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MicroRNA biogenesis pathway from the salmon louse (*Caligus rogercresseyi*): Emerging role in delousing drug response

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ABSTRACT

Despite the increasing evidence of the importance of microRNAs (miRNAs) in the regulation of multiple biological processes, the molecular bases supporting this regulation are still barely understood in crustaceans. Therefore, the molecular characterization and transcriptome modulation of the miRNA biogenesis pathway were evaluated in the salmon louse *Caligus rogercresseyi*, an ectoparasite that constitutes one of the biggest concerns for salmonid aquaculture industry. Hence, RNA-Seq analysis was conducted from six different developmental stages, and also after bioassays with delousing drugs Deltamethrin and Azamethiphos using adult individuals. *In silico* analysis evidenced 24 putative genes involved in the miRNA pathway such as biogenesis, transport, maturation and miRNA-target interaction. Moreover, 243 putative single nucleotide polymorphisms (SNPs) were identified, 15 of which showed non-synonym mutations. RNA-Seq analysis revealed that CCR4-Not complex subunit 3 (*CNOT3*) was upregulated at earlier developmental stages (nauplius I–II and copepodid), and also after the exposure to Azamethiphos, but not to Deltamethrin. In contrast, the subunit 7 (*CNOT7*) showed an inverse expression pattern. Different Argonaute transcripts were associated to chalimus and adult stages, revealing specific expression patterns in response to antiparasitic drugs. Our results suggest novel insights into the regulatory network of the post-transcriptional gene regulation in *C. rogercresseyi* mediated by miRNAs, evidencing a putative role during the ontogeny and drug response.

1. Introduction

MicroRNAs (miRNAs) comprise a large group of small (~21 nucleotide) non-coding RNAs that regulate gene expression at the post-transcriptional level by interfering either in the stability or the translation of a target mRNA (Chekulaeva and Filipowicz, 2009). From the biogenesis of the precursor molecules that constitute the mature miRNAs to gene silencing, miRNAs are supported by a variety of proteins that ensure their correct functioning (Krol et al., 2010). Typically, miRNA genes are transcribed by *RNA polymerase II* into a primary miRNA (pri-miRNA) (Lee et al., 2004) that folds into hairpin(s), serving as substrate for the microprocessor complex composed of *Drosha* and *Partner of Drosha* (*Pasha* or *DGCR8*) (Gregory et al., 2004). Within this complex, pri-miRNAs are processed into a ~70 nucleotide precursor miRNA (pre-miRNA) that later is exported to the cytoplasm, mediated by *Exportin-5* (*XPO-5*) (Yi et al., 2003). Once in the cytoplasm, *Dicer* and the *Protein kinase RNA* (*PKR*) *activator* (*PACT*) process pre-miRNA into a double-stranded RNA (dsRNA) duplex containing a mature miRNA and a passenger strand (Lee et al., 2006). The duplex product is loaded onto an Argonaute protein and the passenger strand is ejected (Wilson and Doudna, 2013), thus conforming the minimal structure of the RNA-induced silencing complex (RISC) required for the recognition and cleavage of the target mRNA (Pratt and MacRae, 2009). Through base pairing, RISC is preferentially directed to the 3′ UTR of the target mRNA wherein the interaction with protein *Gawky* (*GW*) is required for the recruitment of molecular machinery for the deadenylation and translational repression of the target mRNA, such as *poly(A)-binding protein* (*PABP*) and the CCR4-NOT complex (Fabian et al., 2009; Pratt and MacRae, 2009; Hafner et al., 2011; Wilson and Doudna, 2013).

Abbreviations: MiRNA, MicroRNA; SNP, single nucleotide polymorphism; *CNOT1*, *CCR4-Not complex subunit 1*; *CNOT2*, *CCR4-Not complex subunit 2*; *CNOT3*, *CCR4-Not complex subunit 3*; *CNOT4*, *CCR4-Not complex subunit 4*; *CNOT7*, *CCR4-Not complex subunit 7*; *CNOT10*, *CCR4-Not complex subunit 10*; Delta, Deltamethrin; Aza, Azamethiphos; pri-miRNA, primary miRNA; *XPO-5*, Exportin-5; *PACT*, *protein kinase RNA activator*; DsRNA, double-stranded RNA; RISC, RNA-induced silencing complex; *GW*, *Gawky*; *PABP*, *poly(A)-binding protein*; TSA, transcriptome shotgun assembly; NCBI, National Center for Biotechnology Information; RPKM, reads per kilobase of the transcript per million mapped reads; NS, non-synonymous; Ago, Argonaute; *Ago1*, *Argonaute1*; *Ago2*, *Argonaute2*; *Ago3*, *Argonaute3*; PTGR, post-transcriptional gene silencing; DSRM, dsRNA-binding motif; RNAi, RNA interference.

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Due to the widespread distribution of miRNAs and some core miRNA-processing proteins among Metazoans, it has been proposed that this regulatory network corresponds to a conserved mechanism of post-transcriptional gene regulation (PTGR) among animals (Berezikov, 2011). In arthropods, major advances in the characterization of miRNA processing and target genes have been developed in model organism like *Drosophila* (Enright et al., 2004; Miyoshi et al., 2010), however for crustaceans this supporting mechanism is still a barely understood process and even the minimal miRNA-processing genes have not been completely characterized for a same specie. For instance, *Drosha* has been previously described only for the shrimp *Marsupenaeus japonicus* (Huang et al., 2012), meanwhile, *Pasha* only for *Litopenaeus vannamei* (Chen et al., 2012). As the key effectors of the miRNA-based silencing, the Argonaute family has been the most studied genes among the components of the miRNA pathway in crustaceans, nevertheless mainly associated with their modulation in response to viral infections (Dechklar et al., 2008; Huang and Zhang, 2012; Phetrungnapha et al., 2013a; Yang et al., 2014). Thus, a global perspective of the complete miRNA pathway and its modulation during different conditions is still needed. This information would be able to provide the baseline knowledge in order to develop more comprehensive studies into the mechanism of post-transcriptional gene regulation in crustaceans.

Ectoparasitic copepods or sea lice are one of the major concerns for the salmonid farming industry worldwide (Costello, 2009). Although not lethal, sea lice infestations induce stress response in salmonids, which leads to an immune depletion that enhances the host susceptibility to further infections (Nolan et al., 1999; Saksida et al., 2013). Commonly, delousing strategies includes the use of pesticides that are applied by bath treatments, such as the Deltamethrin and Azamethiphos. However, high economic costs associated with the implementation of these treatments (Costello, 2009), as well as their negative effects against non-target species (Ernst et al., 2014) and drug resistance of wild populations (Fast, 2014) require the generation of relevant genomic knowledge to develop integrative strategies against salmon sea lice. In this context, this study reports the transcriptome analysis of genes involved in the miRNA biogenesis pathway of *Caligus rogercresseyi*, and also evidences single nucleotide polymorphisms (SNPs) associated with the miRNA pathway. Moreover, RNA-Seq analysis revealed a transcriptional modulation during different developmental stages of the sea lice and after the exposure to delousing drugs; the Argonaute variants and different CC4-Not complex subunits being the most differentially transcribed genes.

2. Methods

2.1. Databases

Molecular characterization of the miRNA processing genes was conducted using 44,093 sequences from a transcriptome shotgun assembly (TSA) available at the National Center for Biotechnology Information (NCBI) for *C. rogercresseyi* (taxid: 217165) and 83,444 contigs yielded from the *de novo* transcriptome assembly published for this ectoparasite (Gallardo-Escarate et al., 2014). In addition, three different transcriptome sequencing datasets from *C. rogercresseyi* were used to perform RNA-Seq analysis, including (1) different developmental stages (nauplius I, nauplius II, copepodid, chalimus) (Gallardo-Escarate et al., 2014) and adult *C. rogercresseyi* exposed to 3 parts per billion (ppb) of (2) Deltamethrin (Alphamax™) and (3) Azamethiphos (Bayer™) (unpublished data). Briefly, each library was prepared from total RNA pooled from 10 different individuals and cDNA libraries were constructed using the TruSeq RNA Sample Preparation kit v2 (Illumina, San Diego, CA, USA). Two biological replicates for each sample pool were sequenced by the MiSeq (Illumina) platform using sequencing runs of 2×250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.2. Molecular characterization of miRNA processing genes

Contig annotation was performed using the CLC Genomics Workbench software (V7.1, CLC Bio, Denmark). For this, the nucleotide sequences were blasted against non-redundant (nr) protein database (Blastx) using a word size = 3, gap cost existence = 11, gap cost extension = 1 and a BLOSUM62 matrix. Thus, as it has been proposed as conserved mechanism of action among metazoans (Grimson et al., 2008; Berezikov, 2011), the different classical genes supporting the biogenesis, maturation and function of miRNAs were sought among annotated contigs.

In silico protein analysis was conducted using Geneious pro (V5.1.7, Aarhus Denmark) software. From pre-selected contigs, open reading frames were identified and protein sequences were predicted considering the standard genetic code and the ATG start codon. Putative conserved domains within amino acid sequences were blasted using NCBI domain search against de CDD V3.11-45746 PSSMs database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), considering an E-value threshold of 0.001.

2.3. Single nucleotide polymorphism identification

Candidate SNPs were identified with the following settings: window length = 11, maximum gap and mismatch count = 2, minimum average quality of surrounding bases = 15, minimum quality of central base = 20, maximum coverage = 100, minimum coverage = 20, minimum variant frequency (%) = 25.0, and maximum expected variations (ploidy) = 2. Frequencies of SNPs found in all the contigs were calculated using the same software and were tabulated in Excel spreadsheets. Furthermore, to detect whether the SNPs were synonymous or non-synonymous variants, multiple alignments and ORF identifications were used to compare to the position of the respective polymorphism to estimate the implication of amino acids variations.

2.4. Differential expression analysis

RNA-Seq analyses were conducted using the characterized genes involved in the biogenesis, maturation and function of the miRNA-based silencing. For this purpose, raw transcriptome sequencing reads from the developmental stages and adults exposed to antiparasitic drugs were mapped against the reference contigs, and the expression level of each transcript was quantified in reads per kilobase of the transcript per million mapped reads (RPKM). Different RNA-Seq analyses were performed for each library by mapping filtered reads against the identified miRNA processing genes. The considered parameters included a minimum read length fraction = 0.9, minimum read similarity fraction = 0.9 and unspecific read match limit = 10 in relation to the reference. Expression values were estimated as RPKM and then normalized by totals, using state numbers in reads per 1,000,000.

3. Results

3.1. Molecular characterization of the miRNA-processing genes

In order to identify putative genes involved in the miRNA pathway, contigs from previously published data generated through *de novo* transcriptome assembly for six different developmental stages of *C. rogercresseyi* (Gallardo-Escarate et al., 2014) were blasted against non-redundant protein database from NCBI (Blastx). Based on E-values and protein similarity, it was possible to identify 12 different groups of genes that are known to be associated with the processing (*Drosha* and *Pasha*), export (*Exportin-5*), maturation (*Dicer* and *PACT*) and function (*Argonaute*, *GW*, *PABP*, *Decapping*, *Tudor*, *CCR4-Not* and

Mov-10) of miRNAs (Fig. 1). Thus, 24 contigs were distributed among these groups, the Argonaute and the CCR4-Not having the larger number of contigs (Table 1).

To corroborate Blastx results, *in silico* protein analysis was conducted over the different contigs identified for the miRNA pathway. Thus, open reading frames (ORFs) were annotated and protein sequences were predicted for each contig (Table 2). Further, conserved domains were sought within the predicted protein sequences and were compared with previously reported proteins from different species (Table 2). Results showed that except *Pasha* and *Dicer-1*, all predicted proteins shared the conserved domains characteristics for their reference protein (Table 2). However, when multiple protein alignments are performed for both proteins, a high degree of conservation could be observed within both sequences (Figs. S1 and S2). Thereby, our results give strong evidence that the identified contigs would correspond to genes of the miRNA pathway.

3.2. Single nucleotide polymorphism (SNP) discovery

Among the 24 identified contig for miRNA pathway, 243 SNP markers were identified through *in silico* characterization, averaging approximately 10 SNPs per gene. Only 15 SNPs were identified as non-

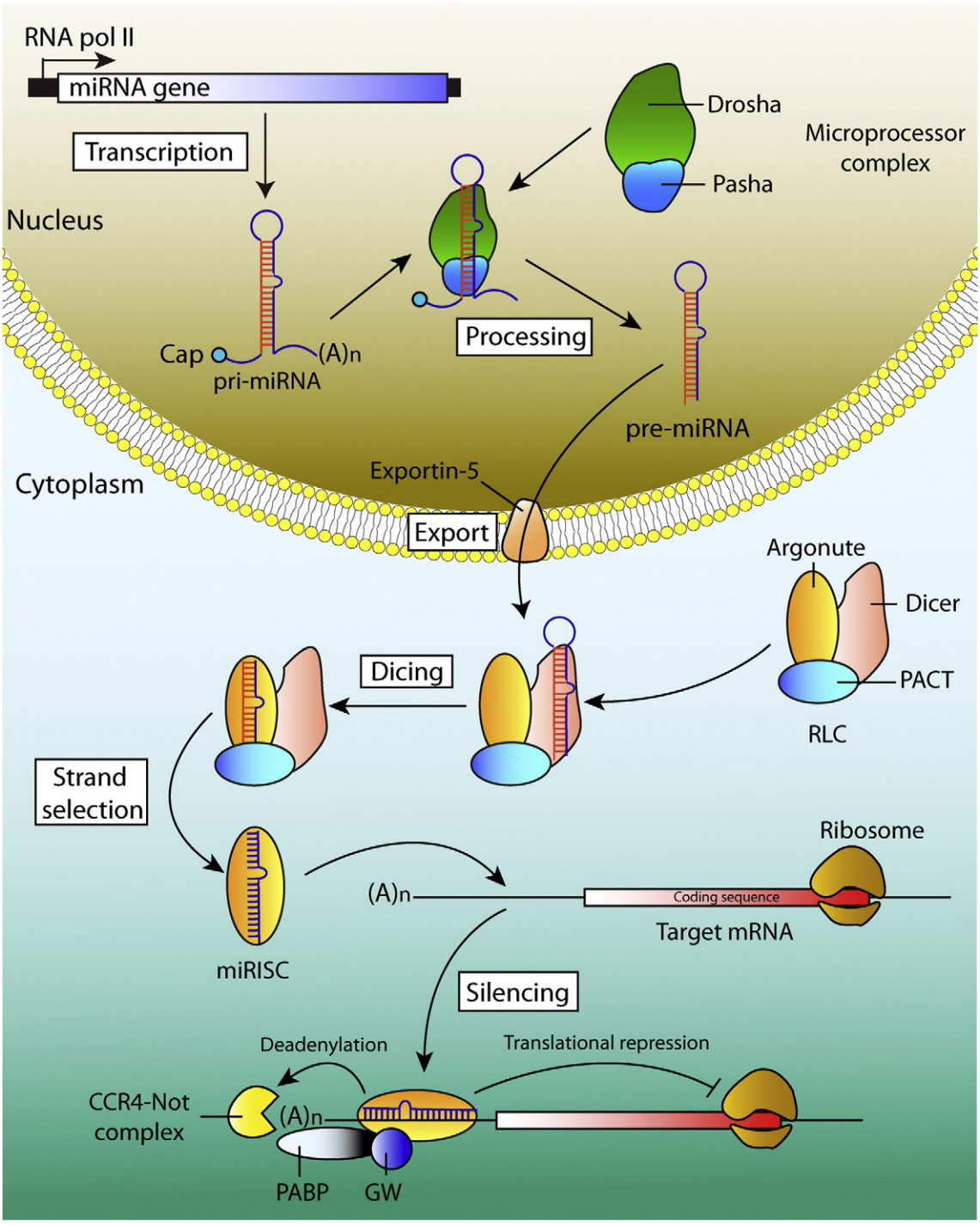

Fig. 1. Schematic representation of the putative post-transcriptional gene regulation mediated by miRNA in *C. rogercresseyi*. Different groups of genes identified in *C. rogercresseyi* and their role in the processing, export, maturation and silencing of mRNA mediated by miRNAs.

Table 1
Blastx results of the different genes identified from *C. rogercresseyi* miRNA biogenesis pathway.

Gene	Blastx hit	E-value	Identity	Accession
Drosha				
Contig3501	Ribonuclease 3 [*Acromyrmex echinatior*]	0	57%	EGI58626.1
DGCR8/Pasha				
Contig11280	Microprocessor complex subunit DGCR8-like [*Megachile rotundata*]	1.50E−134	47%	XP_003707487
Exportin-5				
Contig1401	Exportin-5 [*A. echinatior*]	0	35%	EGI70297
Argonaute				
Contig3113	Argonaute 1, partial [*Locusta migratoria*]	0	90%	AGO85968.1
Contig3668	Argonaute 1, partial [*L. migratoria*]	0	36%	AGO85968.1
Contig294	Argonaute 1 [*L. migratoria*]	5.00E−169	39%	AGH55729.1
Contig12941	Argonaute 2, partial [*L. migratoria*]	1.00E−151	38%	AGO85972.1
Contig9012	Argonaute 3 protein-like protein [*Daphnia pulex*]	1.00E−145	39%	EFX81763.1
Dicer				
Contig3759	Endoribonuclease Dcr-1 [*Camponotus floridanus*]	0	38%	EFN74275.1
Contig11486	Dicer 2 [*Litopenaeus vannamei*]	1.00E−150	30%	AEB54796.1
PACT				
Contig5117	Interferon-inducible double stranded RNA-dependent protein kinase activator A homolog [*Lepeophtheirus salmonis*]	0	87%	ACO13150
Gawky (GW)				
Contig3554	Predicted: similar to gawky CG31992-PA [*Tribolium castaneum*]	4.26E−65	39%	XP_973043
PABP				
GAZX01033907	Predicted: polyadenylate-binding protein 1-like isoform 3 [*Bombus terrestris*]	0	65%	XP_003398393.1
CCR4-Not complex				
Contig4724	Predicted: CCR4-NOT transcription complex subunit 1 isoform 2 [*Megachile rotundata*]	0	61%	XP_003704702
Contig5965	CCR4-NOT transcription complex subunit 2 [*Camponotus floridanus*]	1.25E−88	78%	EFN60402
Contig9301	CCR4-NOT transcription complex subunit 3 [*Harpegnathos saltator*]	1.22E−91	82%	EFN84777
Contig5849	CCR4-NOT transcription complex subunit 4 [*H. saltator]*	1.04E−108	74%	EFN84292
GAZX01027275	CCR4-NOT transcription complex subunit 7 [*Caligus clemensi*]	0	99%	ACO14594.1
Contig3320	Predicted: CCR4-NOT transcription complex subunit 10 isoform 3 [*Tursiops truncatus*]	8.91E−38	30%	XP_004315392
Deccaping				
Contig9709	mRNA-decapping enzyme 2 [*C. clemensi*]	0	81%	ACO14725
GAZX01019649	mRNA-decapping enzyme 1B [*Lepeophtheirus salmonis*]	0	62%	ACO12259.1
Tudor-SN				
GAZX01021891	Tudor staphylococcal nuclease [*Penaeus monodon*]	0	98%	AEK49107.1
Mov-10				
Contig9937	Predicted: putative helicase Mov10l1 [*Ornithorhynchus anatinus*]	1.90E−122	47%	XP_001513658

synonymous (NS) variants, meaning 6.17% of the total number of polymorphisms. These NS-variants were discovered in nine different key genes of the miRNA pathway of the species (Table 3). Interestingly, the greatest frequencies of the NS-variants were identified in *Argonaute* genes, especially in *Argonaute-3*, which had from 44 to 60% of allele frequencies. Also, some of the NS-variants reported in this study include substitutions between amino acids with different biochemical properties, suggesting potential changes in functional activity of the corresponding proteins.

3.3. Transcriptional modulation of miRNA processing genes: an indirect measurement of miRNA activity

Different RNA-Seq analyses were conducted in order to evaluate the transcriptional modulation of miRNA-processing genes from *C. rogercresseyi* during different developmental stages and after the exposure to the antiparasitic drugs Deltamethrin and Azamethiphos. In regard to the different developmental stages (Fig. 2), different transcriptional patterns were observed among the miRNA-processing genes, being remarkable those observed for different subunits of the CCR4-Not complex and the Argonaute family. Thereby, the subunits *CNOT3*, *CNOT7* and in a lesser extent *CNOT2* were upregulated at the earlier developmental stages nauplius-I, nauplius-II and copepodid, decreasing their transcription values in chalimus and adult stages (Fig. 2). Besides, the CCR-Not complex subunits also showed a transcriptional regulation during the exposure of the adult *C. rogercresseyi* to Deltamethrin and Azamethiphos. While *CNOT7* exhibited an upregulation both in males and females in response to Deltamethrin, *CNOT3* was upregulated after the exposure to Azamethiphos, but nevertheless just in adult females (Fig. 3).

3.4. Argonaute gene family

Due to their pivotal role in the miRNA-based gene silencing, an especial emphasis was placed on the Argonaute (Ago) family. Thus, six putative transcripts sharing the PAZ and PIWI domains were annotated to the Ago gene family in *C. rogercresseyi* databases (Table 2). Taking together *in silico* protein analysis, results showed three variants of *Ago1* transcripts, two variants of *Ago2* and one *Ago3*. Therefore, RNA-Seq analyses were conducted in order to explore the implication of miRNAs during the lifecycle and drug response of *C. rogercresseyi*. Herein, different transcription patterns were observed. For instance, during earlier developmental stages (nauplius I, nauplius II and copepodid), *Ago1* (contig3113) and *Ago3* (contig9012) showed higher expression values, *Ago1* (contig3668 and contig 294) and *Ago2* (contig12941) reached higher transcription values at late developmental stages (chalimus and adult) (Fig. 4). Coincidentally, these Ago transcripts with late transcriptional modulation also displayed differential transcription values between males and females. Meanwhile, *Ago1* (contig3668) was

Table 2
Molecular characterization of the predicted proteins from *C. rogercresseyi* miRNA biogenesis pathway.

Gene	Nt	ORF	aa	Ref aa§	Ref Dom*	Specific domains	E-value	Position
Drosha								
Contig3501	5097	3807	1269	1423	3/3	DSRM[cd00048]	5.31e−13	1083–1154
						RIBOc[cd00593]	7.00e−31	948–1028
						RIBOc[cd00593]	1.59e−35	779–889
Pasha/DGCR8								
Contig11280	5514	2412	804	633	1/2	DSRM[cd00048]	3.21e−09	531–591
Exportin-5								
Contig1401	6496	3516	1172	1214	2/2	Xpo1[pfam08389]	9.74e−23	112–189
						IBN_N[smart00913]	1.93e−05	33–101
Argonaute 1								
Contig3113	5349	2673	891	848	3/3	Piwi_ago-like[cd04657]	0	422–846
						PAZ_argonaute_like[cd02846]	9.74e−39	257–377
						DUF1785[pfam08699]	1.91e−21	205–257
Contig294						Piwi-like super family[cl00628]	1.47e−141	257–688
	4644	2190	730	837	3/3	PAZ_argonaute_like[cd02846]	1.38e−15	85–208
						DUF1785[pfam08699]	1.06e−11	34–85
Contig3668	3181	2922	974	848	3/3	Piwi_ago-like[cd04657]	1.45e−142	493–932
						PAZ_argonaute_like[cd02846]	4.67e−20	330–448
						DUF1785[pfam08699]	1.31e−10	280–230
Argonaute 2								
Contig1394	3005	2820	940	822	3/3	Piwi_ago-like[cd04657]	2.42e−179	476–910
						PAZ_argonaute_like[cd02846]	3.51e−18	321–427
						DUF1785 super family[cl07356]	5.83e−03	259–308
Contig12941	3071	2319	773	822	3/3	Piwi_ago-like[cd04657]	4.53e−169	316–743
						PAZ_argonaute_like[cd02846]	1.67e−18	159–266
						DUF1785 super family[cl07356]	3.57e−04	101–148
Argonaute 3								
Contig9012	2841	2538	846	944	2/2	Piwi_piwi-like_Euk[cd04658]	2.59e−94	477–841
						PAZ_piwi_like[cd02845]	1.23e−36	369–468
Dicer 1								
Contig3759	8519	6189	2063	1976	6/7	DSRM[cd00048]	3.63e−05	2020–2056
						RIBOc[cd00593]	2.04e−33	1812–1989
						RIBOc[cd00593]	3.77e−21	1561–1766
						PAZ_dicer_like[cd02843]	5.76e−58	933–1052
						Dicer_dimer[pfam03368]	1.33e−20	613–701
						Helicase_C[pfam00271]	7.23e−07	477–531
Dicer 2								
Contig11486	5235	4740	1580	1502	7/7	DSRM super family[cl00054]	8.54e−03	1505–1568
						RIBOc[cd00593]	1.35e−34	1331–1491
						RIBOc[cd00593]	7.53e−11	1055–1115
						PAZ super family[cl00301]	4.15e−09	867–994
						Dicer_dimer super family[cl04028]	6.67e−19	570–666
						HELICc[cd00079]	1.27e−10	352–501
						DEXDc[cd00046]	2.16e−12	25–170
PACT								
Contig5117	1698	1002	334	334	2/2	DSRM[cd00048]	4.87e−21	119–186
						DSRM[smart00358]	1.84e−08	19–65
Gawky								
Contig3554	4196	4038	1346	1014	4/4	RRM_GW182_like[cd12435]	8.64e−32	1181–1249
						M_domain super family[cl15179]	6.60e−22	752–908
						UBA[cd00194]	4.46e−06	668–699
						ARS2 super family[cl20283]	5.56e−03	539–623
PABP								
GAZX01033907	2472	1974	658	621	5/5	PABP[pfam00658]	6.45e−30	586–645
						RRM4_I_PABPs[cd12381]	6.35e−52	318–397
						RRM3_I_PABPs	5.68e−40	215–294
						RRM2_I_PABPs[cd12379]	1.06e−52	121–197
						RRM1_I_PABPs	1.17e−55	36–115
CCR4-Not complex subunit 1 (*CNOT1*)								
Contig4724	8874	7320	2440	2447	2/2	Not1[pfam04054]	0	2050–2428
						DUF3819[pfam12842]	2.25e−56	1438–1586
CCR4-Not complex subunit 2 (*CNOT2*)								
contig5965	2988	1545	515	461	1/1	NOT2_3_5[pfam04153]	2.97e−50	314–441
CCR4-Not complex subunit 3 (*CNOT3*)								
Contig9301	3048	2220	740	752	2/2	NOT2_3_5[pfam04153]	2.99e−62	604–734
						Not3[pfam04065]	1.08e−102	1–227

(continued on next page)

Table 2 *(continued)*

Gene	Nt	ORF	aa	Ref aa§	Ref Dom*	Specific domains	E-value	Position
CCR4-Not complex subunit 4 (*CNOT4*)								
Contig5849	4313	3168	1056	487	2/2	RRM_CNOT4[cd12438]	3.67e−52	108–208
						zf-RING_4[pfam14570]	3.99e−21	19–64
CCR4-Not complex subunit 7 (*CNOT7*)								
GAZX01027275	2817	1104	368	365	1/1	CAF1[pfam04857]	5.31e−109	28–256
Deccaping 1								
GAZX01019649	1884	1473	491	443	1/1	Dcp1[cd09804]	1.63e−61	4–120
Deccaping 2								
Contig9709	1348	1011	337	356	2/2	Dcp2p[cd03672]	3.03e−63	105–248
						DCP2[pfam05026]	2.26e−32	15–102
Tudor-SN								
GAZX01021891	3308	2706	902	889	5/5	TUDOR[cd04508]	2.07e−10	726–763
						SNc[cd00175]	3.42e−27	523–651
						SNc[cd00175]	4.78e−29	347–484
						SNc[cd00175]	3.68e−26	189–324
						SNc[cd00175]	5.06e−19	20–154
Mov-10								
Contig9937	5121	3396	1132	1292	2/2	AAA_12[pfam13087]	5.46e−51	881–1098
						DEXDc[cd00046]	4.25e−06	712–836

Abbreviations: Nt (nucleotide), ORFs (open reading frames), aa (amino acid). §Ref aa: number of amino acids present in the reference protein. *Ref Dom: represents the number of domains from the predicted protein regarding reference protein. Thus, predicted domains/reference domains.

preferentially transcribed in adult females, *Ago1* (contig294) and *Ago2* showed higher RPKM values in adult males (Fig. 4).

Regarding Ago gene expression during the exposure to delousing drugs, a similar transcriptome modulation was observed among Agos with respect to the antiparasitic drugs used (Fig. 5). Thus, *Ago1* (contig294) displayed upregulation in response to both antiparasitic drugs. Furthermore, the sex-dependent transcription patterns observed for Agos in adults of *C. rogercresseyi* were also maintained in drug challenged individuals (.5).

4. Discussion

Despite that miRNAs are known to be important modulators of regulation (PTGR) in animals, the molecular basis of this regulation in crustaceans is not well understood. The present study identified 24 genes involved in the processing, export, maturation and function of miRNAs from the ectoparasite copepod *C. rogercresseyi*. Among them, *Argonaute* and *Dicer* have been the most studied genes in crustaceans (Dechklar et al., 2008; Su et al., 2008; Yao et al., 2010; Chen et al., 2011; Huang and Zhang, 2012; Li et al., 2013; Phetrungnapha et al., 2013a; Yang et al., 2014), followed by *Drosha*, *Pasha* and *Tudor-SN* (Chen et al., 2012; Huang et al., 2012; Phetrungnapha et al., 2013b). However, according to our knowledge, the transcriptome analysis is the first that reports *Exportin-5*, *GW*, *Mov-10* and the subunits of the CCR4-Not complex *CNOT1*, *CNOT2*, *CNOT3*, *CNOT4* and *CNOT10* in crustaceans.

Table 3
Identification of non-synonym SNPs identified from *C. rogercresseyi* miRNA biogenesis pathway.

Gene	Position (nt)	Ref	Allele	Freq	Position (aa)	Ref	Substitution
Drosha	1347	G	C	31%	430	E	D
Pasha/DGCR8	2474	C	T	47%	183	P	L
Exportin-5	2558	T	G	26%	852	N	K
Argonaute2	636	C	A	40%	86	Q	K
Argonaute2	1417	G	A	25%	346	G	D
Argonaute3	551	T	C	60%	184	V	A
Argonaute3	556	T	C	51%	168	S	P
Argonaute3	2559	T	A	44%	835	N	K
Dicer1	746	A	G	20%	246	I	M
Dicer2	836	G	T	25%	235	E	D
Dicer2	1098	C	A	43%	323	L	I
CCR4-Not subunit 1	1227	A	G	20%	189	K	E
CCR4-Not subunit 1	3324	A	G	26%	894	T	A
CCR4-Not subunit 3	1596	T	C	39%	439	S	P
CCR4-Not subunit 3	1936	T	G	21%	552	V	G

In silico protein analysis of the miRNA-processing genes revealed characteristic conserved domains for each reference sequence. However, *Pasha* and *Dicer*-1 from *C. rogercresseyi* did not match with known domains, contrary to *Pasha* from the shrimp *L. vannamei* in which N-terminal WW domain and C-terminal dsRNA-binding motif (DSRM) were found (Chen et al., 2012). Multiple protein alignments of this protein with different *Pasha* sequences among arthropods showed a high degree of conservation in the WW domain region (data not shown). Notwithstanding, it has also been reported that in several insects *Pasha* lacks the N-terminal WW domain (Shreve et al., 2013). On the other hand, while seven different domains have been reported for *Dicer-1* in *L. vannamei* (Yao et al., 2010), the predicted protein for *C. rogercresseyi* lacked the N-terminal helicase domain, similar to sequences reported from *Drosophila melanogaster* and *Anopheles gambiae* (Shreve et al., 2013).

Despite that further experiments were not performed in order to corroborate the implication of these characterized genes in the miRNA pathway, the presence of these core processing proteins and the functional conserved domains within them suggest a similar mechanism to the common miRNA processing pathway for *C. rogercresseyi*. Besides, it has to be emphasized that *Pasha* has been described as a protein that exclusively function in the miRNA processing pathway (Berezikov, 2011). In addition, the presence of miRNA genes and miRNA-processing genes in species from early branches of the metazoan evolution has suggested a common origin of the miRNA-processing machine in animals (Grimson et al., 2008; Berezikov, 2011) and therefore, similar conserved mechanism of action among them is expected.

SNP variations identified in genes involved in miRNA biogenesis pathway suggest putative functional polymorphisms involved in post-transcriptional gene regulation. As previous studies have revealed the presence of SNPs in this species (Nuñez-Acuña et al., 2014), efforts to identify these molecular markers in a key pathway of *C. rogercresseyi* should give the basis to develop further relevant studies including different genotypes and phenotypes. With respect to SNPs in the miRNA

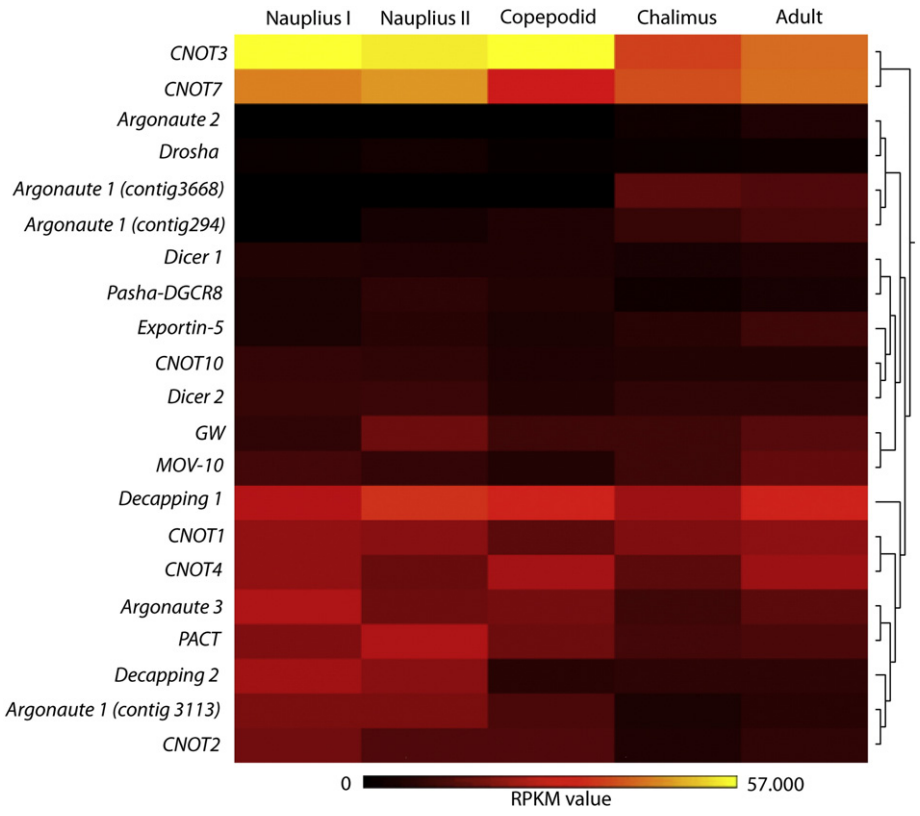

Fig. 2. Transcriptional abundance of the miRNA pathway genes during the developmental stages of *C. rogercresseyi*. Normalized RPKM values for each gene are represented through a color scale, ranging from black (low), red (medium) to yellow (high).

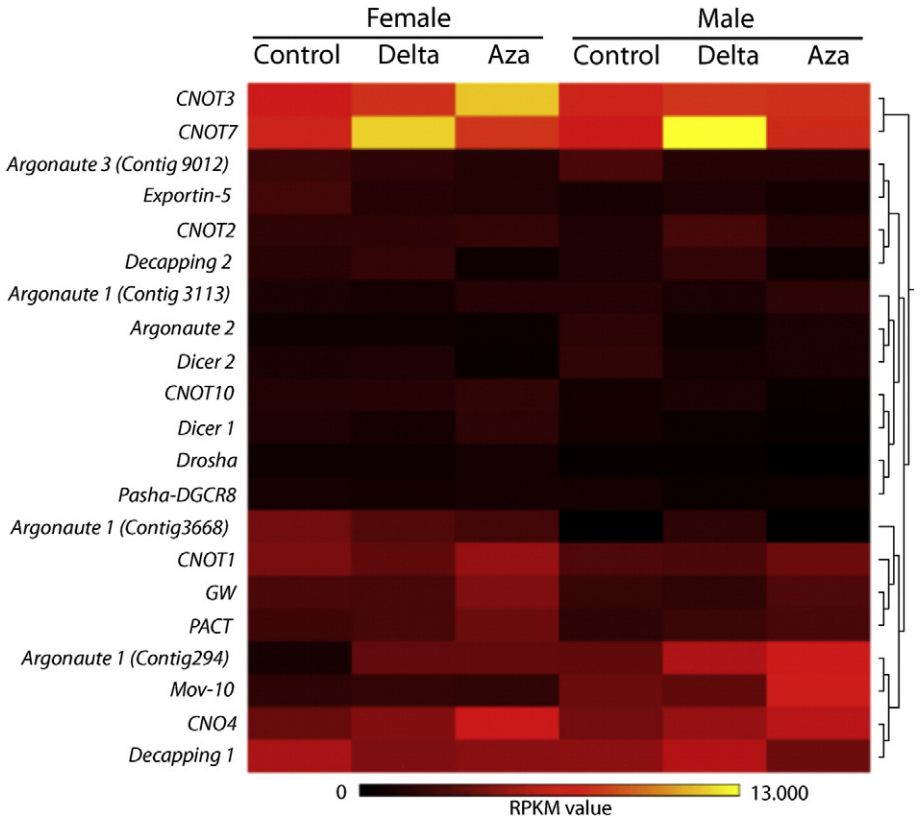

Fig. 3. Sex-dependent transcriptional response of the miRNA pathway genes in *C. rogercresseyi* exposed to Deltamethrin (Delta) and Azamethiphos (Aza). Normalized RPKM values for each gene are represented through a color scale, ranging from black (low), red (medium) to yellow (high).

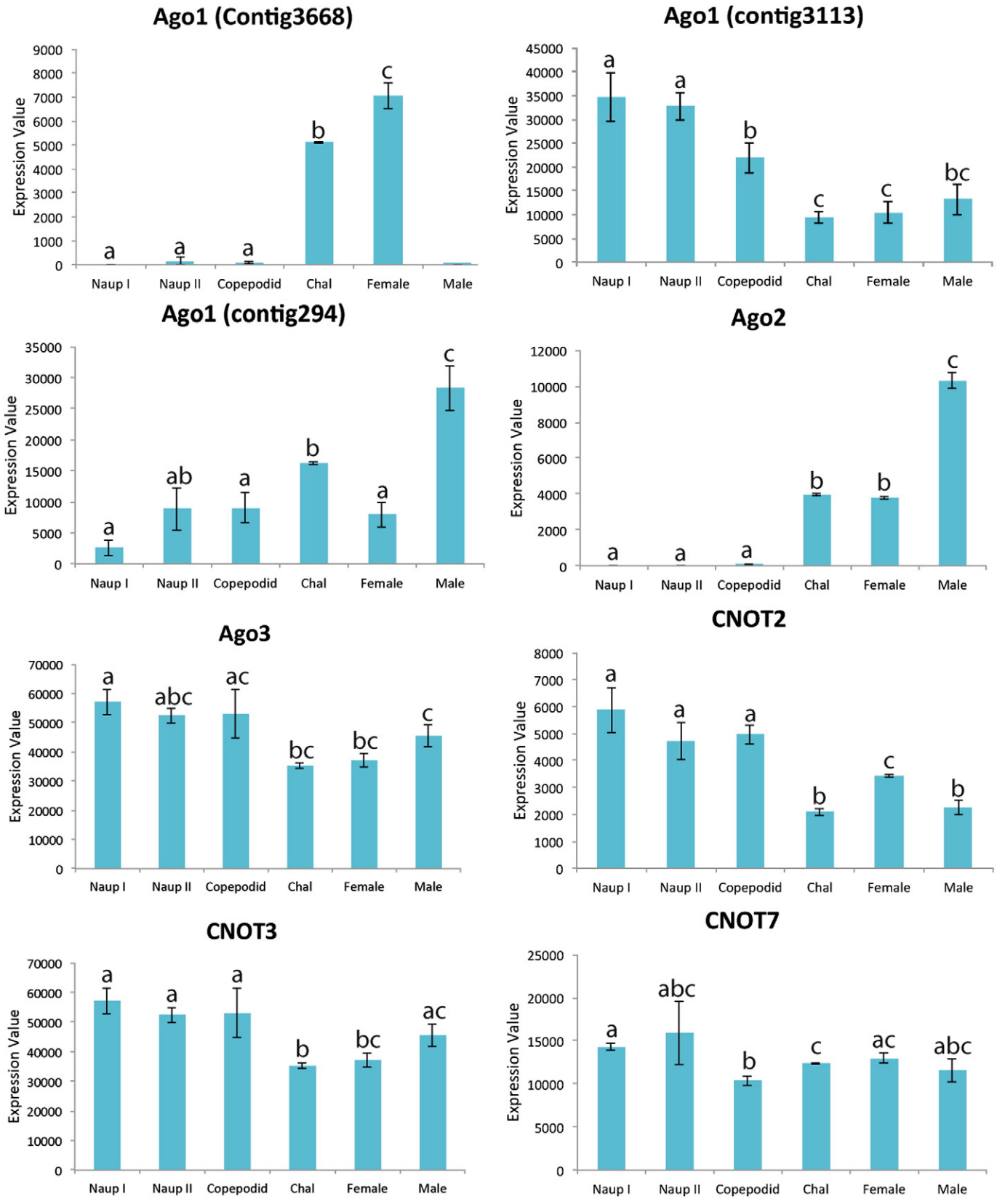

Fig. 4. Mean expression values for different developmental stages. Mean expression values for the most differentially transcribed genes within nauplius I, nauplius II, copepodid, chalimus and adult female and male. Data are presented as mean ± SE and different letters indicate significant differences among groups by Baggerly's *t*-test with $P < 0.05$.

pathway, previous studies have suggested associations between polymorphisms and potential risk of diseases (Ma et al., 2012).

In order to explore the role of miRNAs in the regulation of the development and drug response in *C. rogercresseyi*, the transcriptional modulation of the different miRNA-processing genes was evaluated. Thus, RNA-Seq analyses were conducted over different developmental stages and after the exposure of adults (males and females) to the most common delousing drugs. Although different transcription

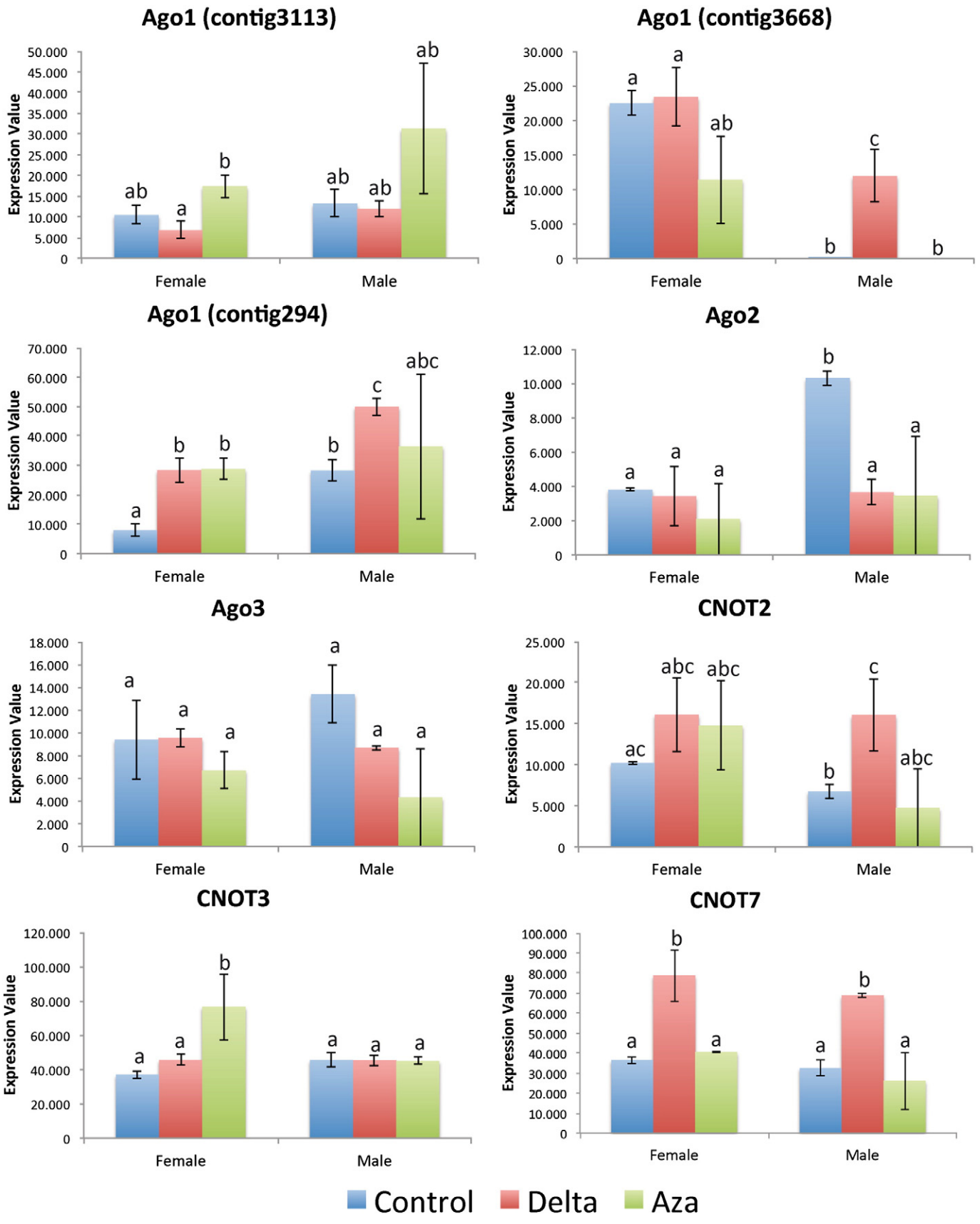

Fig. 5. Mean expression values for *C. rogercresseyi* after drug exposure. Mean expression values for the most differentially transcribed genes within adult female and male exposed to Deltamethrin (red) and Azamethiphos (green). Data are presented as mean ± SE and different letters indicate significant differences among groups by Baggerly's *t*-test with $P < 0.05$.

patterns were observed, higher transcriptional modulations were found within subunits of the CCR4-Not complex (*CNOT3* and *CNOT7*) and members the Ago family. The interaction of CCR4-Not complex with RISC for the deadenylation and translational repression gives the complex the possibility to be a versatile machinery that regulates gene expression over a diverse range of biological processes (Collart and Panasenko, 2012). Among them, it has been shown that in *Drosophila* mutations in the subunits *CNOT3* and *CNOT7* (*NOT3* and *POP2* respectively in *Drosophila*) are lethal at early developmental stages (Neely et al., 2010; Temme et al., 2014). The results of the present study revealed that both subunits were upregulated in nauplius and copepodid stages in *C. rogercresseyi*, suggesting that the CCR4-Not complex may also be an important factor in the development of early instars. With the increasing evidence of the regulatory role of this complex over the expression of multiple gene networks, new processes influenced by the modulation of the CCR4-Not has been described (Chen et al., 2011). For instance, it has been shown that the activity of this complex is related to the modulation of key pathways for virulence in pathogenic species, and even conditioning the antifungal drug susceptibility in yeast (Panepinto et al., 2013). Considering increased transcriptional response of *CNOT3* and *CNOT7* after the exposure to Azamethiphos and Deltamethrin respectively, our results suggest that this complex may also be orchestrating gene expression in drug response pathways in *C. rogercresseyi*. As delousing strategies are mainly based on antiparasitic drugs, further experiments are needed in order to elucidate the role of the CCR4-Not complex during drug response.

Since members of the *Ago* gene family have been described as the key mediators of the miRNA-based gene silencing (Hutvagner and Simard, 2008), an especial emphasis was placed on this family. Thus, five putative Ago contigs were identified within the analyzed databases with their respective predicted proteins sharing the PAZ and PIWI domains characteristic for this family (Hock and Meister, 2008) and clustered them among *Ago1* (3), *Ago2* (1) and *Ago3* (1) variants. Different organisms encode for multiple Ago variants, ranging from one in the yeast *Schizosaccharomyces pombe* to 27 in *Caenorhabditis elegans* (Carmell et al., 2002; Yigit et al., 2006; Hock and Meister, 2008). Meanwhile in arthropods, five different Agos have been previously described for *D. melanogaster* (Williams and Rubin, 2002), the same number of Ago variants identified for *C. rogercresseyi*. Interestingly, different transcription patterns were observed during the development and drug exposure of *C. rogercresseyi*. Besides the similar organization of the PAZ and PIWI domains in the Ago family, it has been suggested that *D. melanogaster* Agos are not redundant sequences, and therefore each variant can be implicated in the regulation of a different biological process (Rehwinkel et al., 2006), and also overlapping regulation can be observed (Okamura et al., 2004). Thereby, the importance of a particular Ago transcript during the ontogeny and through drug response can also be dynamic in *C. rogercresseyi*. Herein, *Ago1* (contig3113) and *Ago3* (contig9012) demonstrated higher transcriptional values at early developmental stages, while *Ago1* (contig3668 and contig294) and *Ago2* (contig12941) were upregulated in late developmental stages. Although the role of miRNAs in the development of sea lice is not well understood, different evidence suggests that the knockdown of genes through RNA interference (RNAi) can directly impact the correct development of the sea lice *Lepeophtheirus salmonis* (Campbell et al., 2009; Dalvin et al., 2009; Eichner et al., 2014). Moreover, in arthropods such as *D. melanogaster*, it has been suggested that miRNAs may play pivotal roles in embryonic development (Aboobaker et al., 2005). Besides, *Ago1* and *Ago3* have been proposed as essential factors for the efficient RNAi response in embryos (Williams and Rubin, 2002) and for the early somatic development in *Drosophila* (Mani et al., 2014), respectively. On the other hand, it has been recently evidenced that *Ago2* had an increased transcriptomic response in late larval stages of the crustacean *Homarus americanus* (Hines et al., 2014), consistent with the late transcription of the gene observed in *C. rogercresseyi*.

Ago variants that exhibited an increased transcriptional response in late developmental stages (*i.e.* chalimus) also showed a sex-dependent transcriptional modulation. While *Ago1* (contig3668) reached higher RPKM values in females, *Ago1* (contig294) and *Ago2* showed a peak of transcription in males. These sex-dependent patterns were also maintained during the exposure to Deltamethrin and Azamethiphos between males and females. Considering that sexual dimorphism in *C. rogercresseyi* starts to appear in the chalimus stage (Gonzalez and Carvajal, 2003), our results suggest that within the spectrum of biological processes regulated by the Ago proteins, some variants such as *Ago1* (contig3668) and *Ago2* could be implicated in the regulation of molecular pathways exclusive for each sex, such as those involved in the sexual maturation and reproduction. Although the sex-dependent transcription patterns were maintained after the exposure to antiparasitic drugs, an increased transcription response of the *Ago1* (contig294) was observed between males and females exposed to Deltamethrin and Azamethiphos. This result indicates that miRNAs could also be involved in the modulation of *C. rogercresseyi* during drug response, congruently with recent evidence that miRNAs fulfill critical roles in drug response through the regulation of drug-related gene expression (Garofalo et al., 2008; Zhang and Dolan, 2010; Rukov and Shomron, 2011; Koturbash et al., 2012). Furthermore, considering the increasing resistance that has been reported for *L. salmonis* to antiparasitic drugs (Eichner et al., 2014) and the emerging role of miRNAs in drug response, it is necessary to elucidate if miRNAs can influence the sensitivity of the sea lice to delousing drugs and how this knowledge could be used to develop novel strategies for *C. rogercresseyi* control.

5. Conclusions

The increasing genetic resources available for the sea lice have allowed developing more comprehensive studies into the molecular biology of these copepods. Herein, a molecular and functional characterization of the supporting mechanism of miRNA-based gene silencing was accomplished. Our results reveal novel insights into the nature of this regulation in crustaceans, and evidence how some of the components of this pathway could be fulfilling pivotal roles in the regulation of the development and drug response in *C. rogercresseyi*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.11.008.

References

Aboobaker, A.A., Tomancak, P., Patel, N., Rubin, G.M., Lai, E.C., 2005. Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development. Proc. Natl. Acad. Sci. U. S. A. 102, 18017–18022.

Berezikov, E., 2011. Evolution of microRNA diversity and regulation in animals. Nat. Rev. Genet. 12, 846–860.

Campbell, E.M., Pert, C.C., Bowman, A.S., 2009. RNA-interference methods for gene-knockdown in the sea louse, *Lepeophtheirus salmonis*: studies on a putative prostaglandin E synthase. Parasitology 136, 867–874.

Carmell, M.A., Xuan, Z.Y., Zhang, M.Q., Hannon, G.J., 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. 16, 2733–2742.

Chekulaeva, M., Filipowicz, W., 2009. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr. Opin. Cell Biol. 21, 452–460.

Chen, Y.H., Jia, X.T., Zhao, L., Li, C.Z., Zhang, S.A., Chen, Y.G., Weng, S.P., He, J.G., 2011. Identification and functional characterization of Dicer2 and five single VWC domain proteins of *Litopenaeus vannamei*. Dev. Comp. Immunol. 35, 661–671.

Chen, Y.H., Zhao, L., Jia, X.T., Li, X.Y., Li, C.Z., Yan, H., Weng, S.P., He, J.G., 2012. Isolation and characterization of cDNAs encoding Ars2 and Pasha homologues, two components of the RNA interference pathway in *Litopenaeus vannamei*. Fish Shellfish Immunol. 32, 373–380.

Collart, M.A., Panasenko, O.O., 2012. The Ccr4-Not complex. Gene 492, 42–53.
Costello, M.J., 2009. The global economic cost of sea lice to the salmonid farming industry. J. Fish Dis. 32, 115–118.
Dalvin, S., Frost, P., Biering, E., Hamre, L.A., Eichner, C., Krossoy, B., Nilsen, F., 2009. Functional characterisation of the maternal yolk-associated protein (LsYAP) utilising systemic RNA interference in the salmon louse (*Lepeophtheirus salmonis*) (Crustacea: Copepoda). Int. J. Parasitol. 39, 1407–1415.
Dechklar, M., Udomkit, A., Panyim, S., 2008. Characterization of Argonaute cDNA from *Penaeus monodon* and implication of its role in RNA interference. Biochem. Biophys. Res. Commun. 367, 768–774.
Eichner, C., Nilsen, F., Grotmol, S., Dalvin, S., 2014. A method for stable gene knock-down by RNA interference in larvae of the salmon louse (*Lepeophtheirus salmonis*). Exp. Parasitol. 140, 44–51.
Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., Marks, D.S., 2004. MicroRNA targets in *Drosophila*. Genome Biol. 5, R1.
Ernst, W., Doe, K., Cook, A., Burridge, L., Lalonde, B., Jackman, P., Aube, J.G., Page, F., 2014. Dispersion and toxicity to non-target crustaceans of azamethiphos and deltamethrin after sea lice treatments on farmed salmon, *Salmo solar*. Aquaculture 424, 104–112.
Fabian, M.R., Mathonnet, G., Sundermeier, T., Mathys, H., Zipprich, J.T., Svitkin, Y.V., Rivas, F., Jinek, M., Wohischlegel, J., Doudna, J.A., Chen, C.Y.A., Shyu, A.B., Yates, J.R., Hannon, G.J., Filipowicz, W., Duchaine, T.F., Sonenberg, N., 2009. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol. Cell 35, 868–880.
Fast, M.D., 2014. Fish immune responses to parasitic copepod (namely sea lice) infection. Dev. Comp. Immunol. 43, 300–312.
Gallardo-Escarate, C., Valenzuela-Munoz, V., Nunez-Acuna, G., 2014. RNA-Seq analysis using de novo transcriptome assembly as a reference for the salmon louse *Caligus rogercresseyi*. PLoS One 9.
Garofalo, M., Condorelli, G., Croce, C.M., 2008. MicroRNAs in diseases and drug response. Curr. Opin. Pharmacol. 8, 661–667.
Gonzalez, L., Carvajal, J., 2003. Life cycle of *Caligus rogercresseyi*, (Copepoda: Caligidae) parasite of Chilean reared salmonids. Aquaculture 220, 101–117.
Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., Shiekhattar, R., 2004. The microprocessor complex mediates the genesis of microRNAs. Nature 432, 235–240.
Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B.J., Chiang, H.R., King, N., Degnan, B.M., Rokhsar, D.S., Bartel, D.P., 2008. Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. Nature 455, 1193–1197.
Hafner, M., Ascano, M., Tuschl, T., 2011. New insights in the mechanism of microRNA-mediated target repression. Nat. Struct. Mol. Biol. 18, 1181–1182.
Hines, D.J., Clark, K.F., Greenwood, S.J., 2014. Global gene expression profiling of *Homarus americanus* (Crustacea) larval stages during development and metamorphosis. Invertebr. Reprod. Dev. 58, 97–107.
Hock, J., Meister, G., 2008. The Argonaute protein family. Genome Biol. 9.
Huang, T.Z., Zhang, X.B., 2012. Contribution of the Argonaute-1 isoforms to invertebrate antiviral defense. PLoS One 7.
Huang, T.Z., Xu, D.D., Zhang, X.B., 2012. Characterization of shrimp Drosha in virus infection. Fish Shellfish Immunol. 33, 575–581.
Hutvagner, G., Simard, M.J., 2008. Argonaute proteins: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. 9, 22–32.
Koturbash, I., Beland, F.A., Pogribny, I.P., 2012. Role of microRNAs in the regulation of drug metabolizing and transporting genes and the response to environmental toxicants. Expert. Opin. Drug Metab. Toxicol. 8, 597–606.
Krol, J., Loedige, I., Filipowicz, W., 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11, 597–610.
Lee, Y., Kim, M., Han, J.J., Yeom, K.H., Lee, S., Baek, S.H., Kim, V.N., 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 23, 4051–4060.
Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., Kim, V.N., 2006. The role of PACT in the RNA silencing pathway. EMBO J. 25, 522–532.
Li, X.L., Yang, L.S., Jiang, S., Fu, M.J., Huang, J.H., Jiang, S.G., 2013. Identification and expression analysis of Dicer2 in black tiger shrimp (*Penaeus monodon*) responses to immune challenges. Fish Shellfish Immunol. 35, 1–8.
Ma, H.X., Yuan, H., Yuan, Z.Y., Yu, C.J., Wang, R.X., Jiang, Y., Hu, Z.B., Shen, H.B., Chen, N., 2012. Genetic variations in key microRNA Processing genes and risk of head and neck cancer: a case–control study in Chinese population. PLoS One 7.
Mani, S.R., Megosh, H., Lin, H.F., 2014. PIWI proteins are essential for early *Drosophila embryogenesis*. Dev. Biol. 385, 340–349.
Miyoshi, K., Miyoshi, T., Hartig, J.V., Siomi, H., Siomi, M.C., 2010. Molecular mechanisms that funnel RNA precursors into endogenous small-interfering RNA and microRNA biogenesis pathways in *Drosophila*. RNA 16, 506–515.
Neely, G.G., Kuba, K., Cammarato, A., Isobe, K., Amann, S., Zhang, L.Y., Murata, M., Elmen, L., Gupta, V., Arora, S., Sarangi, R., Dan, D., Fujisawa, S., Usami, T., Xia, C.P., Keene, A.C., Alayari, N.N., Yamakawa, H., Elling, U., Berger, C., Novatchkova, M., Koglgruber, R., Fukuda, K., Nishina, H., Isobe, M., Pospisilik, J.A., Imai, Y., Pfeufer, A., Hicks, A.A., Pramstaller, P.P., Subramaniam, S., Kimura, A., Ocorr, K., Bodmer, R., Penninger, J.M., 2010. A global in vivo *Drosophila* RNAi screen identifies NOT3 as a conserved regulator of heart function. Cell 141, 142–153.
Nolan, D.T., Reilly, P., Bonga, S.E.W., 1999. Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in postsmolt Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 56, 947–959.
Nuñez-Acuña, G., Valenzuela-Muñoz, V., Gallardo-Escárate, C., 2014. High-throughput SNP discovery and transcriptome expression profiles from the salmon louse *Caligus rogercresseyi* (Copepoda: Caligidae). Comp. Biochem. Physiol. A: Genomics Proteomics 10, 9–21.
Okamura, K., Ishizuka, A., Siomi, H., Siomi, M.C., 2004. Distinct roles for argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. 18, 1655–1666.
Panepinto, J.C., Heinz, E., Traven, A., 2013. The cellular roles of Ccr4-NOT in model and pathogenic fungi — implications for fungal virulence. Front. Genet. 4.
Phetrungnapha, A., Ho, T., Udomkit, A., Panyim, S., Ongvarrasopone, C., 2013a. Molecular cloning and functional characterization of Argonaute-3 gene from *Penaeus monodon*. Fish Shellfish Immunol. 35, 874–882.
Phetrungnapha, A., Panyim, S., Ongvarrasopone, C., 2013b. Penaeus monodon Tudor staphylococcal nuclease preferentially interacts with N-terminal domain of Argonaute-1. Fish Shellfish Immunol. 34, 875–884.
Pratt, A.J., MacRae, I.J., 2009. The RNA-induced silencing complex: a versatile gene-silencing machine. J. Biol. Chem. 284, 17897–17901.
Rehwinkel, J., Natalin, P., Stark, A., Brennecke, J., Cohen, S.M., Izaurralde, E., 2006. Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in *Drosophila melanogaster*. Mol. Cell. Biol. 26, 2965–2975.
Rukov, J.L., Shomron, N., 2011. MicroRNA pharmacogenomics: post-transcriptional regulation of drug response. Trends Mol. Med. 17, 412–423.
Saksida, S.M., Morrison, D., McKenzie, P., Milligan, B., Downey, E., Boyce, B., Eaves, A., 2013. Use of Atlantic salmon, *Salmo salar* L., farm treatment data and bioassays to assess for resistance of sea lice, *Lepeophtheirus salmonis*, to emamectin benzoate (SLICE (R)) in British Columbia, Canada. J. Fish Dis. 36, 515–520.
Shreve, J.T., Shukle, R.H., Subramanyam, S., Johnson, A.J., Schemerhorn, B.J., Williams, C.E., Stuart, J.J., 2013. A genome-wide survey of small interfering RNA and microRNA pathway genes in a galling insect. J. Insect Physiol. 59, 367–376.
Su, J.S., Oanh, D.T.H., Lyons, R.E., Leeton, L., van Hulten, M.C.W., Tan, S.H., Song, L., Rajendran, K.V., Walker, P.J., 2008. A key gene of the RNA interference pathway in the black tiger shrimp, *Penaeus monodon*: identification and functional characterisation of Dicer-1. Fish Shellfish Immunol. 24, 223–233.
Temme, C., Simonelig, M., Wahle, E., 2014. Deadenylation of mRNA by the CCR4-NOT complex in *Drosophila*: molecular and developmental aspects. Front. Genet. 5.
Williams, R.W., Rubin, G.M., 2002. ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos. Proc. Natl. Acad. Sci. U. S. A. 99, 6889–6894.
Wilson, R.C., Doudna, J.A., 2013. Molecular mechanisms of RNA interference. Annu. Rev. Biophys. 42 (42), 217–239.
Yang, L.S., Li, X.L., Jiang, S., Qiu, L.H., Zhou, F.L., Liu, W.J., Jiang, S.G., 2014. Characterization of Argonaute2 gene from black tiger shrimp (*Penaeus monodon*) and its responses to immune challenges. Fish Shellfish Immunol. 36, 261–269.
Yao, X.M., Wang, L.L., Song, L.S., Zhang, H.A., Dong, C.H., Zhang, Y., Qiu, L.M., Shi, Y.H., Jianmin, Z.M., Bi, Y.K., 2010. A Dicer-1 gene from white shrimp *Litopenaeus vannamei*: expression pattern in the processes of immune response and larval development. Fish Shellfish Immunol. 29, 565–570.
Yi, R., Qin, Y., Macara, I.G., Cullen, B.R., 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17, 3011–3016.
Yigit, E., Batista, P.J., Bei, Y.X., Pang, K.M., Chen, C.C.G., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., Mello, C.C., 2006. Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell 127, 747–757.
Zhang, W., Dolan, M.E., 2010. The emerging role of microRNAs in drug responses. Curr. Opin. Mol. Ther. 12, 695–702.