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Characterization of the salmon louse *Lepeophtheirus salmonis* miRNome: Sex-biased differences related to the coding and non-coding RNA interplay

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ABSTRACT

The salmon louse *Lepeophtheirus salmonis* is a marine ectoparasite that has a detrimental impact on salmon farms. Genomic knowledge of adult stages is critical to understand the reproductive success and lifecycle completion of this species. Here, we report a comprehensive characterization of the *L. salmonis* miRNome with emphasis on the sex-differences of the parasite. Small-RNA sequencing was conducted on males and females, and mRNA-sequencing was also conducted to identify miRNA-targets at these stages. Based on bioinformatics analyses, 3101 putative miRNAs were found in *L. salmonis*, including precursors and variants. The most abundant and over-expressed miRNAs belonged to the bantam, mir-100, mir-1, mir-263a and mir-276 families, while the most differentially expressed mRNAs corresponded to genes related to reproduction and other biological processes involved in cell-differentiation. Target analyses revealed that the most up-regulated miRNAs in males can act by inhibiting the expression of genes related to female differentiation such as vitellogenin genes. Target prediction and expression patterns suggested a pivotal role of miRNAs in the reproductive development of *L. salmonis*.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs acting as regulators of diverse biological processes (Stefani and Slack, 2008). Biogenesis of miRNAs has been described in living organisms as an endogenous cellular process that culminates with the synthesis of short mature miRNA sequences of around 22 base pairs in length (Murchison and Hannon, 2004). Mature miRNAs can suppress gene transcription at post-transcriptional level by regulating complex signaling pathways that include diverse key proteins. This suppression causes a decrease in the mRNA abundance of a target gene, inhibiting its translation (Filipowicz et al., 2008). However, knowledge about gene pathways and proteins interfered by miRNAs depends on the knowledge of whole transcriptomes and miRNomes, allowing miRNA-target associations that could explain changes at the expression level. Therefore, current advances in molecular biology and next-generation sequencing technologies have been critical to identify these associations, particularly in non-model organisms (Lee et al., 2010). The current public miRNA sequence databases, such as miRbase, are also responsible for the increase in our understanding of transcriptomic regulations in living organisms (Kozomara and Griffiths-Jones, 2013). Nonetheless, we still have little knowledge about the miRNomes of many species and their implications in pivotal biological processes, including organisms that inhabit the marine environment.

The salmon louse *Lepeophtheirus salmonis* is a marine ectoparasite that infects salmonid species, but mainly the Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* (Costello, 2006). It has a high impact on salmon farms worldwide as well as on wild fish populations (Costello, 2009a, 2009b; Torrissen et al., 2013). The most used treatment methods for sea lice control, mainly in salmon farms, are based on the application of pesticides (Burridge et al., 2010). However, the emerging resistance of salmon lice to these chemicals have implied the need to develop alternative control methods (Aaen et al., 2015). The *L. salmonis* lifecycle has obligate parasitic stages, which live attached to salmon skin and feed mainly on their mucus and blood, causing immunosuppression and tissue damage in the host (Tully and Nolan, 2002). The male and female adult stages of this parasite are responsible for their reproductive success. These stages display sexual dimorphism, including morphological and behavioral features (Brooker et al., 2018). Current knowledge is not precise or vast enough to understand the regulation of the adult stages of *L. salmonis* at the molecular level, which could be important for the generation of novel control treatments for parasitic infection caused by this copepod.

At the genetic level, salmon louse species display differences among both sexes, presenting male or female-dependent single nucleotide polymorphism (SNP) markers (Carmichael et al., 2013). Notably, SNPs

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have been found in the *prohibitin-2* gene in two different species of salmon louse: *L. salmonis* (Carmichael et al., 2013) and *Caligus rogercresseyi* (Farlora et al., 2014). In this latter species, transcriptomic differences exist in the global transcriptome expression depending on the sex of the parasites (Gallardo-Escárate et al., 2014) and also in specific genes related to reproduction such as *vitellogenins*, *Dmrt3* and *aquaporins* (Farlora et al., 2014). In the *L. salmonis* species, sex-biased gene expression has been described in many transcripts, including genes related to reproduction but also those related to other biological process such as coding genes for proteases, kinases, and structural proteins (Poley et al., 2016). With respect to miRNA characterization, the only study conducted in sea lice species has been reported for *C. rogercresseyi*, which included a whole miRNome regulating genes related to reproduction and adult stage development (Gallardo-Escárate et al., 2017). In the *L. salmonis* species, transcriptome regulation related to sex and reproduction remains unknown, particularly because there is no description of any miRNome in this species.

The goal of this study was to characterize the miRNome of the salmon louse *L. salmonis* and establish an association with the reproductive stages of its lifecycle. This could introduce novel knowledge allowing us to explore new treatments against this parasitic species. In perspective, novel miRNA and mRNA transcripts related to reproduction and sex-development could be identified and described. In turn, this genomic knowledge could be applied to develop novel therapeutic tools for controlling sea lice disease in the worldwide salmon industry.

2. Methods

2.1. Salmon lice culture

Adult female and male specimens of *Lepeophtheirus salmonis* were collected from farmed Atlantic salmons (*Salmo salar*) at the Austevoll Research Station of the Institute of Marine Research (IMR), Norway. Collected animals were separated into female and male groups, fixed in RNA Later solution (Ambion®, USA) and stored at −80 °C until RNA extractions. Information about sampling procedure and samples features, following MIxS mandatory information, are found in Table 1.

2.2. Small RNA libraries sequencing

Total RNA was extracted from 10 parasites from both male and female stages using the Trizol reagent method (Invitrogen, USA). Quality of extracted RNAs was measured in a TapeStation 2200 instrument (Agilent Technologies Inc., USA), using the R6K Reagent Kit based on manufacturer's instructions. RNA samples > 9 in RIN number were selected for NGS libraries. TapeStation 2200 profiles for RNAs quality are provided in Supplementary materials 1. Small-RNA libraries were constructed from total RNA samples using the TruSeq Small RNA Kit (Illumina®, USA). Quality of libraries was evaluated by TapeStation 2200 and small-RNA libraries quantification was conducted by qPCR using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs Inc., USA). Libraries were sequenced in an Illumina MiSeq instrument (Illumina®, USA) in the Laboratory of Biotechnology and Aquatic Genomics, INCAR, Universidad de Concepción, Chile. MiSeq runs consisted of 41 single-end cycles for each library.

Table 1
MixS descriptors.

Item	Description
Investigation type	Eukaryote
Project name	miRNome of *Lepeophtheirus salmonis*
Lat_lon	60°05′34″N, 05°13′54″E
Geo_loc_name	Norway: Størebo, Austevoll
Collected_by	Gustavo Núñez-Acuña
Collection_date	April, 10, 2017, T12:15 + 01:00
Environment	ENVO: 01000312
Biome	ENVO_00000447
Feature	ENVO:01000159
Material	UBERON:0007023
Alt_elev	0.2 m
Temp	8 °C
Salinity	32 psu
Sequencing method	Illumina MiSeq
Assembly method	CLC Genomic Workbench
Assembly name	*Lepeophtheirus salmonis* miRNome
Ploidy	Haploid (females and males)

2.3. Identification of miRNAs in L. salmonis and transcript expression analyses

Reads obtained from libraries from both females and males were filtered by quality (> 0.05 in the Phred scale), length (shorter than 15 nt, or longer than 55 nt) and adapter sequence trimming in the CLC Genomics Workbench v10.0 (CLC Bio, Denmark). Adapter sequences and index were search and removed in both directions for the two libraries. Index sequences corresponded to number 2 and 4 provided by the TruSeq Small RNA-seq kit (Illumina®, USA). Multi-BLAST against non-desired non-coding RNA (short mRNAs, rRNAs, tRNAs) databases (e.g. ncRNAs in NCBI, RFam, Repbase) were conducted to keep only putative miRNA sequences. Extract and count tools from the CLC Genomics Workbench were performed to select and count unique miRNA families among reads. Length distribution for trimmed miRNAs reads are in Supplementary materials 2. Putative miRNAs were annotated by BLAST against all the arthropods species available on the miRBase release 22 database (Griffiths-Jones et al., 2006). Parameters for miRNAs annotation were: additional downstream bases = 2; additional upstream bases = 2; maximum mismatches = 2; missing bases downstream = 2; missing upstream bases = 2. Prediction of novel miRNAs were conducted with the miRanalyzer software (Hackenberg et al., 2009). MicroRNA expression analyses were conducted through the RNA-seq tool from the CLC Genomic Workbench. Annotated miRNAs were used as a reference dataset. The abundance of miRNAs in each library was measured by using the trimmed reads obtained from males and females, and the following RNA-seq settings in CLC Genomics: minimum length fraction = 0.6; minimum similarity fraction (long reads) = 0.5. Expression values were calculated as CPM (counts per million), which included normalization by total reads count. Significant differences among sexes were found by Kal's statistical analyses, where cutoffs to consider significantly expressed miRNAs were fold change > |4| and p-value < .05. Differences in miRNA expression were observed through heatmaps based on hierarchical clustering analysis, grouped by Manhattan distances and average linkage.

2.4. Validation of miRNAs expression by qPCR analyses

From 200 ng of each extracted RNA, cDNAs were synthetized using the kit miScript II RT Kit (Qiagen, Germany) following manufacturer's instructions. Buffer 5× miScript HiSpec was used for RT reactions because this reagent it was optimized to conduct qPCR assays of mature miRNAs. RT reactions were conducted by incubation with the enzyme at 37 °C for 60 min, followed by 5 min at 95 °C. A group of miRNAs with sex-biased expression levels obtained in the previous point by miRNA-seq for validation of its expression levels by qPCR. Specific forward-primers were designed for these miRNAs (Table 2). The miScript SYBR Green PCR (Qiagen, Germany) kit was used for qPCR reactions following the manufacturer's protocol. Reactions consisted in the enzyme-SYBR Green mix supplied by the kit, specific primers by miRNA, the universal primer provided by the kit and male and females lice cDNAs as templates. Reactions were conducted in a QuantStudio 3 System (Life Technologies, ThermoFisher Scientific, USA) under the following cycling conditions: enzyme activation at 95 °C by 15 min, followed by 40 cycles of denaturation at 94 °C by 15 s, annealing at 55 °C by 30 s, and extension at 70 °C by 45 s. Quantification was conducted using the

Table 2
Primers used for qPCR validation of miRNAs expression levels.

Name	Sequence	Direction	Reference
bantam-2	ATCAGATTTCACAATGATCTCA	Forward	(Gallardo-Escárate et al., 2017)
mir-1	CCATGCTTCCTTGCATTCAATA	Forward	This study
mir-2796	AGGGGTTTCTATCGGCCTCC	Forward	This study
mir-993	GCGAACATGGATCTAGTGCACG	Forward	This study
mir-276-4[a]	TAGGAACTTCATACCGTGCTCT	Forward	This study
mir-263a-1	AATGGCACTGGAAGAATTCACGGG	Forward	(Gallardo-Escárate et al., 2017)
UT primer	–	Reverse	miScript SYBR Green PCR kit

[a] Used as endogenous control.

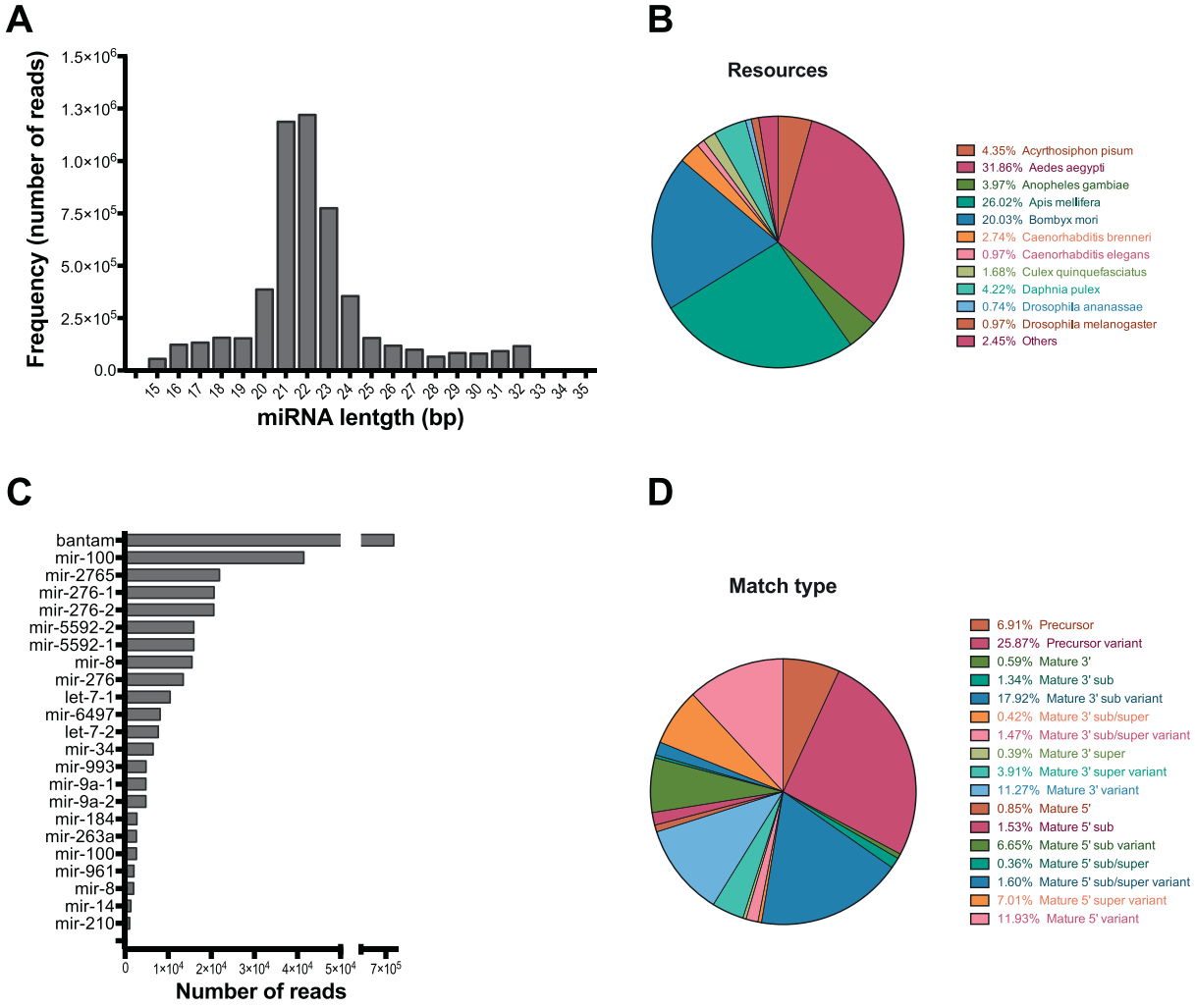

Fig. 1. Characteristics of the annotated *Lepeophtheirus salmonis* miRNome. A: length distribution of annotated miRNAs in *L. salmonis* using miRBase release 22; B: most representative species from which *L. salmonis* miRNAs were annotated (only includes species from miRBase); C: count of annotated reads corresponding to miRNAs by families; D: number of variant types of miRNAs characterized in *L. salmonis*.

comparative $\Delta\Delta C_T$ method. As housekeeping gene validation, three miRNAs were selected from the transcriptome expression data having stable expression patterns (not varying between males and females), which were validated as housekeeping genes by the NormFinder application (Jensen and Ørntoft, 2004). The miRNAs with the best stability index (mir-276-4) was selected as endogenous control and was used as a reference for $\Delta\Delta C_T$ calculation of target genes.

2.5. Transcription analysis of mRNAs related to sex and reproduction

To associate miRNAs with genes related to sex differentiation and reproduction, expression of coding-genes was evaluated through next-generation sequencing. From the total RNA samples extracted per sex, double-stranded cDNA libraries were constructed using the CATS Total RNA-seq kit (Diagenode®, Belgium). Libraries were sequenced in the Illumina MiSeq sequencer in paired-end runs of 500 cycles (251 × 251). Obtained read sequences were trimmed by quality and adapter removal using the Cutadapt software (Martin, 2011), using a python script detailed in the CATS's kit protocol. Trimmed reads were used for a reference-based assembly with the *L. salmonis* genome draft (https://licebase.org) by the Large Gap Read Mapping tool in the CLC Genomics Workbench. The transcripts discovery tool of the same software was used to extract mRNA sequences (only consensus contigs with higher coverage than 20 were considered). RNA-seq analyses were conducted

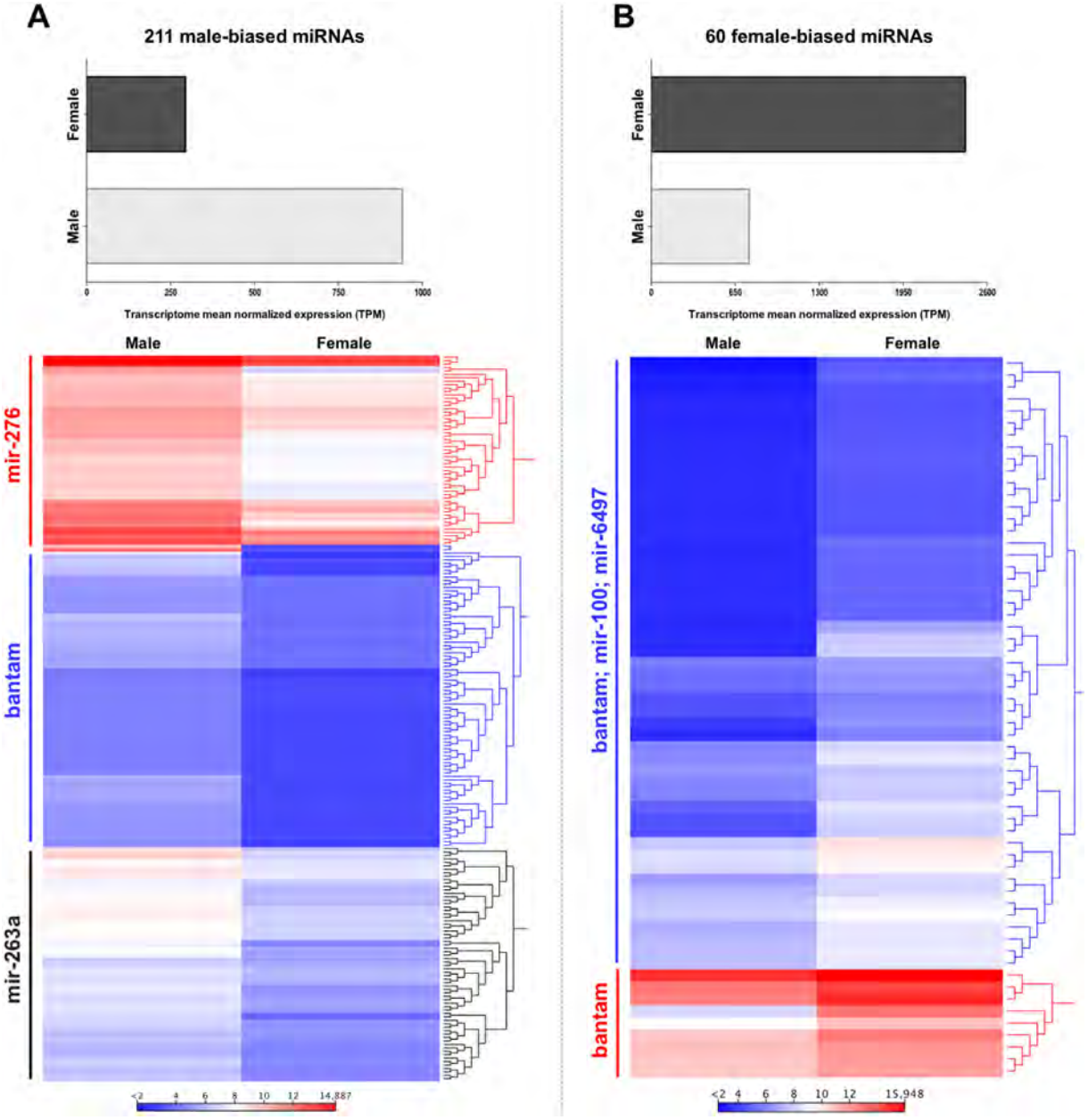

Fig. 2. Transcriptional profiles of miRNAs identified in *L. salmonis* by sexes. A: male-biased miRNAs (211 in total); B: female-biased miRNAs (60 in total). Column bars represent the total mean expression (TPM values) for biased miRNAs. Heatmaps correspond to hierarchical clustering analyses of biased miRNAs expression by Manhattan distances and average linkage.

based on extracted mRNA sequences, with a minimum length fraction of 0.8 and a similarity fraction of 0.9. Statistically significant differences were calculated according to the same method used for miRNAs. Significantly expressed transcripts were extracted and annotated by Gene Ontology criteria using GOanna software (McCarthy et al., 2006). GO terms for biological processes were extracted for these transcripts and the most enriched terms were found by REVIGO analyses (Supek et al., 2011).

2.6. Prediction of target genes for sex-related miRNAs

Differentially expressed miRNAs in both male or female stages were used for target identification, including the annotated differentially expressed mRNAs according to sexes. Two algorithms were used for target prediction: RNAhybrid (Krüger and Rehmsmeier, 2006) and PSRNATarget (Dai and Zhao, 2011). RNAhybrid cut-offs were $\Delta G < -20$, and p-value < .01, while Expect < 5 was used as a threshold for target prediction in PSRNATarget. Those miRNAs that had the same targets in both software were selected and target-miRNA alignments were obtained.

3. Results

3.1. Identification and characterization of Lepeophtheirus salmonis miRNAs

Small-RNA seq libraries resulted in 3101 unique sequences corresponding to putative miRNAs and precursors ranging from 15 to 36 nt in length. Most of the reads aligned to these unique sequences were 22 or 21 bp in length (Fig. 1A). Annotated miRNAs from miRBase resulted in higher amounts of unique sequences that were homologous to insect species, including *Aedes aegypti* (31.86% of sequences annotated against this species), *Apis mellifera* (26.02%) and *Bombyx mori* (20.03%)

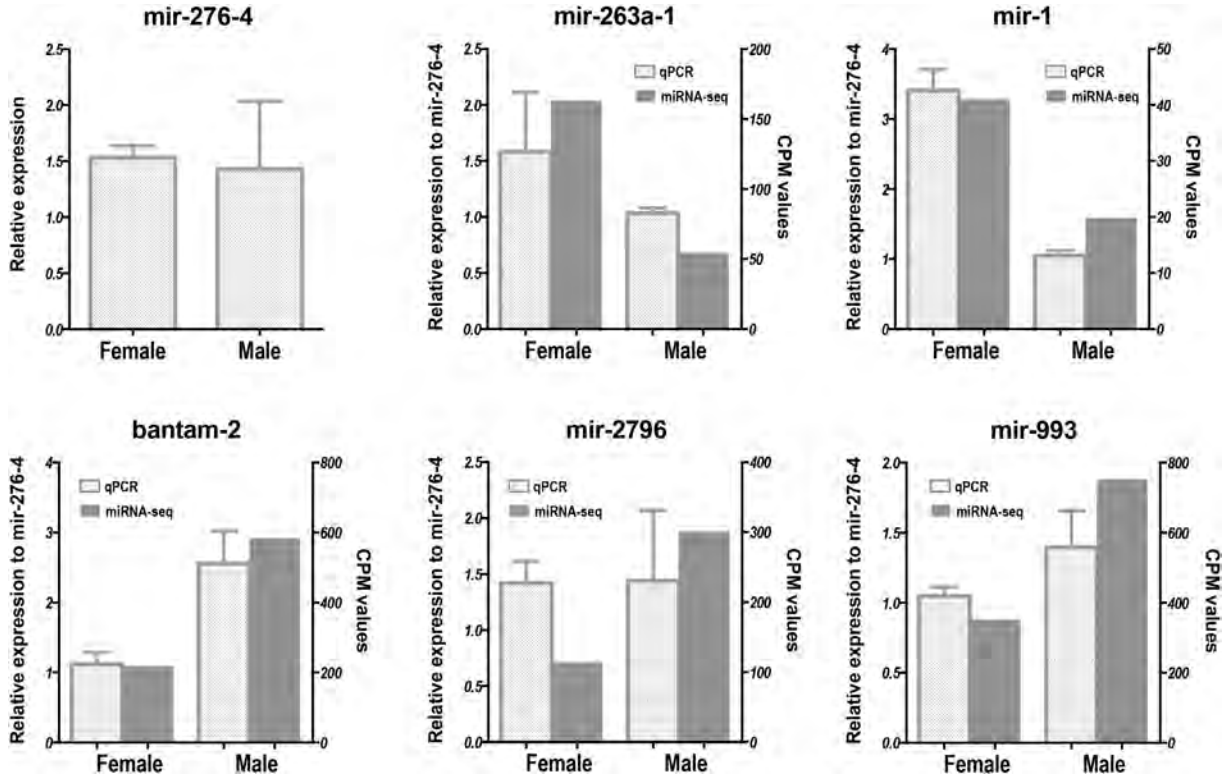

Fig. 3. Validation of miRNAs expression analyses by qPCR. Calculation of expression levels of selected sex-biased miRNAs were conducted by the $\Delta\Delta C_T$ method. The mir-276-4 gene was used as endogenous control. The column graphs show expression levels using two quantification methods: relative expression through qPCR reactions (light grey) and CPM calculations from miRNA-seq data (dark grey). Selected female biased miRNAs: mir-263a-1 and mir-1; male biased miRNAs: bantam-2, mir-2796 and mir-993.

(Fig. 1B). The most abundant conserved miRNAs in *L. salmonis* corresponded to the bantam family, which was one order of magnitude more abundant than the other families (Fig. 1C). Other enriched miRNAs belonged to the mir-100, mir-2765, mir-276, mir-5592 and mir-8 families. According to match type, most of the annotated miRNAs corresponded to Precursor variants (25.87%) and Mature 3′ sub variants (17.92%) (Fig. 1D).

3.2. Sex-biased miRNA expression in L. salmonis

Statistical expression analyses revealed 211 upregulated miRNAs in males and 60 in females. Most miRNAs associated with males were the bantam, mir-263a and mir-276 families; while in females they were associated with the bantam, mir-1, and mir-100 families, among others (see full list at Supplementary materials 3). Heatmap of upregulated miRNAs in males showed three clusters: the first related to the mir-276 family, which comprise largely over-regulated miRNAs; the second to the bantam family and the third to the mir-263a and bantam families, where both presented slighter expression differences than the first one (Fig. 2A). Upregulation of miRNAs associated with the female group comprised two clusters, revealing the first one with slight expression differences that are more related to the bantam, mir-100 and mir-6497 families, and another with stronger expression differences related to the bantam family (Fig. 2B).

Validation of miRNAs expression levels by qPCR showed similar trends than miRNA-seq analyses (Fig. 3). Most of the selected genes, including mir-263-a-1, mir-1, bantam-2, and mir-993 had no differences in the expression patterns obtained by either qPCR of miRNA-seq approaches. The only gene with difference in the expression trend was mir-2796, which were called as male-biased by miRNA-seq analyses, but this was not consistent to qPCR evaluation (Fig. 3).

3.3. Transcriptome sex-differences in L. salmonis coding genes

A total of 1455 differentially expressed genes with significant changes (fold change > |4|; p-value < .05) were found in both sexes: 691 were female-biased and 764 male-biased (Fig. 4A). Heatmap comprising these significant genes revealed four differentiated clusters: one with slight upregulation in males, another with strong upregulation in males, another with slight upregulation in females, and the last one with strong differences in the female group (Fig. 4B). Annotation of genes included in this hierarchical clustering showed that differentially expressed genes belonged to diverse biological processes (Supplementary materials 4). Enrichment analyses of annotated mRNAs by Gene Ontology evidenced that the most abundant biological processes in differentially expressed genes were related to sex and reproduction, such as male gonad development, female meiosis I, female meiotic division and gastrulation (Fig. 5). Other enriched Gene Ontology terms were related to cell differentiation such as cell proliferation, growth, mitotic cell cycle, positive regulation of cell migration and negative regulation of cell differentiation.

3.4. Target gene prediction of sex-related miRNAs in L. salmonis

Target gene prediction for miRNAs was performed with differentially expressed miRNAs for both male and females, and the subset list of genes related to sex differentiation and reproduction. Target prediction with both PSRNATarget and RNAhybrid software showed similar results (Supplementary materials 5), but had more significant hits with RNAhybrid. There were 10 and 3 overlapping miRNAs in the male and female groups, respectively, with significant hits in the same target genes using both software programs (Table 3). Target genes found for upregulated miRNAs in the female group were related to cathepsins, trypsins, estradiol-beta-dehydrogenase, transcription factors and genes

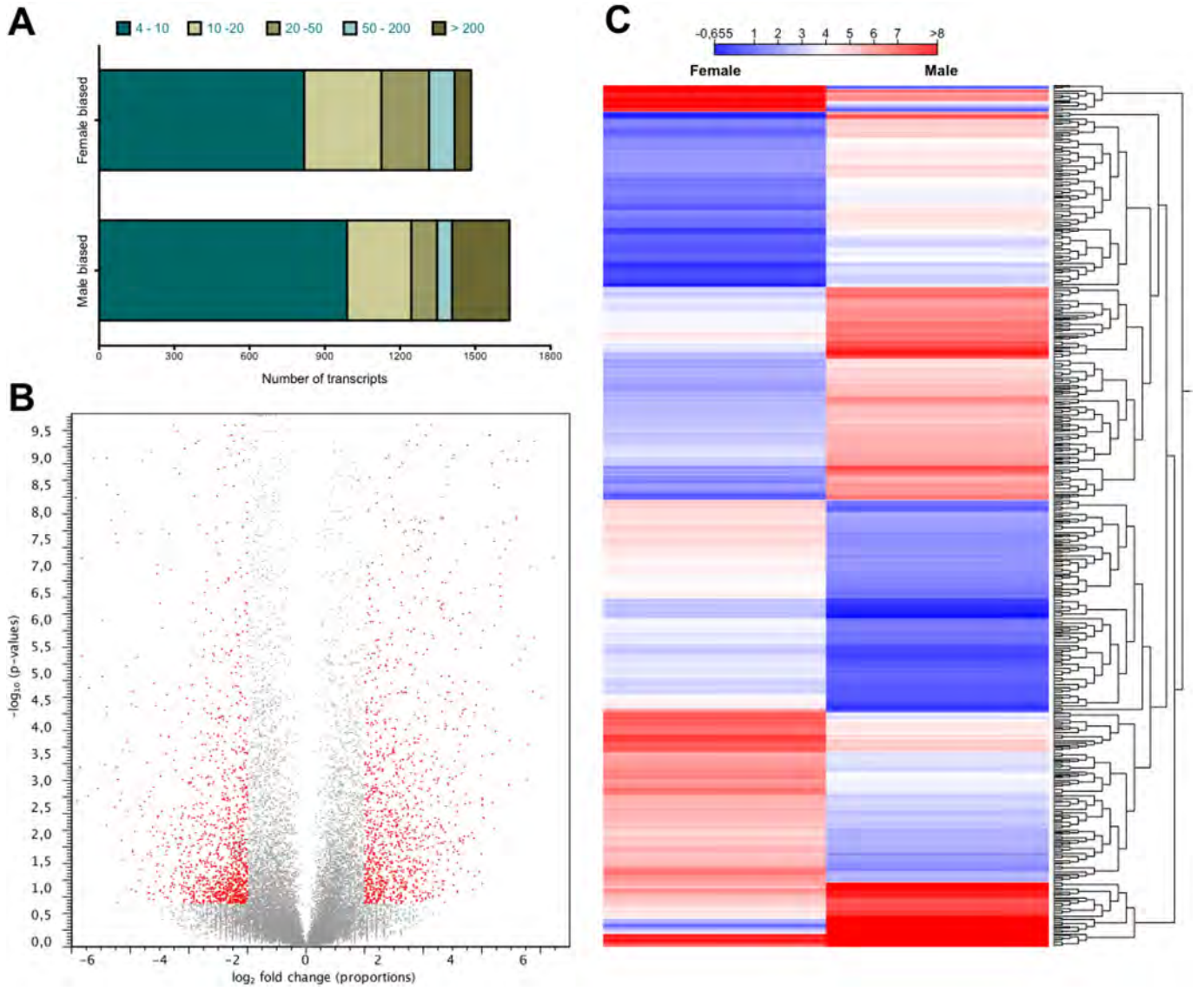

Fig. 4. Transcriptome profiles of mRNAs associated to adult stages in *L. salmonis*. A: number of significant expressed mRNAs by sex (Fold change > 4, p-value < .05); B: volcano plot for significant expressed mRNAs (in red significant transcripts); C: heatmap for hierarchical clustering by Manhattan's distances and average linkage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

related to oxide-reduction process. For the male group, target genes found for upregulated miRNAs included genes coding for cathepsin, trypsin and TSP1-containing protein. Expression patterns of these miRNAs and their targets were consistent with target prediction, which were over-expressed where their targets were down-regulated (Fig. 6).

3.5. Data availability

Transcriptomic data, including small-RNA libraries and total RNA libraries for both male and female parasites, are available in GenBank's Sequencing Read Archive (SRA) under accession ID SRP158708.

4. Discussion

Sea lice *Lepeophtheirus salmonis* is considered as one of the most important marine parasitic species because of its ecological and economic impacts (Liu and vanhauwaer Bjelland, 2014). Sexual dimorphism has been described in this species including physiological, behavioral and morphological differences. At the genetic level, sexual differences have been discovered by SNP markers in target genes (Carmichael et al., 2013), or by transcriptomic changes occurring in the adult stages of this species (Poley et al., 2016). Hence, molecular gene functions have been associated with major biological processes in sea lice species that could trigger sexual dimorphisms (Gallardo-Escárate et al., 2014; Poley et al., 2016). However, regulation of these gene functions is poorly described for sea lice species. Herein, miRNA characterization could be a relevant contribution to the knowledge about gene regulation implied in *L. salmonis* sexual dimorphism. The closest study aimed to describe these regulations in sea lice species was in *Caligus rogercresseyi*, and including not only adult specimens but also all the ontogeny for that species (Gallardo-Escárate et al., 2017). While in other marine crustaceans, miRNA characterization has been conducted to evaluate its involvement in other biological processes such as immunity (Kaewkascholkul et al., 2016; Zhu et al., 2016), somatic growth (Ren et al., 2016), effect of hypoxia (Sun et al., 2016) and phylogenomics in Arthropoda phylum (Rota-Stabelli et al., 2011). Herein, another article has described a global analysis to identify novel miRNAs related to reproduction in a decapod species, with focus on the regulation of oocyte meiosis processes by two miRNA markers (Song et al., 2014). Considering that knowledge about the regulation of sexual

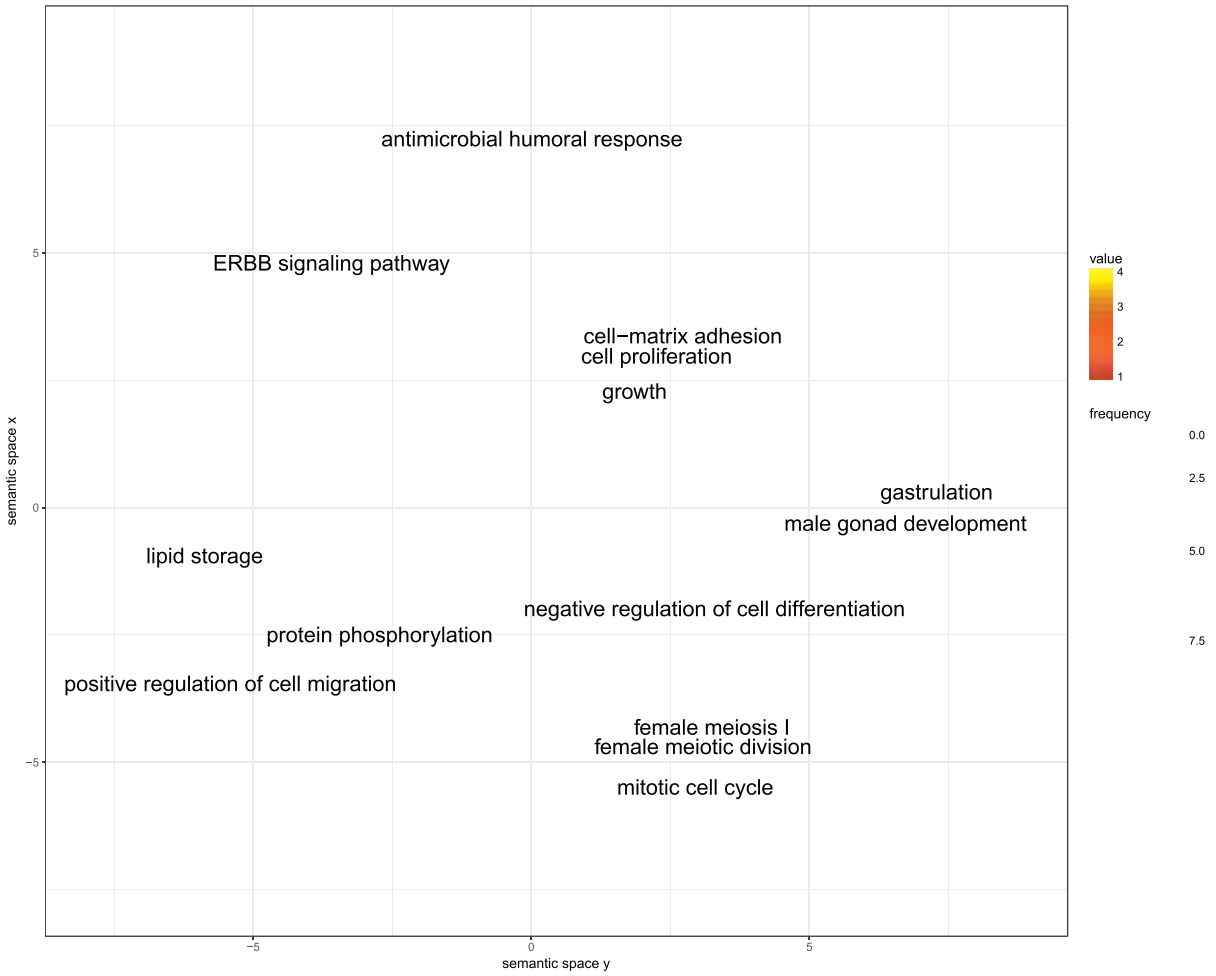

Fig. 5. Enrichment analyses of significantly expressed annotated mRNAs in L. *salmonis*. REVIGO analyses were conducted based on number of Gene Ontology categories found for biological processes. Value scale correspond to the number of times of each GO term, and frequency indicates how many GO terms are in one category.

Table 3
Target sequences for sex-biased miRNAs in *L. salmonis* with significant hits in female and male groups. Expectation was calculated by PSRNAtarget; ΔG and p-value were calculated by RNAhybrid.

Target	miRNA	Expectation	ΔG	p-Value
Female-associated miRNAs				
Chymotrypsin	bantam-1	5.0	−26.2	0.012035
Quinone oxidoreductase	mir-1	2.5	−27.2	0.006822
Troponin C	mir-100	3.0	−29.0	0.036069
Male-associated miRNAs				
60S ribosomal protein L	bantam-2	5.0	−26.4	0.011914
Cathepsin Z isoform 1	mir-263a	5.0	−25.0	0.01775
Cathepsin Z isoform 2	mir-184	5.0	−30.5	0.033076
Embryo cathepsin L-associated protein	mir-263a	5.0	−26.0	0.012793
Estradiol 17-beta-dehydrogenase	mir-276-1	5.0	−25.5	0.037063
Female lethal d-like protein	mir-2796-1	5.0	−28.1	0.014731
ATP synthase alpha subunit precursor	mir-2765-2	5.0	−34.6	0.009683
SPT transcription factor family	bantam-3	4.5	−26.0	0.022609
TSP1-containing protein	mir-993	4.5	−34.0	0.014763
Vitellogenin 2	bantam-4	4.5	−30.0	0.015241

dimorphism at the molecular level by miRNAs in marine invertebrates is yet scarce, we conducted this study to characterize a novel miRNome of *L. salmonis* related to sex-biased transcripts.

In this study, 3101 novel miRNAs were found for *L. salmonis*, which is the largest miRNome characterized for any copepod species. Previously, 673 conserved miRNAs were characterized in the parasitic copepod *Caligus rogercresseyi* (*Gallardo-Escárate* et al., *2017*). According to the last version of the mirBase release 22 (Griffiths-Jones et al., 2006), if variants and precursor sequences are included, this is the largest reported collection for miRNA sequences in any crustacean species. Indeed, if we exclusively count the annotated mature miRNAs, this is the second largest collection of miRNA families, consisting of 154 for *L. salmonis*. The largest collection corresponds to the "living fossile" tadpole shrimp collection, with 160 annotated families (Ikeda et al., 2015). Besides the advances in Next-generation sequencing to characterize novel miRNomes for non-model species (Lee et al., 2010), there is still a low number of miRNA sequences in public databases for highly-relevant taxa such as Crustacea. Our study aimed to contribute to the generation of miRNomes for crustaceans or other marine invertebrates. In this study, most of the differentially expressed genes were associated with the male group, which is congruent with expression patterns found in sex-biased miRNAs from other marine species. In gonad tissues of the yellow catfish, 204 differentially expressed miRNAs were found, where 144 were associated with male gonads and 60 with ovaries (Jing et al., 2014). A similar proportion of sex-biased miRNAs were found in the gonads of the Atlantic halibut *Hippoglossus hippoglossus* (Bizuayehu et al., 2012). Furthermore, in the Amur sturgeon (*Acipenser schrenckii*) 117 miRNAs were differentially expressed in gonads: 71 related to males and 46 to females (Zhang et al., 2018a). Conversely, invertebrate terrestrial species showed an inverse relation. From a library of 476

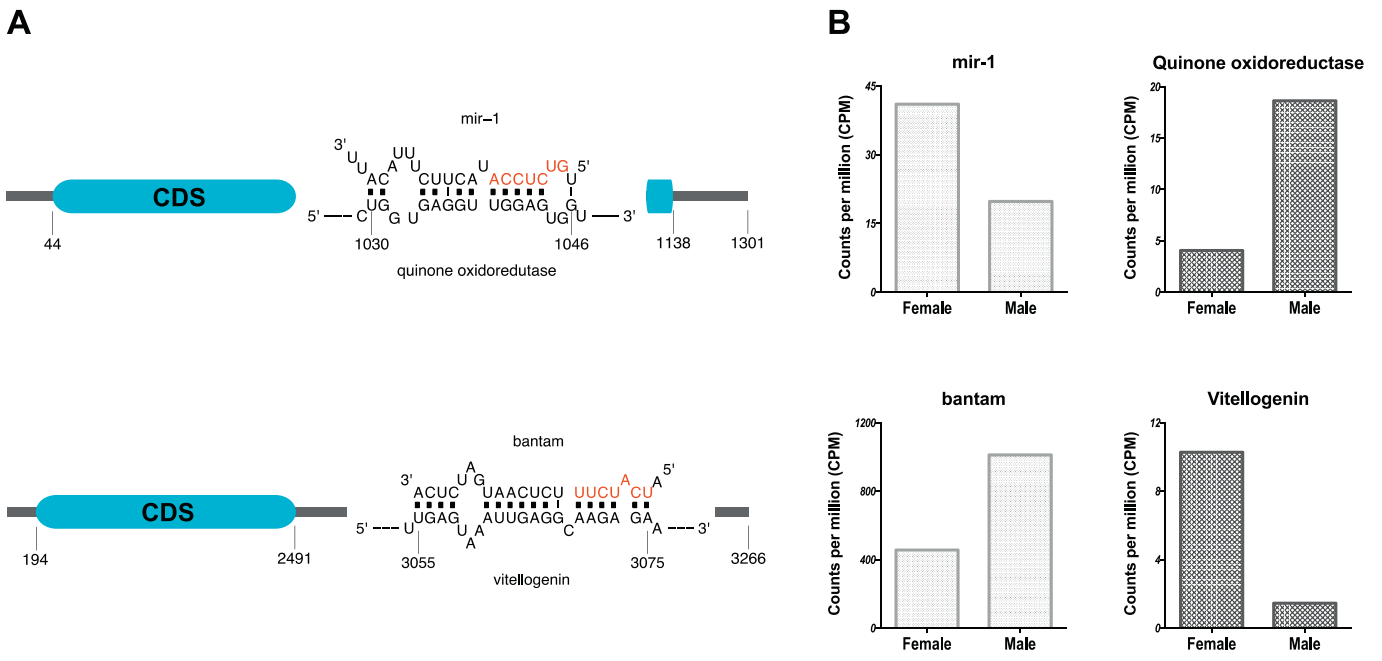

Fig. 6. Target prediction for sex-biased miRNAs in *L. salmonis*. A: schematic representation of sex-biased mRNAs and predicted pairing of miRNAs on coding region or 3′UTR region; B: expression levels (CPM values) of selected miRNAs and targets used for target prediction.

mature miRNAs found in the insect *Drosophila melanogaster*, 37 were associated with females and 28 with males (Marco, 2014). While in marine invertebrates, the only study describing sex-biased miRNAs was in the sea urchin *Strongylocentrotus nudus*, where 184 mature miRNAs were found in the gonads; 47 were male-specific and 51 female-specific (Mi et al., 2014). Regarding sea lice species, *Caligus rogercresseyi* had only two sex-biased miRNAs, which is not enough to establish a comparison with the present study (Gallardo-Escárate et al., 2017).

Regarding coding genes, most of the 1455 differentially expressed genes among sexes were related to biological processes associated with reproduction and development. According to transcriptomic profiles obtained in this study, there are more male-biased transcripts than female-biased, and also a global expression imbalance in the male group. This is congruent to what was found in other studies in sea lice species. Previously, high-throughput sequencing analyses revealed 2206 male-biased transcripts in *Caligus rogercresseyi* versus 1733 female-biased (Farlora et al., 2014). Also in *L.s salmonis* most of the differentially expressed transcripts between sexes were over-regulated in males with respect to females (Poley et al., 2016). In that study, Poley et al. (Poley et al., 2016) reported Protease inhibitor Kunitz_BPTI, Tissue factor pathway inhibitor and Zinc metalloproteinase as part of the male-biased group of transcripts, which were also found in the same condition in our study. The first two genes correspond to protease inhibitors, which in blood-sucking parasite species have been described as anticoagulant factors and defense proteins against other microbial pathogens (Ranasinghe and McManus, 2013; Rimphanitchayakit and Tassanakajon, 2010). But also in diverse arthropod species, a role for these genes in seminal fluids has been suggested, impacting male and female fertility (LaFlamme and Wolfner, 2013; Poley et al., 2016). Regarding female sex-biased transcripts, most of the top differentially expressed genes related to this group corresponded to vitellogenin genes. In most invertebrate species, vitellogenins are largely described as the major yolk proteins, allowing the formation of the vitelline granules in mature eggs (Bellés and Maestro, 2005). However, in arthropod species there are a wide diversity of vitellogenin genes with emerging novel functions (Tufail and Takeda, 2008). This is also possible for sea lice species, because of the wide diversity of vitellogenin genes found in these parasitic animals (Farlora et al., 2014; Gallardo-Escárate et al., 2014). Nonetheless, vitellogenin genes found in this study were highly similar to LsVit1 and LsVit2 genes discovered in *L. salmonis*, which are tightly linked in the oocyte maturation process of adult female lice (Dalvin et al., 2011).

Conversely to mRNA, miRNA evolution dynamics are highly divergent, and have different genomic sources for its origins (from transposable elements, gene duplications, introns, antisense transcripts, pseudogenes, intergenic regions, among others); evolving relatively easily compared to protein-coding genes; and with targets that can be easily lost or acquired (Berezikov, 2011; Campo-Paysaa et al., 2011; Grimson et al., 2008; Necsulea and Kaessmann, 2014). Results obtained in the present study, and also in other sea lice *C. rogercresseyi* species (Gallardo-Escárate et al., 2017), indicate that this also occurs in salmon lice species, showing wide miRNomes including diverse miRNA families. In contrast to what was discovered in *C. rogercresseyi*, in the salmon louse *L. salmonis* the most sex-biased miRNAs in this study corresponded to mir-1 and mir-100 for the male group; and bantam, mir-263a and mir-276 for females. A few studies have described sex-biased miRNA expression in arthropods (Freitak et al., 2012; Zhang et al., 2018b), but this is the first time that this specific cluster of miRNAs was biased for females or males, and at the same time has highly-relevant genes associated with reproduction as targets, such as vitellogenins and estradiol 17-beta-dehydrogenase. This suggests novel roles for these miRNAs in reproduction and sex differentiation in marine invertebrates.

A highly relevant group of miRNAs identified in this study for *L. salmonis* was the bantam family. This was the most diverse group in adult animals of this species, finding 667 variants out of 3101 miRNAs in total (representing > 20% of the total miRNAs found in this species). This is congruent to what was found in *C. rogercresseyi*, where 215 variants were found (Gallardo-Escárate et al., 2017). The bantam miRNA family has been associated with several biological processes in arthropod species: programmed cell death (Brennecke et al., 2003; Carrington and Ambros, 2003), boundary wing formation in insects (Becam et al., 2011); systemic growth (Boulan et al., 2013; Herranz et al., 2012); immune evasion in ectoparasites (Gallardo-Escárate et al., 2017). The only study associating bantam to reproduction was in the insect *Drosophila melanogaster*, where this miRNA suppressed the expression of fragile X mental retardation protein in ovaries, controlling germline stem cell production (Yang et al., 2009). But this is the first

time that sex-biased expression of bantam miRNAs is associated with relevant reproduction proteins in marine arthropods, by interacting with several sex-biased genes including vitellogenins and others.

In this study, we applied bioinformatic tools to infer putative mRNA targets for sex-biased miRNAs. However, our findings will be also validated by further research applying functional analyses to deeply evaluate the role of miRNAs on specific targets through luciferase-assays through application of miRNAs mimics oligonucleotides on transfected cells with vectors expressing luciferase protein and with specific target-genes promoters. In addition, it is also critical to always consider novel sequencing strategies (e.g. long-read sequencing) to deeper infer global expression patterns of true mRNAs and for further target prediction analyses.

5. Conclusions

This is the first comprehensive miRNome characterization in the salmon louse *L. salmonis*. The miRNAs identified were associated with sex-biased transcriptome expression, suggesting putative roles of miRNAs related to sex differentiation and/or reproduction. Relevant non-coding RNAs, such as bantam miRNAs, emerge with novel biological functions of importance in *L. salmonis* adults. This study contributes with valuable genomic information related to gene expression regulation in adult sea lice stages, being critical for the development of novel control methods in the salmon aquaculture.

Declaration of interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.margen.2019.01.005.

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