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RESEARCH

Silencing of the *Vasa* gene by RNA Interference Affects Embryonic Development and Reproductive Output in the Sea Louse *Caligus rogercresseyi*

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Abstract

The sea louse *Caligus rogercresseyi* is a major ectoparasitic copepod that causes significant economic losses in the salmon farming industry. Despite recent advancements, the mechanisms underlying germline and embryo development in this species remain poorly understood. The *Vasa* gene encodes a highly conserved DEAD box helicase that is required for germ cell formation and function in many species. In this study, the *Vasa* gene was characterized in *C. rogercresseyi*, and its expression and function were analyzed. Phylogenetic analysis showed that the *Cr-Vasa* gene product formed clusters in clades with Vasa proteins from closely related species of crustaceans. *Cr-Vasa* gene expression patterns were assessed by qPCR, and the results showed a significantly higher relative expression level in adult females compared to copepodid, chalimus, and adult male stages. Tissue-specific localization of *Cr-Vasa* mRNA in *C. rogercresseyi* was determined using chromogenic in situ hybridization, and strong positive signal was observed in male testes, but also in the intestine and cuticle, while in females, it was observed in the ovaries, oocytes, cuticle, intestine, and egg strings. RNAi-mediated gene silencing of *Cr-Vasa* impacted embryonic development and reproductive output in adult female lice. Females from the ds*Vasa*-treated group displayed unusual phenotypes, including shorter egg strings with numerous extra-embryonic inclusions, irregularly shaped abnormal embryos, and aborted egg strings. This study provides insights into the role of the *Vasa* gene in *C. rogercresseyi* embryonic development and reproductive output, which may have implications for the control of this parasitic copepod in the salmon farming industry.

Keywords *Vasa* · Germline cells · RNAi · Gene expression analysis · *Caligus rogercresseyi*

Introduction

Caligus rogercresseyi, a common sea lice species, causes significant economic harm to the salmon industry (Bravo 2010; Costello 2009; Dresdner et al. 2019). This parasite can lead to skin lesions in the host, making it more susceptible to bacterial and viral infections (Gallardo-Escárate et al. 2019). The negative impact on the host's health and the economic consequences highlights the need for further research on the molecular processes involved in the

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reproduction and development of this species. Transcriptomic studies have reported differences in gene expression between sexes (Farlora et al. 2014, 2016) as well as single nucleotide polymorphisms (SNPs) in sex-biased transcripts (Farlora et al. 2015). Additionally, research has been done on the metabolic pathways involved in ecdysteroid synthesis in this species (Gonçalves et al. 2014). More recently, our laboratory conducted a study in which we silenced the *retinoid X receptor* (*Cr-RXR*) gene in *C. rogercresseyi* adult females. The results showed that this caused a delay in egg string production, reduced fecundity, and abnormal development of the gonads and embryos (Bustos et al. 2023). Despite these recent advancements, the molecular mechanisms underlying germline and embryo development in this species remain poorly understood.

The Vasa protein, a member of the DEAD box helicase family, plays a crucial role in germline and embryo development. Several reviews detail the function of Vasa (Dehghani and Lasko 2017; Lasko 2013; Raz 2000; Wessel 2016; Yajima and Wessel 2011a). Vasa is essential for the proper segregation of maternal and paternal chromosomes during meiosis (Knaut et al. 2000), as well as for the formation and maintenance of the germline stem cell niche (Durdevic and Ephrussi 2019). Additionally, Vasa has been shown to regulate the translation of specific mRNAs during early embryonic development (Carrera et al. 2000; Gavis and Lehmann 1994), suggesting a broader role in the regulation of gene expression. In addition, the *Vasa* gene is a well-established marker of germline cells in various organisms, including invertebrates (Fabioux et al. 2004; Diao et al. 2015; Olsen et al. 1997; Wang et al. 2022; Zhou et al. 2020).

In this study, we assessed the transcriptional profiles of *Vasa* between copepodid, chalimus, and adult male and female stages in *C. rogercresseyi*. Using in situ hybridization, we localized the *Cr-Vasa* transcript in histological sections of adult males and females. Finally, we investigated the effects of RNAi-induced silencing of the *Cr-Vasa* gene on the phenotype and reproduction of adult females of this species. Our results reveal that the *Cr-Vasa* gene is important for ovary maintenance, reproductive output, and embryonic development of *C. rogercresseyi*.

Materials and Methods

Salmon Lice Culturing and Sampling

The Institutional Bioethics Committee for Animal Research from the Universidad de Valparaiso and the National Council of Science and Technology of Chile examined and approved the protocols for sample techniques and experimental manipulations. The culturing conditions for *C. rogercresseyi* specimens have been discussed in other publications (Bustos et al. 2023; Farlora et al. 2014, 2015). From a salmon farm in Puerto Montt, Chile (41.4° S, 72.9° W), recently harvested fish were used to capture ovigerous female *C. rogercresseyi* specimens. Lice were brought to the lab on ice, and their egg strings were cut off and put in buckets with light aeration and a seawater flow at 10 °C. Sea lice were harvested for fish infection after the eggs had been allowed to hatch and mature up until the infestive copepodid stage. Individual fish (*Salmo salar*) were kept in single-pass flow-through 40-L tanks with a 12:12 h light:dark photoperiod cycle and fed daily with a ratio equivalent to 1% of their total biomass. At the Laboratorio de Interacciones Ecológicas, Universidad Austral (Chile), fish were acclimated for a week prior to the infestation. Fish were subsequently infected with a load of 35 copepodids per fish and kept in the dark without water flow for 2 h. Fish were anesthetized with 10% benzocaine in ethanol (0.6 mL/L), and sea lice in the adult male and female developmental stages were harvested from fish. In addition, free-living copepodid stage individuals were also collected. For RNA extraction, samples were fixed in 15 mL of RNAlater™ stabilization solution (Thermo Fisher Scientific, USA). Additionally, ovigerous females were preserved in Bouin's solution for standard histological examinations and 4% paraformaldehyde (PFA) in phosphate-buffered salt solution (PBS) for in situ hybridization.

Phylogenetic Analyses

Multiple sequence alignments of the Vasa protein were created using the MUSCLE 3.8.425 plugin within the Geneious Prime® software 2022.0.2 (Dotmatics, New Zealand). Manual alignment corrections were done when required. For the selected sequences, the presence of distinctive ATP binding sites and DEAD box helicase motifs within the DEAD domain was identified. The phylogenetic tree was constructed using the Geneious Prime® software based on a Jukes–Cantor genetic distance model and the neighbor-joining method. The data were bootstrapped 1000 times to estimate each node's internal stability (719.601 random seeds).

Gene Transcription Analysis of *Cr-Vasa* Transcript by RT-qPCR

The relative gene transcription levels were examined between copepodid, chalimus, and adult male and female *C. rogercresseyi*. Appropriate primers for PCR were generated using the Primer3 Tool (Rozen and Skaletsky 2000) within the Geneious software (Table S2). Following the manufacturer's instructions, total RNA from sea lice ($n = 150$ for copepodid stage; $n = 10$ for chalimi and adult stages) was extracted using TRIzol® (Thermo Fisher Scientific, USA). A NanoDrop Lite spectrophotometer (Thermo Fisher Scientific) was used to measure purity (ratio A260/A280), and an

agarose gel under denaturing conditions was used to determine RNA integrity. Complementary DNA (cDNA) was synthetized using the Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix kit (Agilent Technologies, USA) from 200 ng/µL of total RNA.

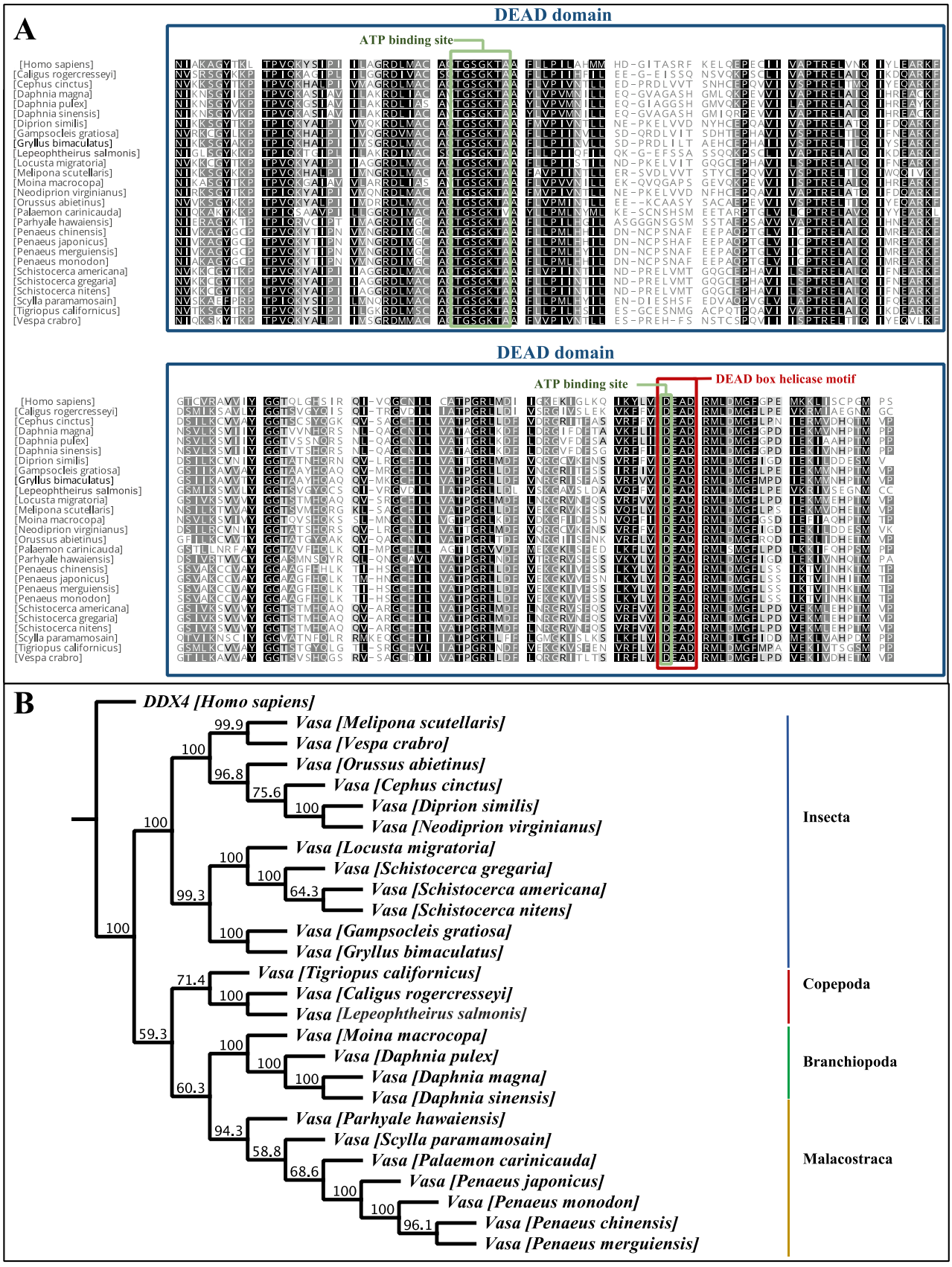
The StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Life Technologies, USA) was used to run the RT-qPCR experiments in triplicate for each sample. The comparative $2^{-\Delta\Delta Ct}$ method was used to assess transcriptional levels (Livak and Schmittgen 2001). The *beta-tubulin* gene was used as a reference gene due to its steady value, which was inferred using the NormFinder algorithm (Andersen et al. 2004). The two other tested reference genes were *beta-actin* and *elongation factor 1 alpha*. Each qPCR reaction was carried out in a total volume of 10 µL, and the conditions for amplification were as follows: 50 °C for 10 min (RT activation), 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s, followed by a dissociation curve analysis at the end of the reaction. The efficiency of the primers was calculated and reported according to MIQE guidelines (Bustin et al. 2009).

Localization of *Cr-Vasa* Transcript

In situ hybridization technique was used to identify the location of *Cr-Vasa* mRNA, as previously reported (Bustos et al. 2023; Hidalgo-Cabrera et al. 2022). For the riboprobe synthesis, separated pools of adult male and female samples previously fixed with RNAlater® (Thermo Fisher Scientific) and stored at − 80 ºC were used for total RNA extraction with the TRIzol® (Thermo Fisher Scientific) according to the manufacturer's protocol. Using the iScript cDNA synthesis kit (Bio-Rad, USA), 1 µg of RNA from each pool was mixed in a 1:1 ratio and converted into cDNA. The specific primers for the Cr-*Vasa* mRNA were then used to amplify the cDNA, producing a PCR product of 501 bp that contained the T3 promoter at the 5′ extreme of the reverse primer. The *Cr-Vasa* antisense probe's forward and reverse primers were forward 5′-CCACGATGTCTCTTCCACCC-3′ and reverse 5′-**GAAATAATTAACCCTCACTAAAGGGAG**TGCGCTCACTTGACTTGCTT-3′. The same forward primer was used as a positive control, and a reverse 5′- **GAAATAATTAACCCTCACTAAAGGGAG**AAGCAAGTCAAGTGAGCGCA-3′ was used as a negative control. The T3 promoter sequence is depicted in bold letters attached to reverse primer. The in vitro transcription procedure was then performed on the amplified products using 4 µL of 10 × DIG-RNA labeling mix (Roche, Switzerland) and 2 µL of T3 RNA polymerase (Roche). The obtained product was precipitated with ammonium acetate and eluted in 31 µL of nuclease-free water along with 1 µL of an RNAse inhibitor (Thermo Fisher Scientific). Formalin-fixed paraffin-embedded (FFPE) *C. rogercresseyi* histological slices were employed to hybridize antisense and sense (negative control) probes for the target gene. Adult male and females were fixed with 4% PFA in PBS for 24 h (Merck, Germany), being subsequently dehydrated in increasing ethanol concentrations, cleaned in xylene, and embedded in Paraplast Plus® (Merck). Using a microtome (Leica RM2255, Germany), serial slices (8 µm thickness) were cut and mounted on Superfrost Plus charged slides (Thermo Fisher Scientific). Sample deparaffinization, rehydration, digestion, probe hybridization, incubation, and colorimetric reaction procedures were carried out as previously reported (Bustos et al. 2023; Hidalgo-Cabrera et al. 2022).

Cr-Vasa Knockdown by RNAi

One primer pair with and without a 5′ T7 promoter extension was used to yield a PCR product of 520 bp of the *Cr-Vasa* open reading frame (forward: 5′-**TAATACGACTCACTATAGG**CCTCTGAAATGCCGTCGATG-3′, reverse: 5′-**TAATACGACTCACTATAGG**CAAACTTCCTTGCCTGGTCC-3′). A *Green Fluorescent Protein* (*GFP*) gene fragment (445 bp) from the crystal jellyfish *Aequorea victoria* was utilized as a control (forward: 5′-GGATCC**TAATACGACTCACTATAGG**GAGCAGGGCGAGGAGCTGT-3′, reverse: 5′-GGATCC**TAATACGACTCACTATAGG**CCTCCTTGAAGTCGATGCCC-3′). Forward and reverse primers are labeled with bold letters representing the T7 promoter sequence. The RNAi protocol was carried out as previously reported (Bustos et al. 2023). Briefly, the PCR products with T7 RNA polymerase were used as templates to generate dsRNA fragments using the T7 RiboMAX™ Express RNAi System kit (Promega, USA), as indicated by the manufacturer. The dsRNA concentration was adjusted to 0.6 µg/µL for both *Cr-Vasa* (ds*Vasa*) and *GFP* control (ds*GFP*), and subsequently, 50 µL of dsRNA was mixed with 5 µL of trypan blue to the final concentration of 600 ng/µL. The mixture of trypan blue-dsRNA solution was delivered by subcuticular injection in the cephalothorax area and visualized using a stereomicroscope (Leica EZ-4). Following injection, the female lice were kept in aerated seawater for 3 h before being reintroduced into anesthetized *S. salar* fish along with male lice that had not received treatment, in 1:1 = female: male ($n = 18$ females per tank). The experiments for *Cr-Vasa* and *GFP* target gene test were carried out in triplicate, utilizing one fish per tank (40 L). To confirm the efficacy of dsRNA-mediated RNA interference, a total of six sea lice individuals were collected from each tank at 48 h post-injection for total RNA isolation and cDNA preparation and were analyzed by qRT-PCR as described in the "Localization of Cr-Vasa Transcript" section. At 19 days after the injection of dsRNA, the adult females in control *GFP* group produced a second batch of egg strings, which marked the end of the experiment. At this point, six individuals for each


A
DEAD domain
ATP binding site
[Homo sapiens]
[Caligus rogercresseyi]
[Cephus cinctus]
[Daphnia magna]
[Daphnia pulex]
[Daphnia sinensis]
[Diprion similis]
[Gampsocleis gratiosa]
[Gryllus bimaculatus]
[Lepeophtheirus salmonis]
[Locusta migratoria]
[Melipona scutellaris]
[Moina macrocopa]
[Neodiprion virginianus]
[Orussus abietinus]
[Palaemon carinicauda]
[Parhyale hawaiensis]
[Penaeus chinensis]
[Penaeus japonicus]
[Penaeus merguiensis]
[Penaeus monodon]
[Schistocerca americana]
[Schistocerca gregaria]
[Schistocerca nitens]
[Scylla paramamosain]
[Tigriopus californicus]
[Vespa crabro]
DEAD domain
ATP binding site
DEAD box helicase motif
B
DDX4 [Homo sapiens]
Vasa [Melipona scutellaris]
Vasa [Vespa crabro]
Vasa [Orussus abietinus]
Vasa [Cephus cinctus]
Vasa [Diprion similis]
Vasa [Neodiprion virginianus]
Vasa [Locusta migratoria]
Vasa [Schistocerca gregaria]
Vasa [Schistocerca americana]
Vasa [Schistocerca nitens]
Vasa [Gampsocleis gratiosa]
Vasa [Gryllus bimaculatus]
Vasa [Tigriopus californicus]
Vasa [Caligus rogercresseyi]
Vasa [Lepeophtheirus salmonis]
Vasa [Moina macrocopa]
Vasa [Daphnia pulex]
Vasa [Daphnia magna]
Vasa [Daphnia sinensis]
Vasa [Parhyale hawaiensis]
Vasa [Scylla paramamosain]
Vasa [Palaemon carinicauda]
Vasa [Penaeus japonicus]
Vasa [Penaeus monodon]
Vasa [Penaeus chinensis]
Vasa [Penaeus merguiensis]
99.9
100
96.8
75.6
100
100
100
100
64.3
99.3
100
71.4
100
59.3
100
100
60.3
94.3
58.8
68.6
100
100
96.1
Insecta
Copepoda
Branchiopoda
Malacostraca

◂**Fig. 1** **A** Amino acid alignments of *Caligus rogercresseyi* Vasa with other known Vasa proteins from arthropods. Conserved and similar amino acid residues are indicated as white letters on a black background and white letters on a gray background, respectively. Non-conserved residues are indicated as gray letters on a white background. The presence of distinctive ATP binding sites (green rectangles) and DEAD box helicase (red rectangle) motifs within the DEAD domain is shown. **B** Phylogenetic tree of *C. rogercresseyi* Vasa including known Vasa amino acid sequences from other arthropods. The numbers at each node indicate the percentage of bootstrapping obtained using the Jukes–Cantor genetic distance model and the neighbor-joining method. *Homo sapiens DDX4* was used as an outgroup. The accession numbers of the sequences used are listed in the supplementary information, Table S1

treatment group were collected for morphological and histological evaluations.

Histological Assessments of RNAi-Treated Individuals

Following fixation in Bouin's solution for 48 h and subsequent rinsing in running tap water, individuals were dehydrated in a series of increasing ethanol concentrations, cleared in butanol, and embedded in Paraplast Plus® (Merck). Serial sections of 5 µm thickness were cut using a Leica RM2255 microtome and mounted on glass slides. The histological sections were then dewaxed and rehydrated via xylene-ethanol to distilled water before being subjected to staining using a trichrome method, wherein hematoxylin (Merck) was used for nuclei, erythrosin-orange G (Merck) for cytoplasm differentiation, and aniline blue (Merck) for connective tissues and secretion granules. Finally, preparations were dehydrated in ethanol, cleared in xylol (Merck), and finally coverslipped using Entellan™ (Merck) mounting medium. The histological sections were photographed under a Leitz-Leica DMRBE microscope equipped with a Leica DFC290 digital camera. When ovarian tissue was present in the histological sections, the total gonad area for each treated female was measured from the photomicrographs by using the ImageJ software 1.53a (National Institute of Health, USA).

Statistical Analyses

All data were tested for normality using the Shapiro–Wilk test. Data not meeting the criteria were normalized through BoxCox transformation (Westfall and Henning 2013). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to evaluate statistical differences in expression data from different developmental stages. To compare expression data between RNAi-treated and control groups, a Student's *t*-test was used. All statistical analyses were performed using Prism 8.0.1 software (GraphPad software Inc., USA), and significant differences were considered when $P < 0.05$.

Results

Molecular Characterization and Phylogenetic Analysis of the Vasa Gene

The *Vasa* partial sequence for *C. rogercresseyi* (GenBank: QQP55941.1) was obtained from its recently published genome (Gallardo-Escárate et al. 2021). The transcript sequence of *Cr-Vasa* displays an open reading frame (ORF) of 1275 nucleotides. The transcript sequence coded for a putative peptide of 424 amino acid residues. The putative coding region was translated into amino acid sequences and compared with other *Vasa* sequences from arthropods using phylogenetic methods. The presence of distinctive ATP binding sites and DEAD box helicase motifs within the DEAD domain was confirmed (Fig. 1A).

The *Cr-Vasa* gene product formed clusters in clades with Vasa proteins from closely related species of crustaceans. The majority of the clades had high bootstrapping values, which ranged from 58.8 to 100%. The Vasa protein from the ectoparasitic copepod *Lepeophtheirus salmonis* was closely related (Fig. 1B). The accession numbers of the sequences used in the phylogenetic tree are listed in the supplementary information, Table S1.

The Vasa Gene Is Overexpressed in *C. rogercresseyi* Females

Cr-Vasa gene relative expression patterns were assessed by qPCR for copepodid, chalimus, and male and female stages. Results showed a significantly higher ($P < 0.0001$) relative expression level in adult females compared to copepodid, chalimi, and adult male stages, with a significant increase of 1719.6-fold, 113.1-fold, and 165.4-fold, respectively (Fig. 2). Moreover, significantly higher ($P < 0.05$) relative expression values were observed for chalimus and adult male stages compared to copepodid stage (Fig. 2).

Tissue-Specific Localization of *Cr-Vasa* Gene in *C. rogercresseyi*

The *Cr-Vasa* gene's specific mRNA was located using chromogenic in situ hybridization (CISH) in male (Fig. 3) and female (Fig. 4) adults of *C. rogercresseyi*. The riboprobe synthesis product had an amplicon size of 600 bp which was visualized by gel electrophoresis and yielded a single band for the antisense probe. Positive signals showing blue coloration were detected with antisense *Cr-Vasa* mRNA riboprobes. Strong positive signal was observed in male testes (Fig. 3A, B, D, E), but also in the intestine (Fig. 3A, C, D) and cuticle (Fig. 3D). Within the testes, presumptive spermatogonia exhibited greater expression for *Cr-Vasa* in

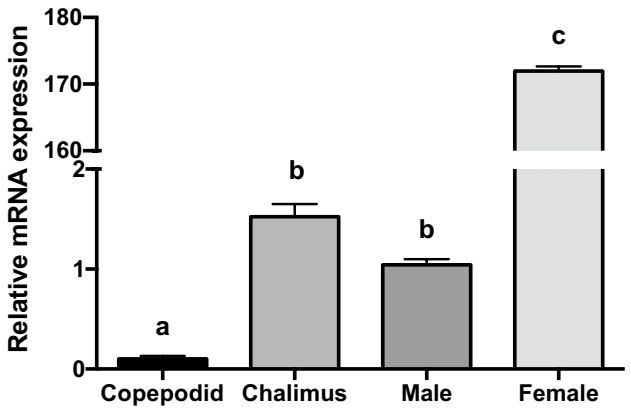


Fig. 2 Relative expression level of the *Cr-Vasa* gene from copepodid, chalimus, and adult male and female *Caligus rogercresseyi* obtained by RT-qPCR according to the $2^{-\Delta\Delta Ct}$ method. Different letters indicate significant statistical differences ($P < 0.005$) according to Tukey's test. Data are shown as mean ± SD. *Beta-tubulin* was used as endogenous control

the proximal region, with a comparatively weaker signal in spermatocytes toward the distal area (Fig. 3B, E). In females, *Cr-Vasa* exhibited strong signal in the ovaries (Fig. 4A, B), previtellogenic oocytes (pvo) in the oviduct (Fig. 4A, C), cuticle (Fig. 4C, D), vitellogenic oocytes in the genital segment (Fig. 4A, D), and intestine (results not shown). Also, the egg strings presented positive chromogenic reaction in the peripheral embryonic cells surrounding the central yolk mass (Fig. 4A, E). CISH using the *Cr-Vasa* negative control sense riboprobes did not reveal any specific signals (Figs. S1 and S2).

RNAi-Mediated Gene Silencing of *Cr-Vasa* Impacted Embryonic Development and Reproductive Output in Adult Female Lice

The *Cr-Vasa* function was assessed by subcuticular administration of ds*Vasa* into freshly molted adult female salmon lice, and ds*GFP* was used as a control group. Female lice were removed from the hosts 48 h after injection, and both RNAi-treated and control lice were analyzed by qPCR. A significant down-regulation ($P < 0.0001$) of 67% was evidenced (Fig. 5). When the adult females treated with ds*GFP* (control group) produced a second batch of egg strings (19 days after injection with dsRNA), the experiment was terminated. The egg-laying cycle was shortened in the ds*Vasa* group to a single egg string extrusion cycle, but viable eggs were not produced. In contrast, two egg string extrusion cycles occurred for the ds*GFP* control group (Table 1). Representative female lice bearing their egg strings from both the ds*GFP* control group and the ds*Vasa*-treated group were collected, and photographs were taken. Females from the ds*Vasa*-treated group displayed

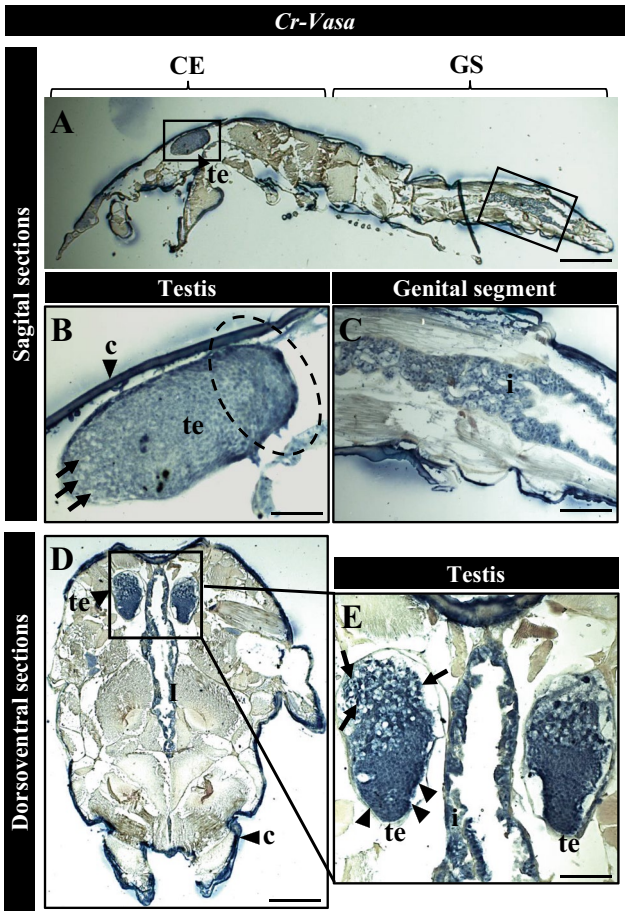


Fig. 3 Chromogenic in situ hybridization (CISH) for *Cr-Vasa* gene in adult male *C. rogercresseyi* individuals. **A**–**C** Adult male sections in sagittal plane. **D**, **E** Adult male sections in dorsoventral plane. Negative controls are depicted in supplementary Fig. S1. **A** Sagittal section of the whole individual. Strong staining for *Cr-Vasa* mRNA is detected in the testis (te), cuticle (c), and intestine (i). Bar = 400 µm. **B** Higher magnification of the whole testis (te). Detailed view showing stronger chromogenic reaction in the proximal area of the testis (circle with dashed line). Arrowheads: spermatogonia. **C** Sagittal section of the genital segment (GS). Strong staining for *Cr-Vasa* mRNA is detected in the intestine (i). Bar = 40 µm. **D** Dorsoventral section of the whole individual. Strong staining for *Cr-Vasa* mRNA is detected in the testis (te), cuticle (c), and intestine (i). Bar = 400 µm. **E** *Cr-Vasa* expression is observed in spermatogonia (arrowheads) and spermatocytes (arrows). Bar = 40 µm. CE, cephalothorax

unusual phenotypes, including shorter egg strings with numerous extra-embryonic inclusions, irregularly shaped abnormal embryos, and aborted egg strings (Fig. 6A–D). Normal antero-posteriorly compressed embryos and longer egg strings were observed in control ds*GFP*-treated female lice (Fig. 6E–H). Embryo counts, egg string lengths, and ovarian areas in ds*Vasa* group were all drastically reduced by 56.27%, 55.71%, and 27.9%, respectively, when compared with the control ds*GFP* group. Importantly, ovarian and vitellogenic development was seriously impaired, with ds*Vasa*-treated females exhibiting smaller ovaries (Fig. 7A,

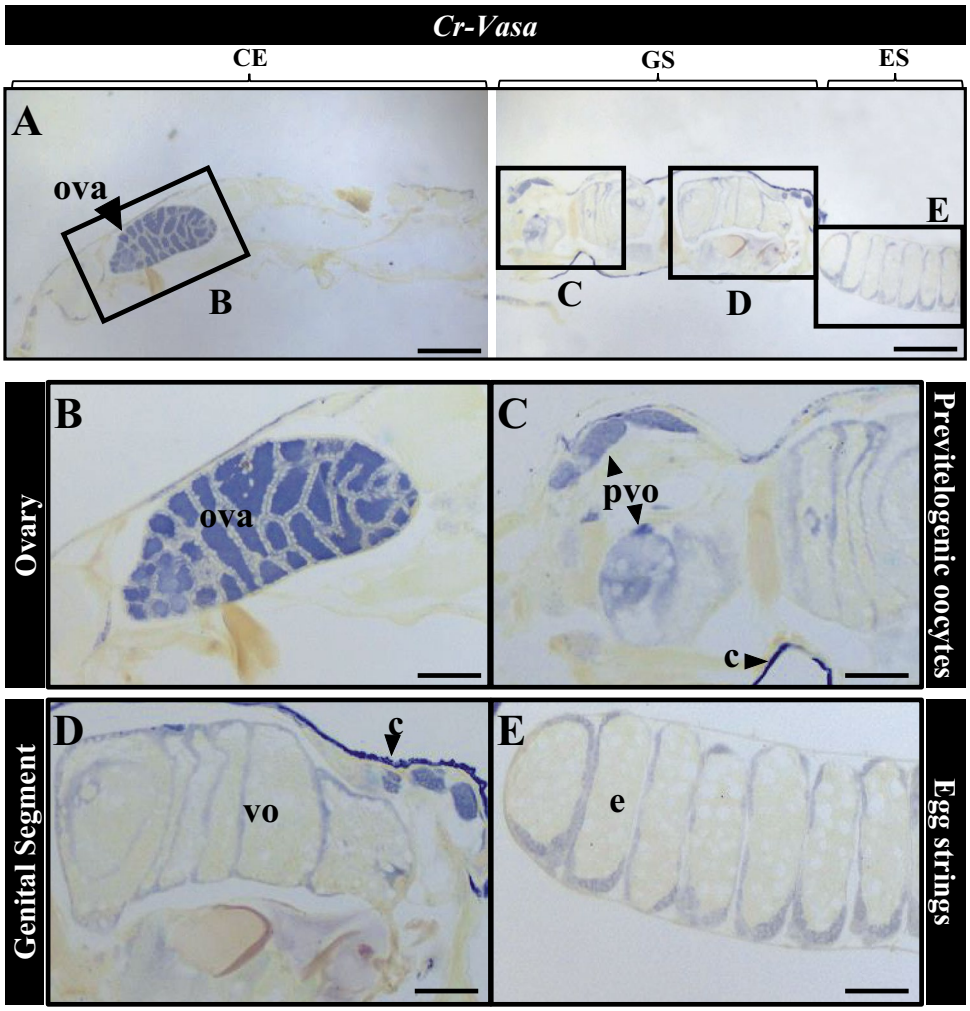


Fig. 4 Chromogenic in situ hybridization (CISH) for *Cr-Vasa* gene in adult females of *C. rogercresseyi*. Sense probe negative controls are depicted in supplementary Fig. S2. **A** Sagittal sections of the whole individual. Intense staining for *Cr-Vasa* mRNA is detected in the ovaries (ova), previtellogenic oocytes in the oviduct (pvo), vitellogenic oocytes (vo), cuticle (c), and embryos (e) in the egg strings. **B** Higher magnification of the whole ovary (ova). Detailed view showing chromogenic aggregations toward the anterior area of the ovary. **C** Higher magnification of the previtellogenic oocyte (pvo) expressing *Cr-Vasa*. **D** Higher magnification of a genital segment (GS) section containing the vitellogenic oocyte (vo) exhibiting *Cr-Vasa* gene expression. Strong staining for *Cr-Vasa* mRNA is detected in the genital segment cuticle (c). **E** Higher magnification of the egg string embryos (e) shows expression high in the outer limits of the embryo. Bar = 40 µm

B) and abnormally shaped vitellogenic oocytes (vo) in the genital segment with a marked decrease in the abundance of yolk granules (Fig. 7C, D). In contrast, ds*GFP*-negative control presented larger ovaries (Fig. 7E, F) and normal vitellogenic oocytes (Fig. 7G, H).

Discussion

Salmon farming worldwide incurs substantial financial losses in managing parasitic copepods. Unfortunately, there is limited knowledge about the molecular mechanisms involved in gametogenesis and embryo development in these parasites. Therefore, it is essential to enhance our current understanding of the biological and molecular foundations of reproduction in sea lice. This is particularly important since such research could lead to novel treatments for parasite control by manipulating the mechanisms governing germ cell production and embryo development. The presented results provide valuable insights into the molecular characterization, expression, localization, and function of the *Vasa* gene in the salmon louse *C. rogercresseyi*. Phylogenetic analysis confirmed the presence of distinctive ATP binding sites and DEAD box helicase motifs within the DEAD domain of *Cr-Vasa*. *Cr-Vasa* gene expression was significantly higher in adult females compared to copepodid, chalimus, and adult male stages, indicating a potential role in female reproductive development. Tissue-specific localization of *Cr-Vasa* mRNA showed strong expression in the testes and ovaries of male and female lice, respectively, as well as in other tissues such as the cuticle and intestine. Finally, RNAi-mediated gene silencing of *Cr-Vasa* resulted in abnormal embryo development and impaired reproductive output, indicating the crucial role of *Cr-Vasa* in *C. rogercresseyi* embryonic and ovarian development.

The results of this study provide significant evidence that *Cr-Vasa* is a crucial gene for reproductive development and gametogenesis in *C. rogercresseyi*. The higher expression of *Cr-Vasa* in adult females compared to other life stages suggests that this gene has a vital role in female reproductive development, possibly including oogenesis, egg maturation, and oviposition. It has been previously reported that

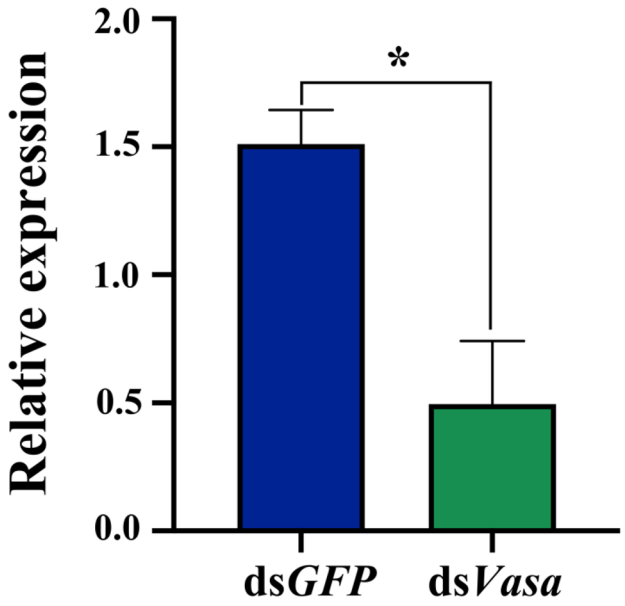


Fig. 5 Relative expression of *Cr-Vasa* injected with ds*Vasa* (green bar) compared to ds*GFP* (blue bar), considered negative control of specific gene silencing. Values are represented as the mean ± SD from pools of $n = 5$ lice collected from three separated replicate tanks (unpaired test: $P < 0.0001$)

Vasa is required for pole plasm assembly and function, for growth of germline cysts, and also for completion of oogenesis in *Drosophila* (Styhler et al. 1998), being also involved in various stages of oogenesis, including the establishment of polarity (Tomancak et al. 1998). Vasa is a well-known germ cell marker and has a highly conserved role in different organisms (Raz 2000). The localization of *Vasa* in the gonads has been reported for other species, including *L. salmonis* (Dalvin et al. 2013), *Caenorhabditis elegans* (Gruidl et al. 1996), *Schistosoma japonicum* (He et al. 2018), *Scylla paramamosain* (Wang et al. 2012), and *Macrobrachium nipponense* (Qiu et al. 2013). In *C. rogercresseyi*, *Cr-Vasa* was strongly expressed in the testes and intestine in adult males, as well as in ovaries, previtellogenic oocytes, vitellogenic oocytes, intestine, and embryos in adult females. In males, the expression of *Cr-Vasa* was higher in spermatogonia. In *S. japonicum*, a progressive decrease of the *Vasa* expression from spermatogonia toward spermatocytes inside the testes was observed (He et al. 2018). Similarly, in the present study, we observed a slight decrease in the colorimetric reaction of the *Cr-Vasa* from spermatogonia toward spermatocytes. In females, *Cr-Vasa* was expressed in previtellogenic oocytes and vitellogenic oocytes as reported in *L. salmonis* (Dalvin et al. 2013), *Litopenaeus vannamei* (Aflalo et al. 2007), *Drosophila melanogaster* (Hay et al. 1988), *Crassostrea gigas* (Fabioux et al. 2004), and *Ctenopharyngodon idella* (Li et al. 2010). The tissue-specific localization of *Cr-Vasa* mRNA in the testes and ovaries of male and female lice, respectively, further supports the notion that *Cr-Vasa* is involved in the gametogenesis process. Moreover, the observed localization of the *Cr-Vasa* in the embryonic cells within the egg strings may indicate a transfer of *Vasa* derivatives from the mother to eggs as it has been reported earlier (Aflalo et al. 2007; Durdevic et al. 2018; Özhan-Kizilet et al. 2009; Qiu et al. 2013). Interestingly, the strong positive signals detected in the cuticle and intestine suggest that *Cr-Vasa* may have additional non-reproductive functions in these tissues. Supporting this notion, previous research has shown that Vasa plays a role in regulating somatic cells, including embryonic cells of different types (Schwager et al. 2015; Yajima and Wessel 2011b), as well as in regenerative tissues (Wagner et al. 2012; Yajima and Wessel 2015).

The RNAi-mediated gene silencing of *Cr-Vasa* led to the severe impairment of embryonic development and reproductive output in female lice. The shorter egg strings with numerous extra-embryonic inclusions, irregularly shaped abnormal embryos, and aborted egg strings observed in the ds*Vasa*-treated group indicate that *Cr-Vasa* is crucial for embryonic development and egg maturation. The reduced ovarian area and vitellogenic development in the ds*Vasa* group compared to the control ds*GFP* group indicate the importance of *Cr-Vasa* in ovarian development and vitellogenesis. The function of the *Vasa* gene has been also associated with the development of female germ cells in other species such as in *Macrobrachium rosenbergii* (Nakkarasae and Damrongphol 2007), *Drosophila* (Hay et al. 1988), *C. gigas* (Fabioux et al. 2004), *M. nipponense* (Qiu et al. 2013), *Oreochromis niloticus* (Kobayashi et al. 2000), *Xenopus* (Komiya et al. 1994), and the mouse (Toyooka et al. 2000).

Table 1 Summary of the results from the knockdown of *CrVasa*

Group	Embryos per oviposition	Egg strings length (mm)	Number of ovipositions	Ovary area (μm²)
ds*GFP*	110; SD = 50.23	7; SD = 1.84	2	319.15; SD = 24.75
ds*Vasa*	61.9; SD = 32.18	3.9; SD = 1.17	1	232.66; SD = 13.41

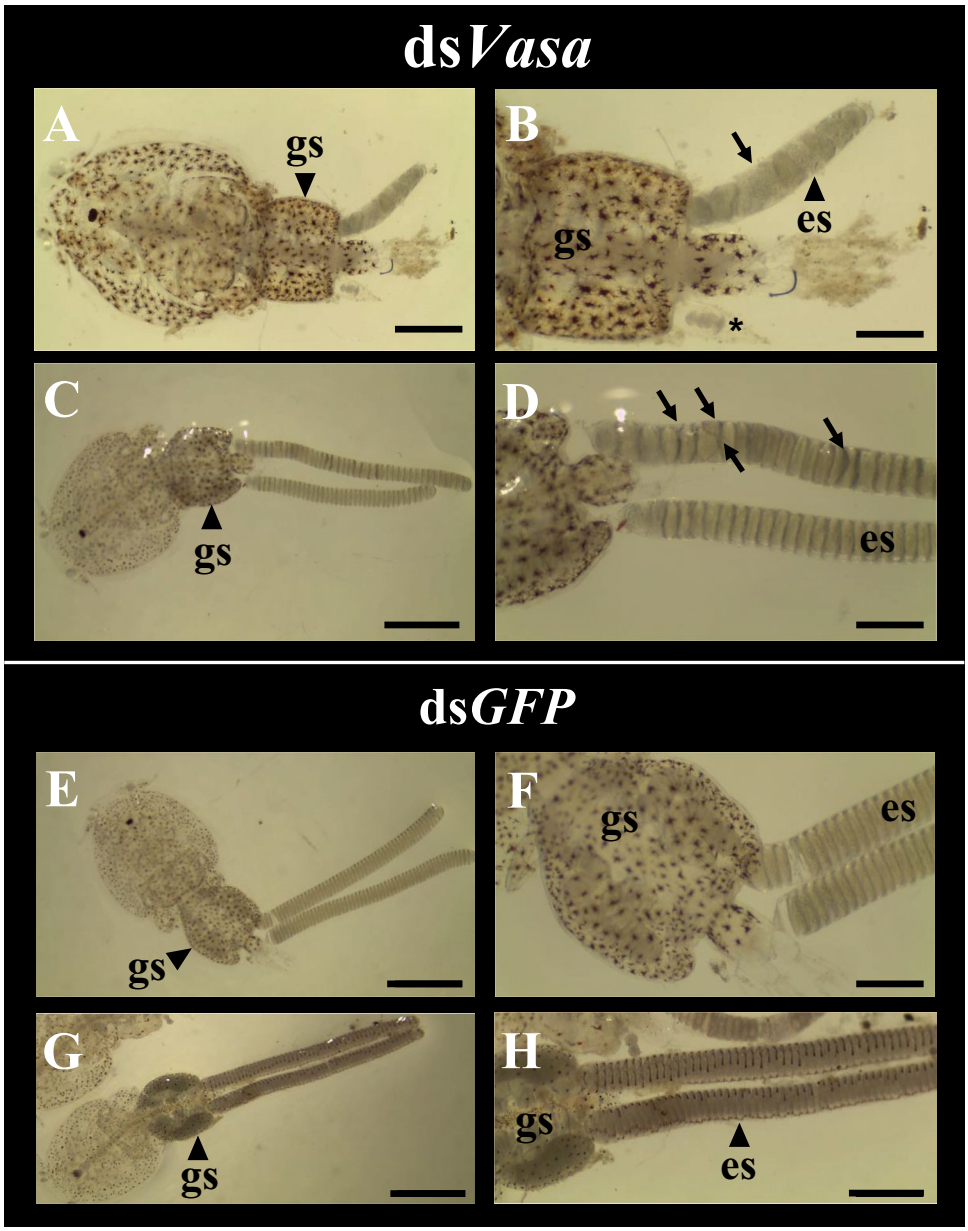


Fig. 6 External phenotype of adult females of *C. rogercresseyi* treated with RNAi injection. Females microinjected with ds*Vasa* (**A**–**D**) and ds*GFP* (**E**–**H**) were observed. Short egg strings (es) with abundant inclusions (arrow) were commonly observed in females microinjected with ds*Vasa* (**B**). Females with aborted egg strings were also appreciated (**B**; asterisk), and egg strings with abnormalities (arrows) were detected (**D**). In contrast, females microinjected with ds*GFP* exhibited longer, regular egg strings with well-delimited antero-posteriorly compressed embryos (**F**, **H**). The genital segment (gs) is shown. Scale bars are 3.5 mm (**A**, **C**, **E**, **G**) and 0.6 mm (**B**, **D**, **F**, **H**), respectively

Overall, this study provides valuable insights into the molecular characterization, expression, localization, and function of the *Vasa* gene in *C. rogercresseyi.* The findings suggest that *Cr-Vasa* is a vital gene for reproductive development, gametogenesis, embryonic development, and ovarian development in *C. rogercresseyi*. Further research is required to elucidate the precise roles of *Cr-Vasa* in these processes and develop effective strategies for controlling salmon lice infestations in salmon farms.

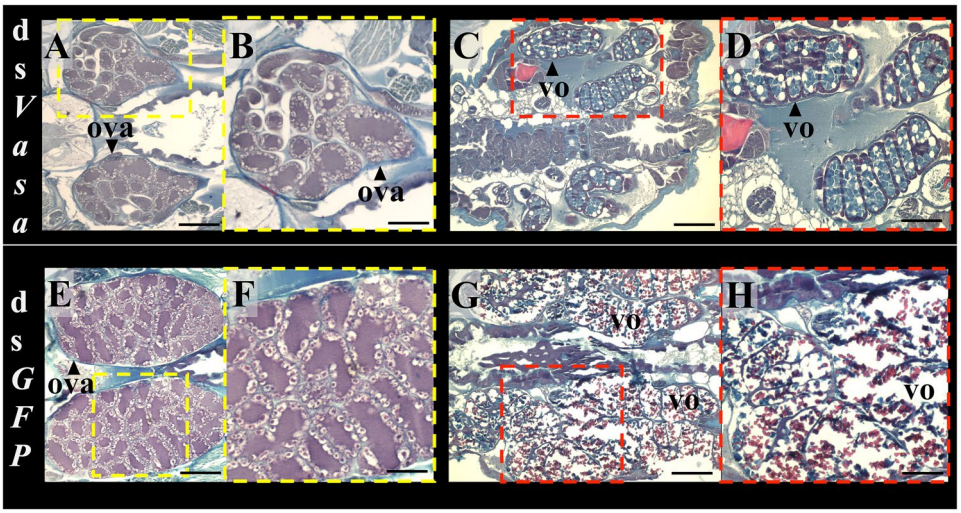


Fig. 7 Histology of *Caligus rogercresseyi* adult females after ds*Vasa* (**A**–**D**) and ds*GFP* (**E**–**H**) injections (trichrome staining). **A**, **B**, **E**, **F** Photomicrographs depicting ovaries. Females treated with ds*Vasa* (**A**, **B**) exhibited smaller ovaries than control ds*GFP*-treated louse (**E**, **F**). ova = ovaries. **C**, **D**, **G**, **H** Photomicrographs of the genital segment revealed abnormal development in vitellogenic oocytes/early embryos (vo) from ds*Vasa*-treated females (**C**, **D**) compared to ds*GFP*-treated louse (**G**, **H**). **A**, **E**, **C**, **G** Bar = 40 µm. **B**, **F**, **D**, **H** Bar = 100 µm. Photomicrographs **B** and **F** correspond to the higher magnification of regions enclosed in yellow discontinuous lines in **A** and **E**, respectively. Photomicrographs **D** and **H** correspond to the higher magnification of regions enclosed in discontinuous red lines in **C** and **G**, respectively

Conclusion

Our results demonstrate that the *Cr-Vasa* gene is highly expressed in adult females and is localized in the testes and ovaries, suggesting a crucial role in gametogenesis. Furthermore, the RNAi-mediated silencing of *Cr-Vasa* gene expression led to significant reproductive defects, including shortened egg-laying cycles, abnormal embryo development, and reduced fecundity, indicating that *Cr-Vasa* is essential for embryonic and ovarian development in the sea louse. These findings highlight the importance of the *Vasa* gene in crustacean reproductive biology and provide a basis for developing new strategies for controlling lice infestations in the aquaculture industry. Further studies are needed to elucidate the precise molecular mechanisms and regulatory pathways involved in the *Cr-Vasa* gene's functions in the salmon louse.

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Authors' Contribution Paulina Bustos: Conceptualization, methodology, investigation, data curation, formal analysis, writing—original draft, visualization. Paulina Schmitt: Conceptualization, resources, writing—review and editing. Donald Irving Brown: Conceptualization, methodology, resources, writing—review and editing. Rodolfo Farlora: Conceptualization, methodology, resources, writing—review and editing, supervision, project administration, funding acquisition. All authors contributed to the article and approved the submitted version.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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