction. All laboratory infections and culture procedures were carried out according to the guidelines approved by the Ethics Committee of the University of Concepción.

2.2. Illumina sequencing of small RNAs

The lifecycle of *C. rogercresseyi* is comprised of the following eight developmental stages: free swimming nauplius I–II and copepodid, and host attached chalimus I–IV and adults (Gonzalez and Carvajal, 2003). For sequencing, three biological replicates, each one containing 20 individuals from each developmental stage were separately collected from different infected fish. Then, each pool was fixed in 1 mL of RNAlater Stabilization Solution (Ambion®, USA) and stored at −80 °C. Total RNA was extracted from each pool using the Trizol™ Kit (Ambion®) following the manufacturer's instructions. Quantity, purity, and quality of isolated RNA were measured in the TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K Reagent Kit according to the manufacturer's instructions. Samples with a RIN > 8.0 were used for library preparation with TruSeq Small RNA Kit (Illumina®, San Diego, CA, USA). All biological replicates were sequenced by the MiSeq (Illumina®) platform using sequencing runs of 41 cycles at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Chile.

2.3. Discovery of miRNAs in C. rogercresseyi

Low-quality reads from the Illumina sequencing data, reads with a quality score of <0.05 on the Phred scale, with a short length, or with three or more ambiguous nucleotides were strictly removed using the CLC Genomics Workbench Software (Version 9.1, CLC Bio, Denmark). Furthermore, any cleaned sequences matching metazoan mRNA, rRNA, tRNA, snRNA, snoRNA, repeat sequences, or other ncRNAs deposited in the NCBI databases (http://www.ncbi.nlm.nih.gov/), RFam (http://rfam.janelia.org/), or Repbase (http://www.girinst.org/repbase/) were discarded. Subsequently, the retained nucleotide reads were mapped onto the EST sequences of crabs and shrimp (http://www.ncbi.nlm.nih.gov/ and http://www.marinegenomics.org/) and onto the *Daphnia pulex* genome (http://genome.jgi-psf.org/Dappu1/) based on the public releases. Then, the various mapped and cleaned sequences were counted, and a unique sequence list was generated. These sequences were aligned against pre-miRNA and mature miRNA (5′ and 3′) sequences listed in the miRBase 21 (Kozomara and Griffiths-Jones, 2011; Kozomara and Griffiths-Jones, 2014). Then putative miRNA sequences were checked for fold-back secondary structure predictions using the CLC Genomics Workbench Software with default folding conditions. Finally, to discover putative new miRNAs in the *C. rogercresseyi* transcriptome, the miRanalyzer tool was tested according the default parameters (Hackenberg et al., 2011).

2.4. RNA-seq analysis during the C. rogercresseyi ontogeny

The miRNAs identified were used as references for RNA-seq analyses through different developmental stages in *C. rogercresseyi*. The RNA-seq settings were chosen as follow: a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5. The expression values were set as transcripts per million (TPM), a modification of reads per kilobase of transcript per million mapped reads (*i.e.* RPKM) designed to be consistent across samples. More specifically, transcripts per million values are normalized by total transcript count, instead of read count, and by average read length. These normalizations allow assessments of overregulated transcripts among different groups (Wagner et al., 2012). The distance metric was calculated with the Manhattan method, with subtraction of the mean expression level in 5–6 rounds of *k*-means clustering. Finally, a Kal's statistical analysis test was used to compare gene expression levels in terms of $\log_2$ fold-change (P = 0.0005; false discovery rate [FDR] corrected). In order to identify co-expression patterns among miRNAs differentially expressed and

Table 1
Summary of sequenced *Caligus rogercresseyi* small RNA libraries.

Stage	Number of reads	Avg. length	Number of reads after trim	Avg. length after trim
Nauplius I–II	6,933,126	14.6	4,277,547	24.0
Copepodid	9,030,047	15.4	5,332,866	22.0
Chalimus I–II	8,219,631	12.0	3,585,290	21.6
Female	10,522,633	12.3	3,593,129	21.6
Male	13,475,608	14.6	6,490,497	21.5
Total	48,181,045	13.8	22,279,329	22.1

genes modulated at different developmental stages, Pearson correlation coefficients were estimated and plotted using the Corrplot library in R (https://cran.r-project.org/web/packages/corrplot/index.html).

2.5. Prediction of genes targeted by miRNAs from C. rogercresseyi

To predict the genes targeted by miRNAs, the following three computational target prediction algorithms were used: PITA (Kertesz et al., 2007), miRanda (John et al., 2004), and STarMir (Rennie et al., 2014). The datasets used were the assembled EST sequences reported by Gallardo-Escarate et al. (2014a) for eight developmental stages of *C. rogercresseyi*. STarMir was used to search for miRNA seed matches (nucleotides 2–8 from the 5′ end of miRNA) in the 3'UTR sequences, and miRanda and PITA were used to match entire miRNA sequences. The STarMir parameters was set at a free energy < −20 kcal/mol and a score > 50. The results predicted by the two algorithms were combined, and the overlaps was calculated. Additionally, CLC Genomics Workbench Software was used to integrate read pre-processing, alignment, mature/precursor/novel miRNA detection and quantification, data visualization, and variant detection in the miRNA coding region between the developmental stages of the sea louse.

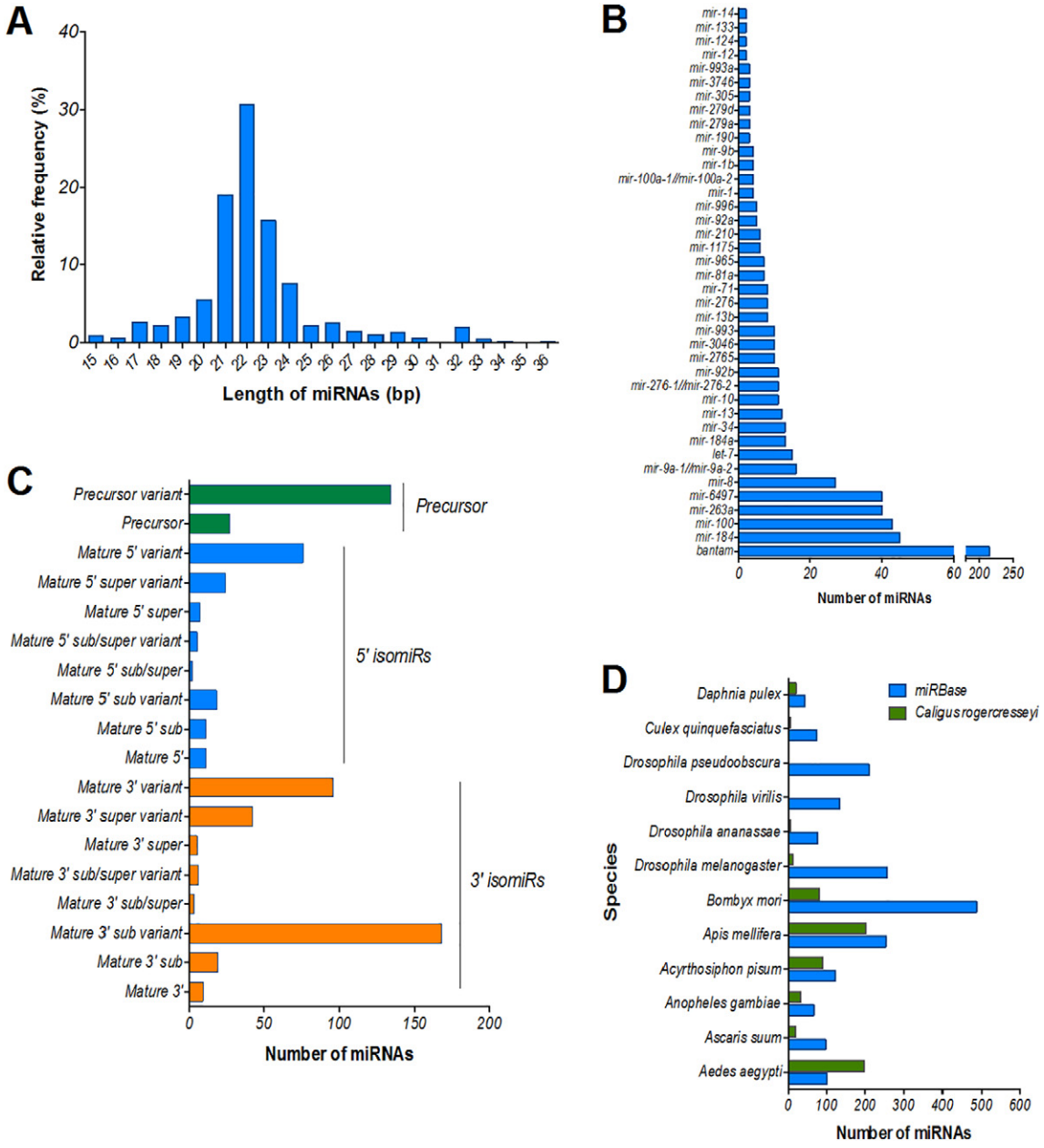

Fig. 1. Identification and annotation of *Caligus rogercresseyi* miRNAs using sRNA-sequencing. A) Size distribution of *C. rogercresseyi* miRNAs after trimming and mapping against miRBase 21 (that is representative of all sea louse stages), B) Family annotation of *C. rogercresseyi* miRNAs, C) Number of isomiRs 5′-3′ and precursor variants identified in the *C. rogercresseyi* transcriptome, and D) Comparison of *C. rogercresseyi* miRNA variants with miRNAs annotated for several arthropod species using miRBase 21.

2.6. Transcription analysis of putative miRNA target genes

To investigate the transcription expression of putative miRNA target genes, two different groups of genes were selected according the bioinformatic target gene prediction previously carried out in this study. The first group was sex-related genes identified with putative miRNA regulation and the second one was immune-related genes with putative binding sites with small RNAs identified in sea lice. Here, adults of sea lice were collected after 14 days post-infestation (dpi) and skin tissue samples from Atlantic salmon (*Salmo salar*) infected with *C. rogercresseyi* were collected at 0, 7 and 14 dpi. The genes involved in sex-related mechanism in *C. rogercresseyi* were *vasa*, *vitellogenin 1* and *vitellogenin 2* and the immune-related genes for infected Atlantic salmon skin tissue were *cluster of differentiation 83* (*CD83*), *interferon gamma* (*IFNg*) and *toll-like receptor 22a* (*TLR22a*) (Table S1). RT-qPCR procedures were conducted according to Valenzuela-Muñoz et al. (Valenzuela-Muñoz et al., 2016) and Farlora et al. (Farlora et al., 2014). Briefly, Total RNA from either sea lice ($n = 20$) or skin tissue ($n = 20$) was isolated using the TRI Reagent® (InvitrogenTM, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity was determined (ratio A260/A280) with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Copenhagen, USA), and the integrity was determined by agarose gel under denaturant conditions. From 200 ng/µl of total RNA, cDNA was synthetized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA). The RT-qPCR runs were performed in triplicates for each sample using the StepOnePlus™ (Applied Biosystems®, Life Technologies, USA). To evaluate the transcriptional level, comparative $2^{-\Delta\Delta Ct}$ method was applied according Livak and Schmittgen (2001) using Elongation factor alpha as reference gene for both species due to its stable value as inferred through the NormFinder algorithm (Table 1S). The other reference genes assayed were beta tubulin and beta actin. Each reaction was conducted with a volume of 10 µL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). The amplification cycle was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, followed by a disassociation curve under these same conditions. The efficiency of each primer was calculated and reported according MIQE guidelines (Bustin et al., 2009). Finally, all data was checked for normality using the Shapiro-Wilk test. Data not meeting this criteria were normalized through BoxCox transformation (Westfall and Henning, 2013). Statistical differences in expression data were evaluated using one way ANOVA (analysis of variance), followed by Tukey's multiple comparison test using the JMP v9 software (SAS Institute Inc., USA). Statistically significant differences were accepted with a $p < 0.05$.

3. Results

3.1. Identification and characterization of miRNAs in C. rogercresseyi

To investigate the relationships between miRNA expression and the morphological changes during the ontogeny of *C. rogercresseyi*, we conducted a deep sequencing analysis of small RNAs libraries from nauplius I–II, copepodid, chalimus I–II, female and male adults. Illumina sequencing yielded 46 millions of small RNA reads, annotating into 663 unique sequences of conserved miRNAs with an average length after trimming

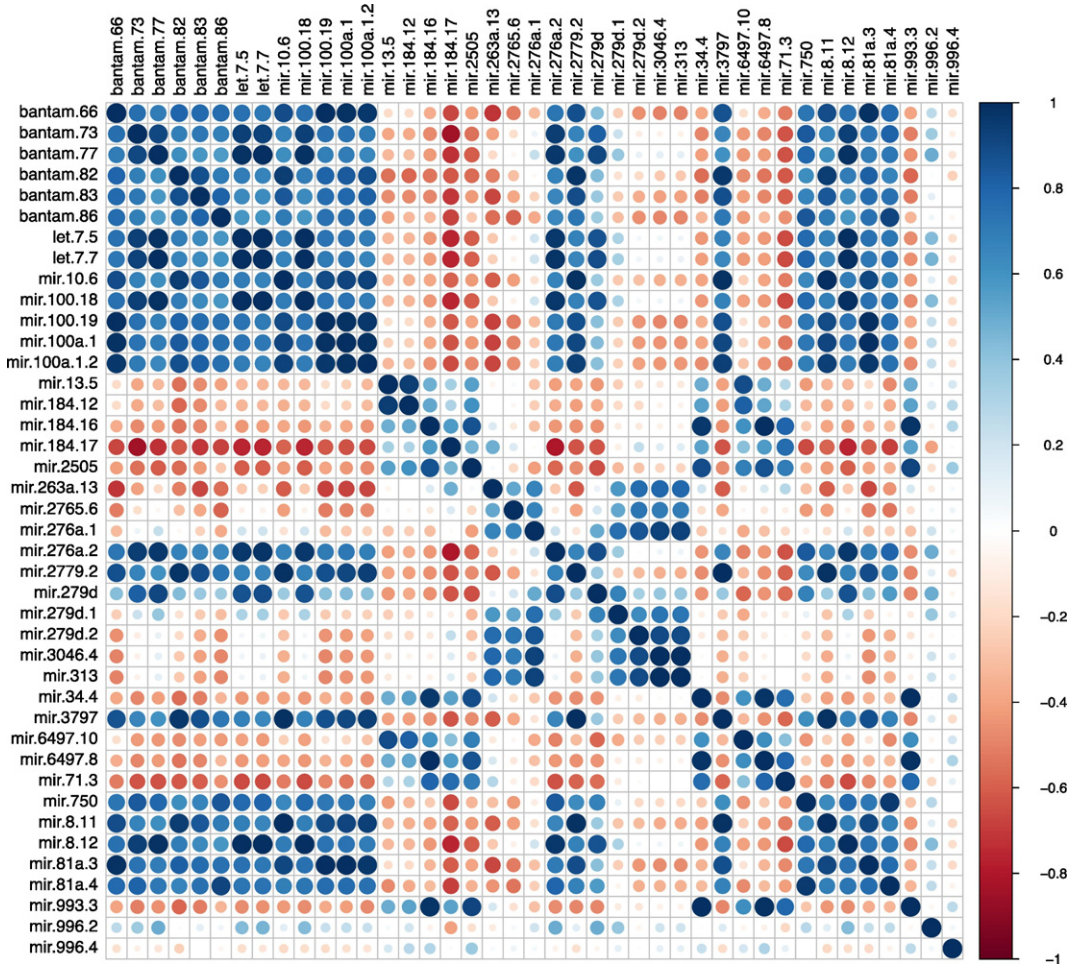

Fig. 2. Correlations plot matrix among differentially expressed *C. rogercresseyi* miRNAs. Pearson correlations among data were calculated for 41 miRNAs that showed modulation during the sea louse development. Color scale represents the correlation values.

of 22 nucleotides for all developmental stages sequenced (Table 1 and Fig. 1A). Furthermore, to identify potential miRNAs in the sea louse *C. rogercresseyi*, the screened dataset was blasted against the known miRNAs precursors and mature miRNAs of Arthropoda species in miRBase release 21 (http://www.mirbase.org). The results identified 40 evolutionary conserved miRNAs families in the *C. rogercresseyi* miRNome, where the top six annotated families were mir-8, mir-6497, mir-263a, mir-100, mir-184 and bantam. Interestingly, the miRNAs annotated to bantam family displayed the highest number of putative miRNAs (>200 sequences) (Fig. 1B). Several isoforms or isomiRs in sequence and size for precursors, mature 5′ and mature 3′ were found in each miRNA family (Fig. 1C). Here, mature 3′ variants were highly expressed compared with mature 5′ and precursor variants. The most expressed isomiRs was the mature 3′ subvariant, meanwhile the isomiR mature 5′ variant was primary detected. With respect to precursors, 134 variants were found, whereas 27 sequences showed high level of conservation with known precursor sequences. The comparative analysis with conserved miRNAs from several Arthropod species revealed that the *C. rogercresseyi* miRNome was more similar with miRNAs reported for species such as *Aedes aegypti*, *Apis mellifera*, *Acyrthosiphon pisum*

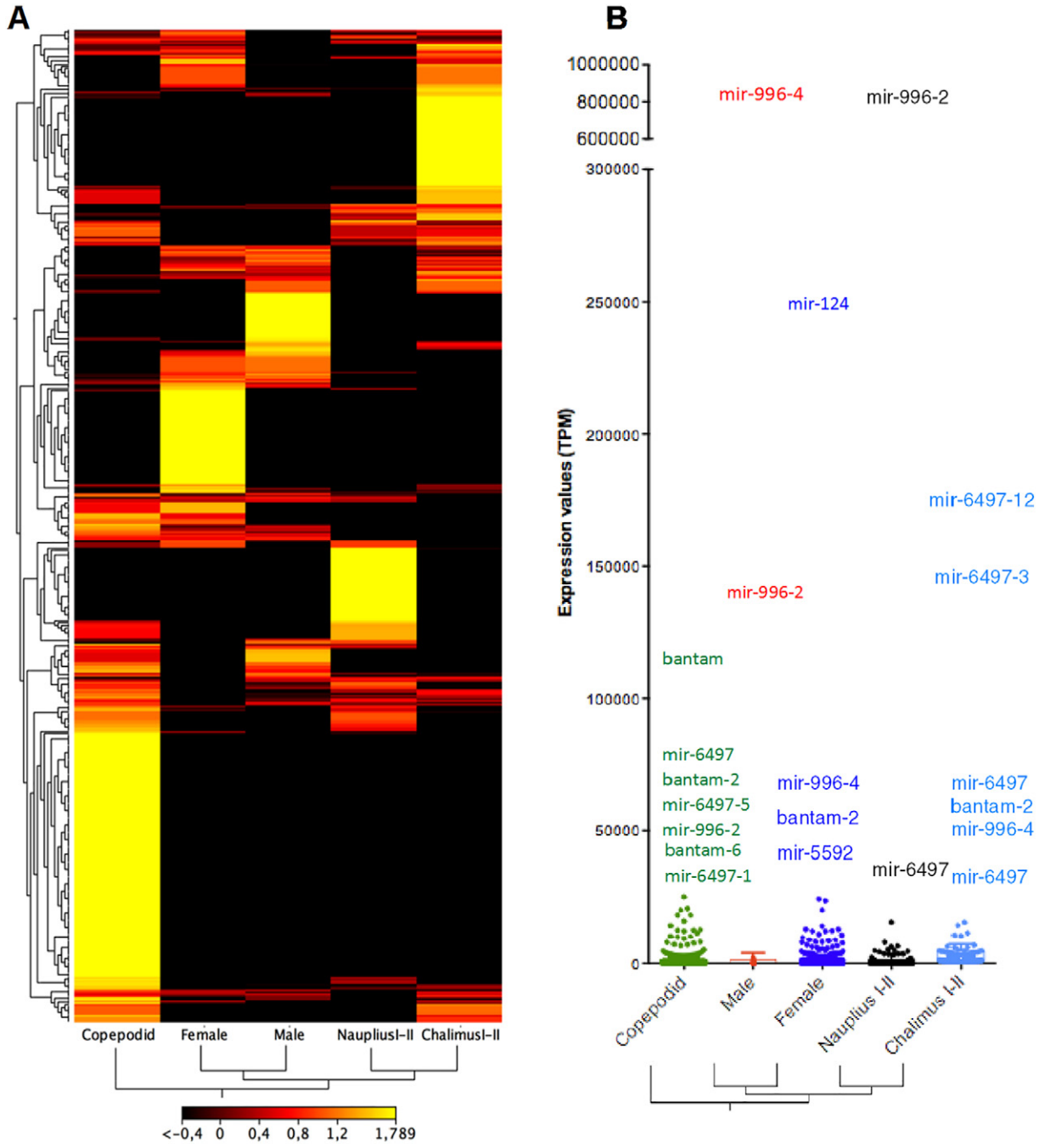

Fig. 3. Transcriptome profiles of miRNAs during the developmental stages of the sea louse *C. rogercresseyi*. A) Heatmap of identified miRNAs during *C. rogercresseyi* ontogenesis. The heatmap was obtained using CLC Genomics Workbench 9.1 (Euclidean distance, average linkage algorithm), and B) Transcription expression of *C. rogercresseyi* miRNAs during the ontogenesis. Total per million (TPM) was used to calculate the mean expression values with 95% confidence interval. The miRNAs with different colors represent outlier values for each estimated distribution.

and *Bombix mori* (Fig. 1D). Interestingly, 197 isomiRs annotated for *C. rogercresseyi* matched with 101 miRNAs reported for the parasite hematophagous species *A. aegypti.*

3.2. Transcription profiling of miRNAs during the C. rogercresseyi development

To analyze co-expression patterns of specific miRNAs during the ontogeny of *C. rogercresseyi*, correlations plot matrix among differentially expressed sea lice miRNAs were estimated. Pearson correlations among data were calculated for 41 miRNAs that displayed high modulation during all developmental stages (Fig. 2). High correlation values were found among bantam, let-7, mir-10 and mir-100 miRNAs families ($r > 0.8$). Pairs of clusters showed high correlation values with these miRNAs, with the first cluster was including the mir-276a, mir-2779 and mir-279d, and the second cluster with members of the mir-750, mir-8 and mir-81a families. Moreover, two further small cluster showing significant correlation values were detected, the first group was composed of mir-263a, mir-2767, mir-276a, mir-279d, mir-3046 and mir-313, and the second group involved the miRNAs, mir-276a, mir-2779, mir-279, mir-750, mir-8 and mir-81a (Fig. 2).

Transcription profiles of miRNAs annotated for the sea louse *C. rogercresseyi* were evaluated through all developmental stage (Fig. 3). Here, clustering analysis was conducted to reveals groups of miRNAs differentially expressed among sea lice instars by Euclidian distances using TPM values. The analysis showed two main cluster composed by nauplius I-II/chalimus I-II and female/male, leaving the copepodid miRNA expression values as a highly regulated outgroup (Fig. 3A). The miRNA expression values for each sea lice instar showed twenty-one miRNAs highly expressed (Fig. 3B). Among them, mir-996-2, mir-6497-12, bantam, mir-996-4 and mir-124 were annotated for nauplius I-II, copepodid, chalimus I-II, female and male, respectively. Interestingly, mir-996 was the most expressed in all developmental stages analyzed. With respect to the cluster analysis for high expressed miRNAs, members of bantam family were the most abundant during the *C. rogercresseyi* ontogeny, meanwhile members of mir-100 and mir-263a were primary transcribed through chalimus I–II/male/female and nauplius I–II/copepodid, respectively (Fig. 4). Here it is important to note that the instars chalimus/adults are the stages attached to the host skin, whereas nauplius and copepodid are free-swimming instars.

Statistical analyses of differentially expressed miRNAs among developmental stages were conducted to reveal exclusive and sharing conserved miRNAs during the *C. rogercresseyi* ontogeny (Fig. 5). The analysis between copepodid *vs.* nauplius I–II and chalimus I–II *vs.* copepodid showed that the infective stage of copepodid and the juvenile stages of chalimus I-II displayed the highest number of exclusively expressed miRNAs equivalent to 20 transcripts, whereas the comparison between copepodid and nauplius I–II identified 7 exclusively expressed miRNAs. The number of shared miRNAs expressed among these three stages was 10 transcripts. The most differentially expressed miRNAs were bantam in copepodid, mir-996-2 in nauplius I–II, mir-9a and mir-263a in chalimus I–II. Among the miRNAs shared for these developmental stages, mir-6497, mir-2505, bantam-6 and mir-13 were the most transcriptionally activated (Fig. 5A). The comparative analysis among juveniles and sea lice adults found a minor number of exclusive differentially expressed miRNAs corresponding to early developmental stages (Fig. 5B). Interestingly, two miRNAs, mir-996-4 and mir-124, were exclusively up/downregulated between male and female adult sea lice, showing putative sex-biased transcription expressions.

3.3. Target gene prediction of C. rogercresseyi miRNAs during the ontogeny

In a previous work our research group reported for the first time a comprehensive study on the sea louse *C. rogercresseyi* transcriptome (Gallardo-Escarate et al., 2014a). In this study, RNA-seq analysis was conducted using *de novo* assembly as a reference for identifying

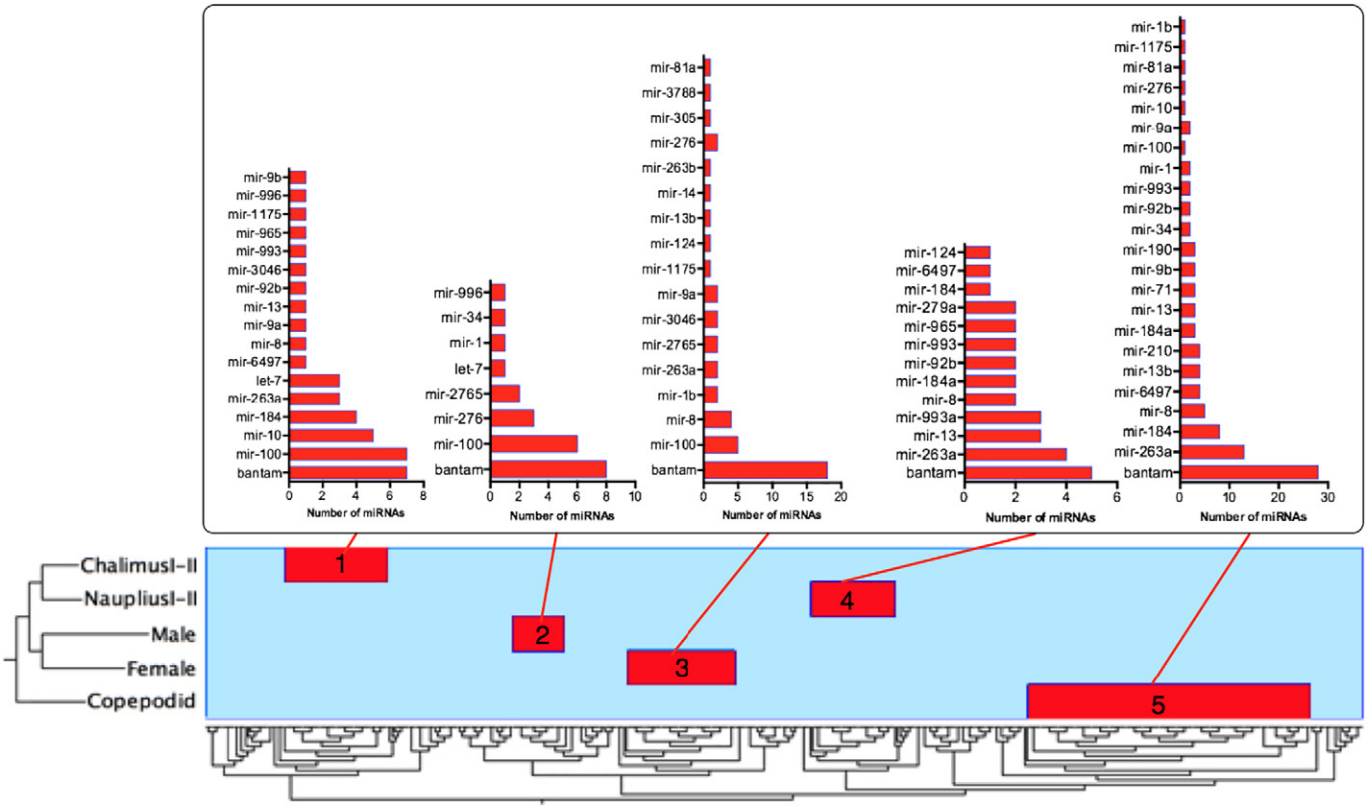

Fig. 4. Identification of miRNA clusters highly expressed for each developmental stages in the sea louse *C. rogercresseyi*. Heatmap in bottom position represents miRNAs with TPM values >2000. The number of miRNAs identified for each cluster is showed in the bar charts in upper position.

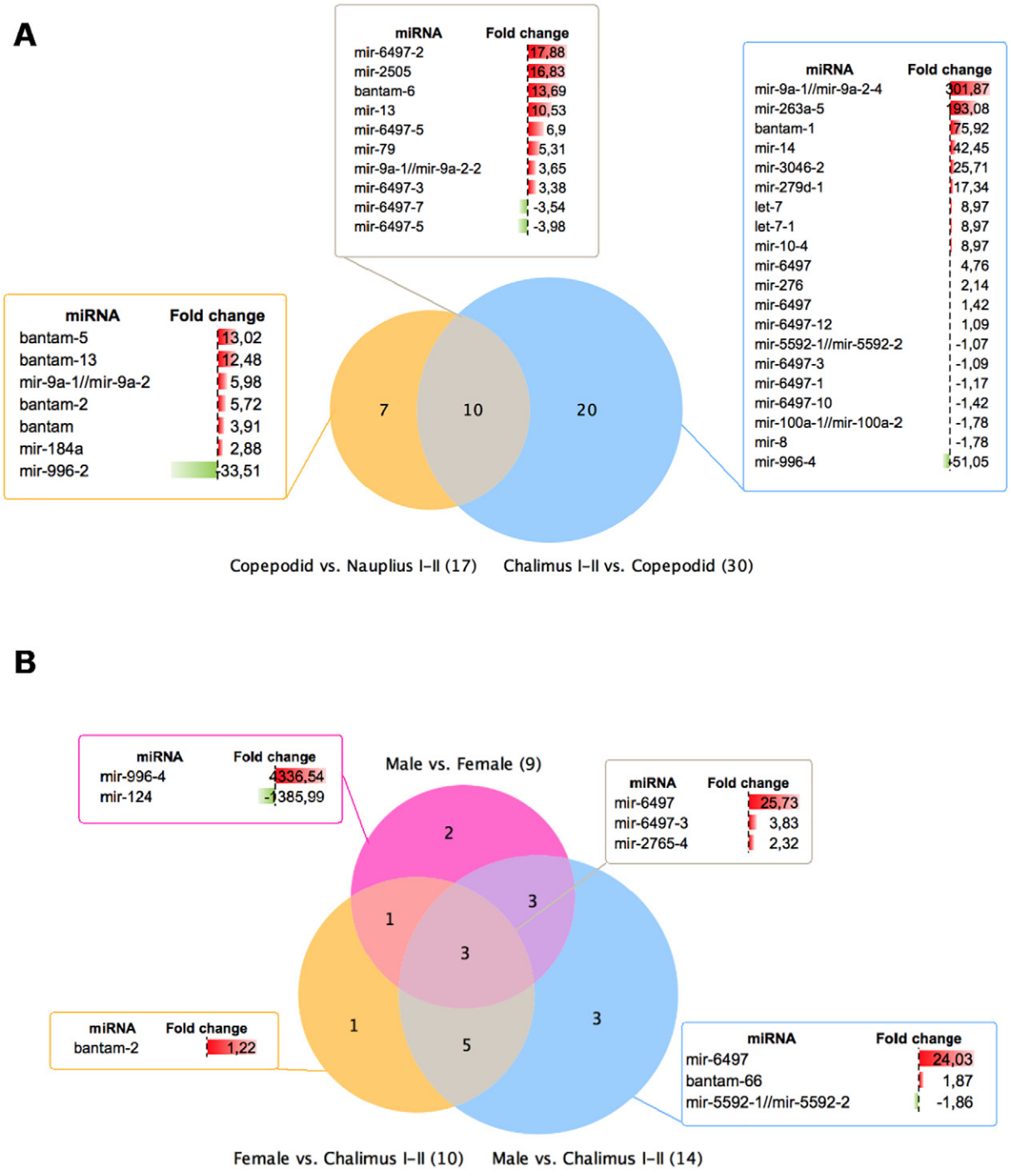

Fig. 5. Veen diagrams of differentially expressed miRNAs among developmental stages of the sea louse *C. rogercresseyi*. A) Comparison between Nauplius I–II *vs.* Copepodid, and Copepodid *vs.* Chalimus stages, respectively, and B) Comparison between Male *vs.* Female, Male *vs.* Chalimus, and Female *vs.* Chalimus, respectively. Each box shows the miRNAs exclusives and shared for each pairwise comparison. Red and green bars represent the fold-changes values, up and downregulated, respectively.

transcriptional changes through developmental stages. Transcriptome information was generated from the nauplius I, nauplius II, copepodid and chalimus stages and from female and male adults using Illumina sequencing. A total of 83k contigs were subsequently annotated into roughly 24k genes based on known proteins. Gene ontology analysis revealed that the nauplius I–II, copepodid and chalimus stages are mainly annotated to amino acid transfer/repair/breakdown, metabolism, molting cycle, and nervous system development. Additionally, genes showing differential transcription in female and male adults were highly related to cytoskeletal and contractile elements, reproduction, cell development, morphogenesis, and transcription-translation processes. This information and the genes modulated for each biological process during the sea lice ontogeny was used as database for miRNAs target predictions. For this, we applied three bioinformatic approaches; (1) Correlation analysis among differentially expressed genes and miRNAs for each instar pairwise comparison (nauplius I–II/copepodid,

Fig. 6. Correlation plot matrix between miRNAs and mRNAs differentially expressed during the *C. rogercresseyi* ontogeny. Genes selected for each developmental stages were previously reported by Gallardo-Escárate et al. (2014). Color scale represents the correlation values.

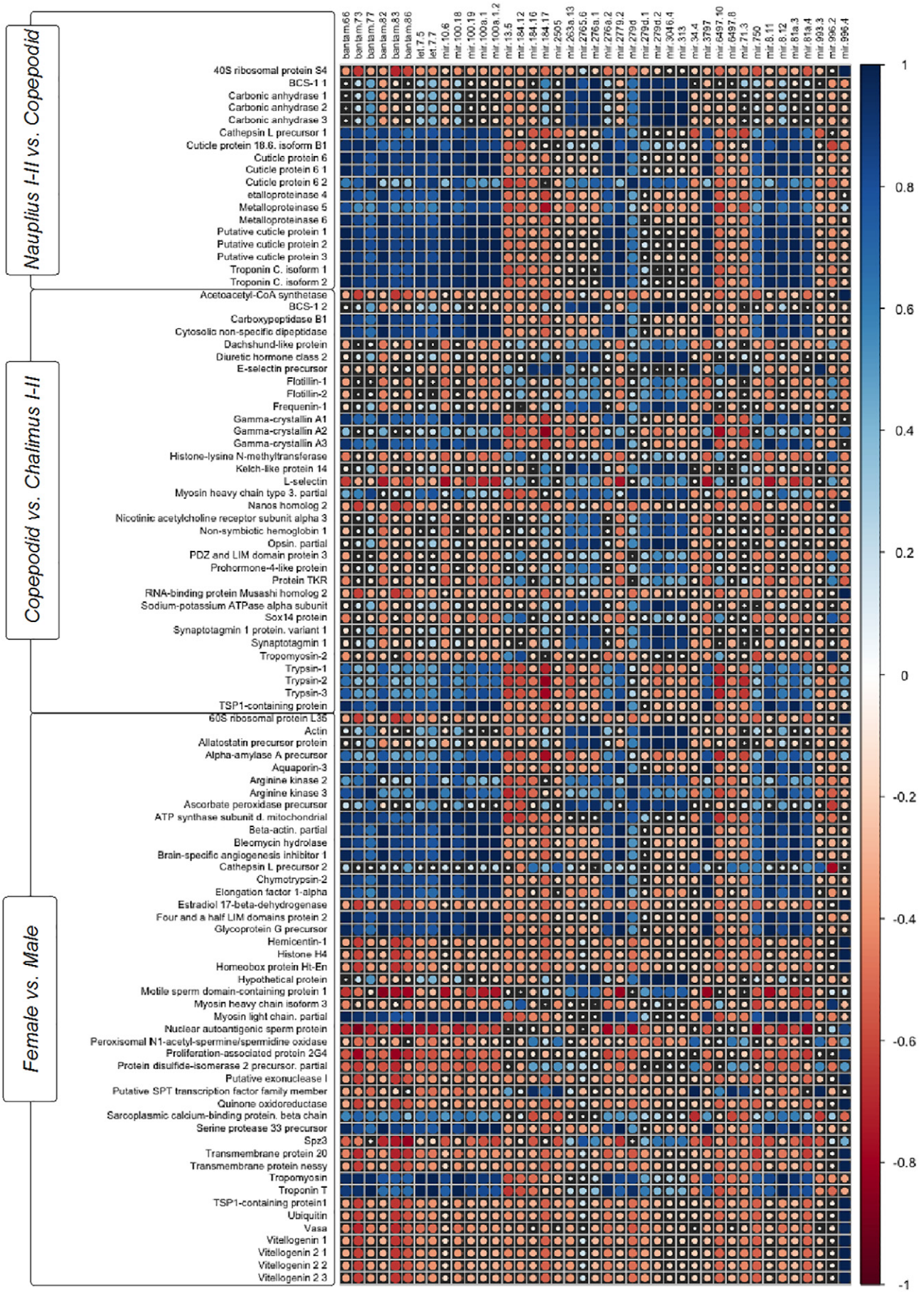
Nauplius I-II vs. Copepodid
Copepodid vs. Chalimus I-II
Female vs. Male
40S ribosomal protein S4
BCS-1 1
Carbonic anhydrase 1
Carbonic anhydrase 2
Carbonic anhydrase 3
Cathepsin L precursor 1
Cuticle protein 18.6. isoform B1
Cuticle protein 6
Cuticle protein 6 1
Cuticle protein 6 2
etalloproteinase 4
Metalloproteinase 5
Metalloproteinase 6
Putative cuticle protein 1
Putative cuticle protein 2
Putative cuticle protein 3
Troponin C. isoform 1
Troponin C. isoform 2
Acetoacetyl-CoA synthetase
BCS-1 2
Carboxypeptidase B1
Cytosolic non-specific dipeptidase
Dachshund-like protein
Diuretic hormone class 2
E-selectin precursor
Flotillin-1
Flotillin-2
Frequenin-1
Gamma-crystallin A1
Gamma-crystallin A2
Gamma-crystallin A3
Histone-lysine N-methyltransferase
Kelch-like protein 14
L-selectin
Myosin heavy chain type 3. partial
Nanos homolog 2
Nicotinic acetylcholine receptor subunit alpha 3
Non-symbiotic hemoglobin 1
Opsin. partial
PDZ and LIM domain protein 3
Prohormone-4-like protein
Protein TKR
RNA-binding protein Musashi homolog 2
Sodium-potassium ATPase alpha subunit
Sox14 protein
Synaptotagmin 1 protein. variant 1
Synaptotagmin 1
Tropomyosin-2
Trypsin-1
Trypsin-2
Trypsin-3
TSP1-containing protein
60S ribosomal protein L35
Actin
Allatostatin precursor protein
Alpha-amylase A precursor
Aquaporin-3
Arginine kinase 2
Arginine kinase 3
Ascorbate peroxidase precursor
ATP synthase subunit d. mitochondrial
Beta-actin. partial
Bleomycin hydrolase
Brain-specific angiogenesis inhibitor 1
Cathepsin L precursor 2
Chymotrypsin-2
Elongation factor 1-alpha
Estradiol 17-beta-dehydrogenase
Four and a half LIM domains protein 2
Glycoprotein G precursor
Hemicentin-1
Histone H4
Homeobox protein Hlt-En
Hypothetical protein
Motile sperm domain-containing protein 1
Myosin heavy chain isoform 3
Myosin light chain. partial
Nuclear autoantigenic sperm protein
Peroxisomal N1-acetyl-spermine/spermidine oxidase
Proliferation-associated protein 2G4
Protein disulfide-isomerase 2 precursor. partial
Putative exonuclease I
Putative SPT transcription factor family member
Quinone oxidoreductase
Sarcoplasmic calcium-binding protein. beta chain
Serine protease 33 precursor
Spz3
Transmembrane protein 20
Transmembrane protein nessy
Tropomyosin
Troponin T
TSP1-containing protein1
Ubiquitin
Vasa
Vitellogenin 1
Vitellogenin 2 1
Vitellogenin 2 2
Vitellogenin 2 3
bantam.66
bantam.73
bantam.77
bantam.82
bantam.83
bantam.86
let.7.5
let.7.7
mir.10.6
mir.100.18
mir.100.19
mir.100a.1
mir.100a.1.2
mir.13.5
mir.184.12
mir.184.16
mir.184.17
mir.2505
mir.263a.13
mir.2765.6
mir.276a.1
mir.276a.2
mir.2779.2
mir.279d
mir.279d.1
mir.279d.2
mir.3046.4
mir.313
mir.34.4
mir.3797
mir.6497.10
mir.6497.8
mir.71.3
mir.750
mir.8.11
mir.8.12
mir.81a.3
mir.81a.4
mir.993.3
mir.996.2
mir.996.4
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Table 2
Ontogeny-associated target genes of differentially expressed miRNAs in *Caligus rogercresseyi* using correlation transcription analysis.

Target Gene	MicroRNA
Nauplius I–II *vs.* copepodid	
Carbonic anhydrase 2	mir-2765-6, mir-276a-1
Cuticle protein 1	mir-8-11, mir-8-12, mir-81a-4, mir-100-18, mir-81a-4, bantam-66, bantam-82, mir-100-19, mir-100a-1, bantam-77, bantam-83, bantam-83
Cuticle protein 2	mir-8-11, mir-100a-1, mir-8-12, bantam-73, mir-10-6, mir-100-19, bantam-66, bantam-82, mir-10-6, bantam-77, mir-100-19, mir-100-18, bantam-86, bantam-83, bantam-73, mir-100a-1
Metalloproteinase_6	mir-81a-3, mir-10-6, bantam-83, bantam-82, bantam-86, mir-100-19, le-7-5, mir-100a-1, mir-100-18, bantam-73, bantam-77
Cuticle protein 6	bantam-66, mir-81a-4, mir-100a-1, mir-8-11, mir-8-12, bantam-86, bantam-77, let-7-7
Cuticle protein 7	bantam-83, mir-100-18, mir-100-19, mir-100-18, bantam-82, mir-10-6, bantam-77, bantam-82, bantam-86, bantam-83, let-7-7
Troponin C isoform 2	bantam-66, bantam-73, bantam-77, bantam-82, bantam-83, bantam-86, let-7-5, let-7-7, mir-10-6, mir-100-19, mir-100a-1, mir-100a-1, mir-8-11, mir-81a-3, mir-81a-4
Copepodid *vs.* chalimus I–II	
Carboxypeptidase B	bantam-77, mir-100-19, mir-100-18, mir-81a-3, mir-8-12, let-7-7, mir-100a-1, let-7-5, mir-8-11
BCS-1	mir-2779-2, mir-279d-1, mir-279d, mir-279d-2, mir-3046-4, mir-313
Gamma-crystallin A1	let-7-5, mir-8-12, bantam-66, mir-81a-3, mir-81a-4, bantam-86, mir-100-19, mir-10-6, mir-8-11, bantam-77
Gamma-crystallin A2	bantam-66, bantam-77, mir-81a-4, mir-8-11, mir-750, mir-100-18, mir-10-6, mir-81a-3
Nicotinic acetylcholine receptor	mir-279d-1, mir-2779-2, mir-279d-2, mir-2765-6, mir-276a-2, mir-279d, mir-3046-4, mir-313
Opsin	mir-279d-1, mir-279d-2, mir-3046-4, mir-313
Trypsin-3	mir-100-18, mir-8-12, mir-81a-3, mir-10-6, bantam-82, bantam-83, mir-8-11, let-7-5
Female *vs.* male	
Aquaporin-3	bantam-66, mir-100a-1_2, bantam-82 let-7-5, mir-100-19, mir-81a-4, mir-100a-1, mir-8-11, bantam-86, mir-81a-3, mir-10-6, bantam-77, bantam-73, mir-100-18, let-7-7, mir-8-12, bantam-83
Chymotrypsin-2	mir-100-18, let-7-5, bantam-77, bantam-82, mir-10-6, bantam-73, bantam-83, mir-81a-4, bantam-86, bantam-66
Serine protease	mir-100a-1_2, mir-100a-1, bantam-73, bantam-82, bantam-83, mir-100-19, bantam-66, bantam-77, bantam-86, mir-10-6, mir-100-18, let-7-5, let-7-7
Vasa	mir-996-4
Vitellogenin 1	mir-996-2, mir-996-4
Vitellogenin 2	mir-996-2, mir-996-3, mir-996-4

Table 3
Reproductive-associated target genes of differentially expressed miRNAs in *Caligus rogercresseyi* using correlation transcription analysis.

Target gene	MicroRNA
Aquaporin-3	bantam-66, mir-100a-1_2, bantam-82 let-7-5, mir-100-19, mir-81a-4, mir-100a-1, mir-8-11, bantam-86, mir-81a-3, mir-10-6, bantam-77, bantam-73, mir-100-18, let-7-7, mir-8-12, bantam-83
Couch potato (CPO) protein	bantam-73, let-7-5, bantam-77, mir-993-3, let-7-7, mir-996-4, bantam-86, bantam-83, mir-996-2, bantam-82, bantam-66
Ecdysone receptor (EcR)	bantam-82, let-7-7, bantam-77, let-7-5, mir-100-18, mir-10-6, bantam-66, bantam-86, bantam-73, bantam-83
Farnesyl pyrophosphate synthase (FPS)	mir-100a-1_2, bantam-66, bantam-73, bantam-82, mir-100-19, bantam-86, mir-10-6, mir-13-5, mir-100a-1, bantam-83, bantam-77, let-7-7, mir-100-18, let-7-5, mir-993-3, mir-81a-4, mir-8-12, mir-81a-3, mir-8-11, mir-996-4, mir-996-2
Peroxinectin	mir-996-2, mir-996-3, mir-996-4
Prohibitin-2	mir-279d, mir-100a-1, mir-2779-2, mir-276a-1, mir-13-5, mir-184-16, mir-276a-2, mir-184-17, mir-100a-1_2, bantam-73, bantam-82, bantam-77, bantam-86, let-7-7, mir-10-6, bantam-83
Prostaglandin E synthase 2	bantam-66, bantam-73, bantam-77, bantam-83, bantam-86, let-7-5, mir-10-6, mir-100-19, mir-6497-10, mir-8-11, mir-8-12, mir-81a-3, mir-81a-4, mir-993-3, mir-996-2, mir-996-4
Retinoid X receptor (RXR)	mir-6497-8, mir-750, mir-6497-10, mir-10-6, mir-81a-3, bantam-82, mir-34-4, bantam-73, bantam-66, mir-8-12, mir-81a-4, bantam-77, mir-3797, mir-993-3, bantam-83, bantam-86, mir-996-2 let-7-7
Vasa	mir-996-4ln
Vitellogenin 1	mir-996-2, mir-996-4
Vitellogenin 2	mir-996-2, mir-996-3, mir-996-4

copepodid/chalimus I-II and female/male), (2) Gene target prediction, and (3) Gene and miRNA expression *in silico* analysis for candidates selected.

Correlation analysis of pairwise comparisons among candidate genes and miRNAs (Fig. 6) revealed conspicuous clusters with correlation values > 0.8. For instance, nauplius I-II/copepodid show high correlation values between bantam, let-7, mir-100, mir-750, mir-8, mir-81a and cathepsin, cuticle, metalloproteinase and troponin C, respectively. Additionally, carbonic anhydrase was highly correlated with mir-279d, mir-3046 and mir-313 for early developmental stages. For copepodid/chalimus I–II, mir-10, mir-8 and mir-100 were linked to carboxypeptidase, cytosolic non-specific dipeptidase, gamma crystallin A and trypsin genes. Pairwise comparison between sea lice adults showed that mir-100, mir-2779, mir-3797 and mir-8 were primarily correlated with *aquaporin*, *beta actin*, *bleomycin*, *chymotrypsin*, *elongation factor 1-alpha*, *glycoprotein G*, *myosin light chain*, *serine protease*, *tropomyosin* and *troponin*. Interestingly, high correlation values were achieved between the mir-996-4 and important sex-related genes such as vasa and vitellogenin.

Bioinformatic gene target predictions were conducted on mRNAs and miRNAs with significant correlation values. Here, miRNA targets were predicted for each miRNAs highly modulated during the *C. rogercresseyi* ontogeny (Table 2) using a non-parameter model that computes the difference between the free energy gained from the formation of the miRNA-target duplex and the energetic cost of unpairing the target to make it accessible to the miRNA was applied (Kertesz et al., 2007; Rennie et al., 2014). This analysis found that *carbonic anhydrase 2*, *cuticle*, *troponin C isoform 2*, *carboxypeptidase B*, *BCS-1*, *gamma-crystallin A*, *nicotinic acetylcholine receptor*, *opsin* and *trypsin* genes were primarily predicted with putative regulation by miRNAs during early sea louse developmental stages. With respect to male and female adults, target prediction analysis revealed that *aquaporin*, *chymotrypsin*, *serine protease*, *vasa* and *vitellogenin* genes were mainly identified. Furthermore, to investigate the putative roles of miRNAs highly transcribed at late developmental stages and exhibiting sex-biased expression between male and female adults, we conducted a similar analysis using sex-related genes previously identified for *C. rogercresseyi* (Farlora et al., 2014; Farlora et al., 2015; Nuñes-Acuña and Gallardo-Escárate, 2015; Farlora et al., 2016). The analysis used either the complete mRNAs or the identified UTR sequences to identity predicted miRNA binding regions. Consequently, *aquaporin*, *couch potato (CPO) protein*, *ecdysone receptor (EcR)*, *farnesyl pyrophosphate synthase (FPS)*, *peroxinectin, prohibitin-2*, *prostaglandin E synthase 2*, *retinoid X receptor (RXR)*, *vasa* and *vitellogenin 1–2* genes were identified as putative genes regulated by miRNAs (Table 3).

Without doubt one interesting findings was the discovery of two miRNAs with sex-biased expression in *C. rogercresseyi*. Here, mir-996-4 and mir-124 were highly transcribed in male and female sea louse adults, respectively. Indeed, correlation plot matrix between *vasa, vitellogenin 1* and *vitellogenin 2* genes and mir-996-4 evidenced the highest

correlation values (Fig. 7A). Schematic representation of sex-related mRNAs and predicted pairing of miRNAs on 3′ UTRs is showed in Fig. 7B. Furthermore, *in silico* transcription expression of miRNA-996-4 in female and male adults of *C. rogercresseyi* evidenced opposite expression patterns between miRNA-996-4 and their putative predict target genes (Fig. 7C).

3.4. Target gene prediction of C. rogercresseyi miRNAs with immune-related genes in infected Atlantic salmon

To explore a putative and novel mechanism to evade the host immune response during the sea lice infestation process, we conducted a bioinformatic approach to evaluate if the most regulated immune related-genes in Atlantic salmon involve potential *C. rogercresseyi* miRNA binding regions. Here, the highly expressed miRNAs in copepodid, chalimus I–II and adult stages were tested against highly modulated genes in infected salmon skin samples. The candidate genes were selected according previous studies conducted by our research group (Nunez-Acuna et al., 2015; Boltana et al., 2016; Valenzuela-Muñoz et al., 2016). The results revealed that most highly expressed miRNAs during the infestation on Atlantic salmon present specific binding region at 3´UTRs (Table 4). For instance, *CD83*, *IFNg* and *TLR22a* displayed lower duplex free energy values (ΔG) such as −12.7, −20.7, −19.5 kcal/mol, respectively. This thermodynamic information suggests theoretical binding regions with members of the bantam family (Fig. 8A). Interestingly, the transcription activity of bantam members was higher from the infective stage of copepodid to the adult stages, except for male adults that showed a low expression of bantam. Female adults exhibit a high transcription expression of bantam small RNAs, suggesting sex-specific miRNA expression patterns (Fig. 8B). To evaluate the relative expression profiles of candidate target genes, RT-qPCR of *CD83*, *IFNg* and *TLR22a* genes was performed at 0, 7 and 14 dpi in skin of Atlantic salmon infected with sea lice. Importantly, all genes were significantly upregulated at 7 dpi and then downregulated at 14 dpi, congruently with the lice stages of chalimus and mobile adults (Fig. 8C). In turn, these results show an increase of the bantam transcription expression during the *C. rogercresseyi* developmental stages and in parallel a modulation of immune genes in Atlantic salmon as response to the sea lice infestation.

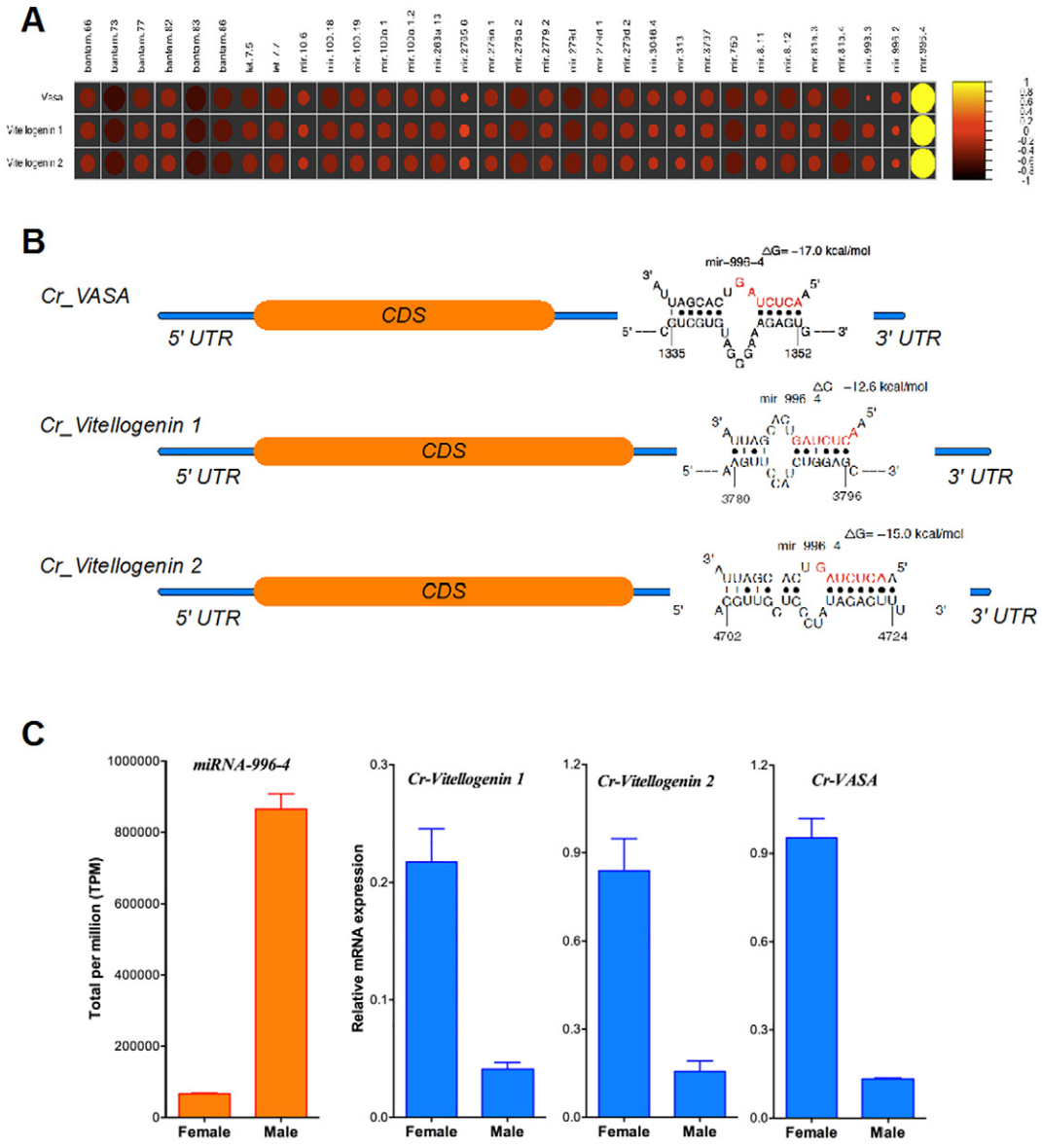

Fig. 7. miRNA target prediction for sex-related genes in the sea louse *C. rogercresseyi*. A) Correlation plot matrix between differentially expressed miRNAs and *Vasa*, *Vitellogenin 1* and *Vitellogenin 2* genes, B) Schematic representation of sex-related mRNAs and predicted pairing of miRNAs on 3′ UTRs, and C) *In silico* transcription expression of miRNA-996-4 in Female and Male adults of *C. rogercresseyi* and profiling by RT-qPCR of their putative predict target genes.

Table 4
Immune genes associated with macrophage activation, Th_1/Th_2 and TLR responses in Atlantic salmon with predicted *C. rogercresseyi* miRNA binding regions.

Target gene	MiRNA
Macrophage	
CD83	mir-34-4, mir-993-3, mir-750, mir-996-4, mir-100-18, mir-2505, mir-184-12, mir-3797, bantam-77, bantam-83
COX2	mir-996-2, bantam-82, mir-34-4, bantam-86, mir-71-3, bantam-66, mir-81a-4, mir-8-11, mir-8-12, bantam-77, bantam-83, mir-8-12
MMP13	mir-3046-4, mir-313, bantam-77, mir-8-11, mir-8-12
MHCII	mir-3046-4, mir-313, mir-750, mir-2779-2, mir-184-16, bantam-66, mir-100-18, mir-10-6, mir-996-2, bantam-73, bantam-77, mir-3797, let-7-7, mir-81a-4, mir-996-2, bantam-82, mir-3046-4, mir-313, bantam-86, bantam-73, mir-184-17, mir-996-4, mir-276a-2, mir-8-11, mir-276a-1, mir-100-19
Th_1	
STAT-4	mir-750
IFNg	mir-184-12, bantam-82, bantam-86, bantam-83, let-7-5, mir-2779-2, mir-279d-2, mir-279d
T-bet	mir-2779-2, mir-2505, mir-34-4, mir-6497-10
Th_2	
IL-10	mir-3046-4, mir-313, mir-996-2, mir-184-12, mir-13-5, mir-100a-1,mir-2505, mir-750, bantam-73, mir-276a-1
IL-4	mir-2765-6, mir-71-3, bantam-77, let-7-5, mir-750, mir-6497-10, bantam-82, mir-2505, bantam-86, bantam-83, mir-2779-2, mir-184-17, bantam-73, mir-184-16, mir-8-11, mir-3797
CCR3	mir-100-19, mir-100-18, mir-8-11, mir-3797, let-7-5, mir-10-6, mir-276a-1
TLR	
TLR13	mir-10-6, mir-276a-1, mir-3046-4, mir-2779-2, mir-313, mir-184-12, mir-2505
TLR19	bantam-83, mir-2505, mir-750, bantam-82, mir-276a-1, mir-8-11, bantam-86, mir-279d-2, mir-3046-4, mir-313, mir-279d, bantam-77, mir-71-3, bantam-73, mir-2779-2, mir-100a-1, mir-2765-6
TLR21	mir-750, mir-184-16, mir-3046-4, mir-996-2, mir-313, mir-3797, mir-184-17, mir-100-19, mir-8-11, mir-8-12, mir-100-18, let-7-5
TLR22	let-7-7, mir-6497-10, mir-3046-4, mir-313, bantam-73

Interestingly, copepodid and adult females evidenced the higher transcription activity of bantam miRNA ($p < 0.05$). However, future functional studies will provide experimental evidence to test the proposed miRNA/mRNA interactions between *C. rogercresseyi* and Atlantic salmon.

4. Discussion

MiRNAs regulate the expression of target mRNA depending on the degree of complementarity between them. An imperfect complementarity leads to translational repression, whereas perfect or nearly perfect complementarity leads to the cleavage of target mRNA. Animal miRNAs are often imperfectly complementary to their target sites, so translational repression is the predominant mechanism by which miRNAs inhibit mRNA expression in animal cells (Zhuo et al., 2013). Thus, by modulating the stability and translational efficiency of target mRNAs, miRNAs play a key role in regulating gene expression, therefore influencing a range of physiological processes, including metabolism, the nervous system development, immune response, and reproduction (Patil et al., 2014). Major advances in transcriptomic research in non-model organisms through NGS are increasing the speed and accuracy of gene and metabolic pathway discovery, as well to elucidate how gene transcription profiling is modulated (Wilhelm and Landry, 2009; Gallardo-Escarate et al., 2013; Nunez-Acuna and Gallardo-Escarate, 2013; Valenzuela-Munoz et al., 2014). However, the interactions between transcriptome profiles and miRNA expression patterns during the ontogenic development of crustaceans are still unknown. Our results revealed that the *C. rogercresseyi* miRNome is comprised by 663 unique miRNA sequences annotated into 40 evolutionary conserved families. At present, according to miRBase release 21 (October 2016), over 28,645 miRNAs have been identified in various taxa and specifically for Arthropoda, including 6098 from Hexapod, 290 from Chelicerata, 101 from Crustacea, and 7 from Mandibulata. Regarding crustacean's miRNAs few crustacean species miRNAs have been identified (Rota-Stabelli et al., 2011; Chen et al., 2012; Song et al., 2014; Kaewkascholkul et al., 2016; Ren et al., 2016; Sun et al., 2016; Zhu et al., 2016a). Thus, the results of this study elucidate the miRNA-based regulatory system not only for *C. rogercresseyi* but also for non-model ecdysozoan species.

So far, transcriptome databases have been reported for a few copepod ectoparasites (Yasuike et al., 2012), and recently for *C. rogercresseyi* (Gallardo-Escarate et al., 2014a). In that study, RNA-seq analysis was performed using *de novo* transcriptome assembly as a reference for evidenced transcriptional changes during the *C. rogercresseyi* ontogeny. Importantly, transcripts annotated as *metalloproteinase*, *arginine kinase*, and the *cuticle protein* were primarily upregulated in early developmental stages, evidencing participation in the digestion of intake proteins, tissue development, cuticle remodeling, and in specific cleavage events that activated or inactivated proenzymes and bioactive peptides (Singh, 2010). In regards to the development of the nervous system, some relevant genes such as the *nicotinic acetylcholine receptor*, *flotillin*, *synaptotagmin*, *allatostatin* and opsin were also highly expressed in copepodid and congruent with previous studies (Christie et al., 2008; Christie et al., 2013a; Christie et al., 2013b). With respect to the current study, our results showed a high number of differentially expressed miRNAs during early developmental stages belonging to the bantam, mir-8, mir-9, mir-996 and mir-184 families. Here, miR-8 has been identified as a regulator of insulin signaling in *Drosophila* fat body and has been shown to be putatively involved in regulating body size and promoting cellular growth, meanwhile large-scale analysis of microRNAs expression revealed that miR-9 is highly enriched in both the developing and mature nervous system of vertebrates and insects (Heimberg et al., 2010). Functional analyses in model species have highlighted a prominent role of miR-9 in regulating the behavior of neural progenitors, as well as the differentiation of some neuronal populations (Coolen et al., 2013). With respect to miRNA-184, it was originally identified by expression cloning from the small RNA fraction of *Drosophila* embryos, but is conserved from insects to humans (Aravin et al., 2003). It has been reported in embryos, larvae and adults, and its expression pattern displays dynamic changes at different embryo stages, suggesting that miR-184 may have an important function during the ontogeny (Li et al., 2011; Faggins, 2012). Furthermore, a central question in arthropods is how chemoreception is mediated through transmembrane-domain receptors and which molecular mechanisms provide “fine-tuning” regulation. Interestingly, two small non-coding RNAs closely located at genome level in *Drosophila*, mir-279 and mir-996, have emerged as key regulators of olfactory neurons (Sun et al., 2015). In turn, these results can be associated with the increased expression of olfactory-related genes during the infective stage of copepodid in *C. rogercresseyi* (Nunez-Acuna et al., 2014b). Moreover, several transcripts related to *cuticle proteins*, *metalloproteinases* and *carbonic anhydrases* were putatively found with miRNA binding regions for bantam, mir-8, mir-100 and mir-2765, suggesting a key role of these miRNAs during the molting process in sea lice.

For the chalimus stage, *trypsin*, *alpha amylase*, and *carboxipeptidase* genes were upregulated in *C. rogercresseyi*. Peptidases from different families may be involved in a wide range of cellular and biological processes, making it difficult to infer specific functions throughout sea louse development (Firth et al., 2000; Fast et al., 2003). For instance, blood degradation has been reported in the salmon louse *Lepeophtheirus salmonis* (Brandal et al., 1976), associating its peptidase activity with an hematophagous behavior (Kvamme et al., 2004; Kvamme et al., 2005). With respect to miRNAs reported with putative post-transcriptional regulation on peptidases, Jayachandran et al. (Jayachandran et al., 2013) identified a miRNA, har-miR-2002b, that is specifically expressed during larval stages of the cotton bollworm *Helicoverpa*

armigera. Importantly, a trypsin-like serine protease (*Ha-TLP*) was identified as a target of har-miR-2002b. *In vivo* and *in vitro* studies revealed that in the presence of the miRNA mimics, the Ha-TLP transcript/protein levels were significantly reduced. In contrast, inhibition of har-miR-2002b led to significant up-regulation of *Ha-TLP* transcript levels. Our results evidenced several miRNAs such as bantam, mir-8, mir-100, mir-81 and let-7 with peptidases as predicted target genes.

Genes showing differential transcription expression between female and male adult of sea lice have been reported (Farlora et al., 2014; Farlora et al., 2015; Nuñes-Acuña and Gallardo-Escárate, 2015). For female adults, sex-related genes such as *vitellogenins* and *estradiol 17-beta-dehydrogenase* were identified. However, the effects of the vertebrate-like steroid hormones on reproductive processes, such as oocyte maturation in crustaceans still remain unidentified. Vitellogenins are the major yolk proteins in most invertebrates, and several different vitellogenins typically give rise to vitelline granules in mature eggs (Belles and Maestro, 2005). The role of multiple vitellogenin genes in some organisms, such as insects, is unknown (Tufail and Takeda, 2008). The data obtained for *C. rogercresseyi* showed several vitellogenin-like proteins and a wide diversity of vitellogenins (Farlora et al., 2014), including *LsVit1* and *LsVit2*, as reported in L. *salmonis* (Dalvin et al., 2011). Moreover, genes associated with cell development, including homologues of vasa, homeobox, Argonaute, cell division protein kinase, and the centromere-associated protein, were also primarily expressed in female adults. The study of sex-biased expression of gene products in model species has produced a number of insightful observations (Marco et al., 2013). However, this effect may be different to that observed for protein-coding genes, as proteins and microRNAs differ in their evolutionary dynamics. For instance, gene duplication is the main mechanism by which novel protein-coding genes emerge, whereas a majority of microRNAs emerge by *de novo* formation within existing transcripts (Campo-Paysaa et al., 2011). Consequently, novel microRNAs are more likely to be lost than protein-coding genes in a short evolutionary period. Although microRNAs have been extensively studied in *Drosophila* (Marco, 2014), the effect of sex-biased expression in miRNA in non-model species remains largely unexplored. Our results

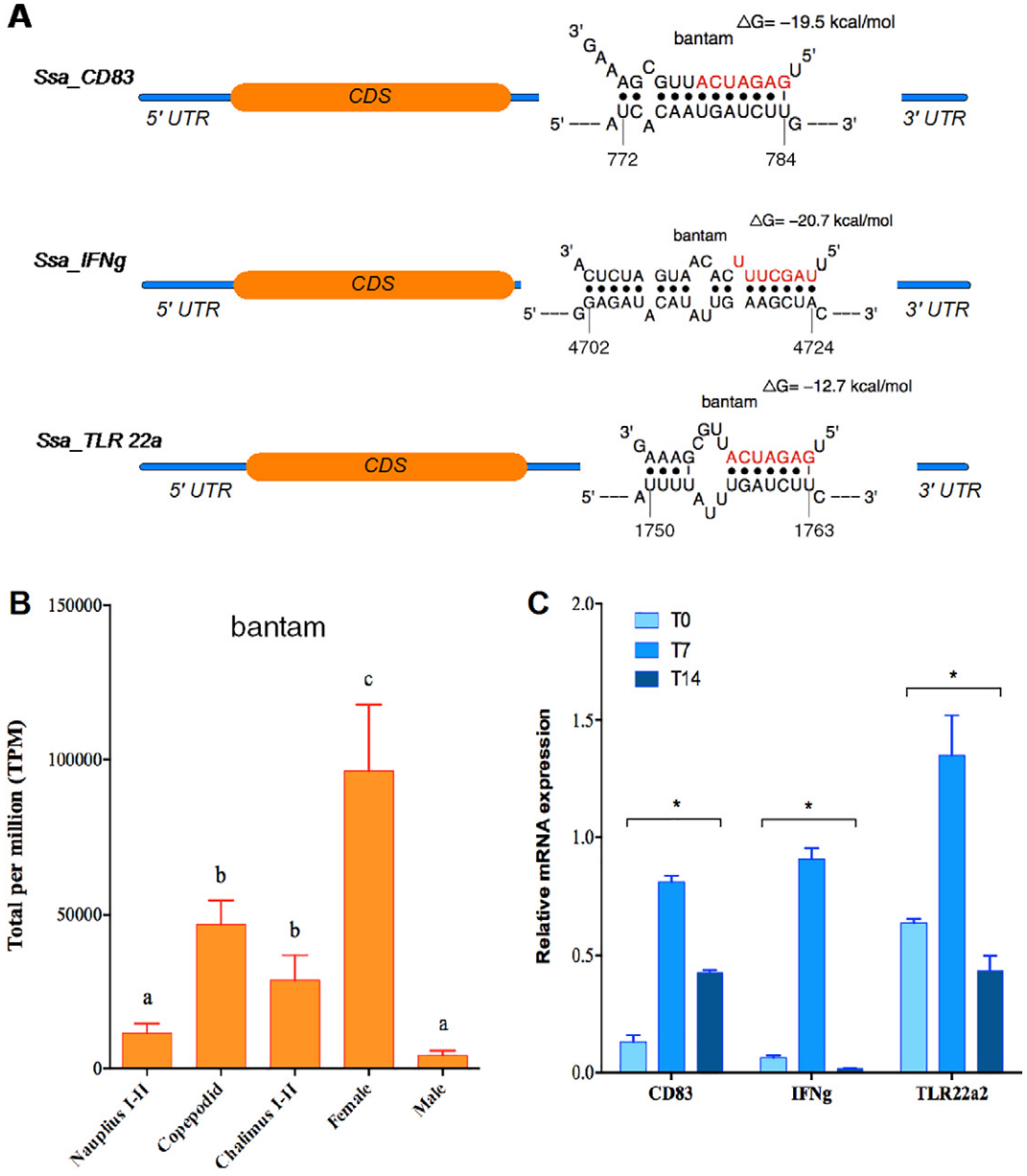

Fig. 8. miRNA-bantam target predictions with immune genes in Atlantic salmon (*Salmo salar*) differentially expressed during the infestation with the sea louse *C. rogercresseyi*. A) Schematic representation of immune-related mRNAs and predicted pairing of miRNAs on 3′ UTRs, b) *In silico* transcription expression of bantam in the sea louse stages, C) Profiling by RT-qPCR of their putative predict target genes in infected skin at 0, 7 and 14 dpi. Bars and letters/asterisks indicate standard deviation and significant differences $P < 0.05$, respectively.

show sex-biased expression in two small RNAs, mir-996-4 and mir-124 for male and female sea lice, respectively. Interestingly, the same miRNAs have been reported in *Drosophila*, but inversely expressed, where mir-996 has been associated to females and the mir-124 to male flies (Weng et al., 2013; Marco, 2014). Furthermore, bioinformatic target prediction suggests that mir-996-4 in *C. rogercresseyi* can potentially knockdown *vitellogenin I-II* and *vasa* genes in male adults.

Finally, it is important to note the high number of bantam variants in the *C. rogercresseyi* miRNome comprising 215 isomiRs. Bantam was the first miRNA characterized in *Drosophila* and was identified in gain-of-function screens for genes promoting organ growth (Hipfner et al., 2002; Brennecke et al., 2003). Further research in imaginal tissues indicated that bantam is an effector of several signaling pathways during development, namely the Hippo, Notch, Dpp, and epidermal growth factor receptor (EGFR) pathways (Oh and Irvine, 2011). Deletion of the bantam locus results in pupae with a 50% reduction in size and associated with unusual levels of ecdysone at the time of metamorphosis (Boulan et al., 2013). Thus, bantam plays a key role in regulating growth at the systemic level. However, our results suggest a novel function putatively related to immune evasion in *C. rogercresseyi* during the infestation in Atlantic salmon. Using bioinformatic approaches, we evidenced that bantam binds with lower duplex free energy on the 3´UTRs of the most highly modulated transcripts in Atlantic salmon in response to sea lice infestation such as macrophage activation, Th1/Th2 pathways and TLRs genes. Notably, several parasite species secrete extracellular vesicles or exosomes that play an important role in pathogen-host interactions (Mathivanan et al., 2010). These vesicles are synthesized through reverse budding of the late endosomal membrane, resulting in the formation of multivesicular bodies to be released into the extracellular environment (Colombo et al., 2014). Here, exosomes comprise functional proteins, lipids and miRNAs, which can transduce signals in target cells. Interestingly, a recent study evidenced that the *Schistosoma japonicum* exosome-like vesicles is involved in the transfer of their miRNAs cargo to host. Here, bantam emerges among the top five most expressed miRNAs (Zhu et al., 2016b).

5. Conclusions

The present study is the first to characterize the miRNome in a crustacean species during the ontogeny. Herein, 663 miRNA sequences were discovered and annotated into 40 evolutionary conserved miRNAs families, that in turn, displayed stage-specific transcription patterns putatively associated with genes highly regulated for each developmental instar. Importantly, high diversity of isomiRs annotated to bantam were evidenced through the sea louse ontogeny, and some of them suggest putative miRNA binding regions with genes associated to immune responses highly transcribed during the infestation with sea lice. This finding suggests novel mechanisms of immune evasion displayed by copepod ectoparasites using miRNAs for gene silencing through RNA interference. Moreover, this study reports two sex-biased non-coding RNAs, mir-996-4 and mir-124 primary expressed in male and female, and with predicted sex-related target genes. The present study opens novel research perspectives with applications for sea lice research. For instance, in addition to the siRNA approach, in which dsRNA is expressed in host-fish, miRNAs could also be applied in formulating diets that use nanoparticles to inhibit key genes in ectoparasitic copepods, thus interrupting further copepodid development and/or preventing additional damage to cultivated fish. Moreover, developing novel control mechanisms with a low impact on the marine environment is a critical issue for establishing a sustainable salmon aquaculture industry in Chile. Given this background, the present study contributes valuable genomic information, through deep sequencing analysis, for one of the most prevalent and economically harmful ectoparasites in Chile.

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Abbreviations

miRNAs	microRNAs
pri-miRNA	primary miRNA
RISC	RNA inducing silencing complex
TPM	transcripts per million
FDR	false discovery rate
CD83	cluster of differentiation 83
IFNg	interferon gamma
TLR22a	toll-like receptor 22a
	CPO protein couch potato
EcR	ecdysone receptor
FPS	farnesyl pyrophosphate synthase
RXR	retinoid X receptor

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