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Investigation of proteins identified in the secretory and excretory products (SEPs) of the infectious copepodid stage of the salmon louse *Lepeophtheirus salmonis*

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ABSTRACT

The salmon louse (*Lepeophtheirus salmonis* (Krøyer, 1837)) is a caligid ectoparasite of salmonids that feeds on host blood, mucus, and skin. While secreted virulence factors from later life stages have been studied, the protein composition of secretory and excretory products (SEPs) from copepodids, the initial infectious stage of *L. salmonis*, remains uncharacterized.

Copepodids were hatched and incubated at 10°C until 7 days post-hatch. Batches (n = 4) were then exposed to either 0.45 µm filtered seawater or 0.1 mg mL^{-1} isophorone to stimulate SEP production. Adult males and females (n = 2 replicates) were similarly treated for comparison. SEPs were filtered, precipitated, trypsin-digested, and analyzed via LC-MS/MS. Proteins were identified using an *L. salmonis* database and further analyzed with SignalP and InterPro.

In total, 433 distinct proteins were detected in copepodid samples (mean 95.5 ± 146.74), and 117 in adult samples (mean 56 ± 12.70). Signal peptide analysis revealed 164 copepodid and 69 adult proteins as secretory. Among adults, 31 secretory proteins were female-specific and 10 male-specific. Twenty-one secretory proteins were shared across life stages, including 8 proteases, 2 protease inhibitors, and 2 uncharacterized proteins. Of proteins with GO annotations, 75 % were involved in proteolysis and 50 % localized extracellularly. However, secretory profiles differed markedly between life stages. Notably, 67 % of adult-specific secretory proteins were extracellular versus 30.7 % in copepodids. Copepodid and adult SEPs also contained 23 and 4 unique uncharacterized proteins, respectively.

These findings highlight a complex repertoire of copepodid SEPs potentially involved in host invasion and immunomodulation, providing new targets for therapeutic development.

1. Introduction

The salmon louse (*Lepeophtheirus salmonis* (Krøyer, 1837)) is a caligid copepod with a distribution across the Northern hemisphere and represents an economically significant ectoparasite of salmonids, most notably Atlantic salmon (*Salmo salar*). It constitutes a key constraint for the Atlantic salmon aquaculture industry, costing approximately €790 million annually (Boxaspen et al., 2022).

L. salmonis has eight distinct life cycle stages separated by molts: nauplius I, nauplius II, copepodid, chalimus I, chalimus II, pre-adult I, pre-adult II, and adult (Hamre et al., 2013). The naupliar stages are exclusively free-living and lecithotrophic, whereas all stages following the copepodid stage are obligate parasitic. The copepodid is initially a free-living lecithotrophic stage, but is parasitic following infection, requiring a host to facilitate the molt into chalimus I.

These parasitic life stages feed upon the host's mucus, skin, and blood, creating open lesions that increase the fish's risk of secondary infection. This risk is further aggravated by the presence of immunomodulatory proteins present in *L. salmonis* secretions, which may simultaneously facilitate the louse's feeding and survival on the host

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while weakening the fish's defenses against opportunistic pathogens. The presence of such immunomodulators in salmon louse secretions was first explored by Fast et al. (2003), in which low molecular weight proteases were detected in the *L. salmonis* SEPs. The next year, the vasodilator prostaglandin E2 was detected in louse SEPs, which was hypothesized to play a role in promoting feeding, modulating host inflammation by downregulating the cytokines interleukin-1_β (IL- 1_β) and tumor necrosis factor alpha (TNF_α), and/or increasing host mucus secretion (Fast et al., 2004). It was later demonstrated that the treatment of an Atlantic salmon head kidney cell line (SHK-1) with *L. salmonis* secretory and excretory products (SEPs) results in the downregulation of IL-1_β and major histocompatibility complex I (MHC-I) expression (Fast et al., 2007). Later *in vitro* work also found that *L. salmonis* SEPs induce macrophage phagocytosis in SHK-1 cells, as well as inhibiting chemotaxis in *S. salar* leukocytes (Lewis et al., 2014; Piesz et al., 2020).

To better understand the composition of the *L. salmonis* secretome, Hamilton et al. (2018) employed liquid chromatography-electrospray ionization-tandem mass spectrometry to investigate the protein composition of the SEPs of adult and pre-adult salmon lice, and detected multiple putative virulence factors, including serine proteases, which may contribute to the digestion of host tissue or to the cleavage of host proteins implicated in the immune response, as well as peroxiredoxins, which may serve to protect the louse against host reactive oxygen species. Later investigations by Midtbø et al. (2024a, 2024b) focusing on *L. salmonis* labial gland proteins have allowed for the elucidation and characterization of a number of secreted virulence factors, including LG3P, which causes the death of host leukocytes, LG4P, which has an anti-coagulant effect on host plasma, and the triad of LGP2, LGPS1, and LGP1 which dampen the host immune response.

However, while proteomic studies have been conducted on *L. salmonis* SEPs, much about this species' secretome remains opaque. For example, most studies of *L. salmonis* SEPs (*e.g.*, Fast *et al.* in 2007 and Piez *et al.* in 2020) have relied on the usage of the neurotransmitter dopamine to induce secretory activity. While induction with dopamine was observed to yield SEP protein production in these studies, other attractive candidates for secretory inducers remain unexplored. For example, the ketone isophorone is present naturally in the mucus of *S. salar* and is known to serve as a semiochemical and chemoattractant for *L. salmonis* copepodids (Bailey et al., 2006). This role of isophorone as a key semiochemical in driving host-parasite interactions *in vivo* thus makes it a promising candidate as an inducer of secretory activity in *L. salmonis*.

Furthermore, these previous studies have focused on SEPs from later *L. salmonis* life stages (*i.e.*, adults and pre-adults), and there remain no documented studies of copepodid SEPs. It is therefore unclear to date how the *L. salmonis* copepodid secretome differs in composition and function from that of later life stages, and, accordingly, whether potential control strategies based on louse secretions might require differential approaches for different life stages.

2. Methodology

2.1. Maintenance of Salmon Lice

Adult male and female *L. salmonis* (strain IoA 00) were removed from Atlantic salmon hosts at the Marine Environmental Research Laboratory at Machrihanish, Scotland, and were transported to the University of Stirling's Institute of Aquaculture in Stirling, Scotland, where they were maintained in clean, aerated, 0.45 μm filtered seawater at 10°C. Egg strings were sequestered from gravid females and transferred to a sieve in a large beaker containing seawater held under identical conditions. The sieve allows for the retention of unhatched egg strings, while hatched nauplii were able to pass through and enter the seawater medium. This sieve was transferred to a new beaker daily, allowing for the determination of hatching time and ensuring that all individuals within a beaker would progress through the life cycle at predictable intervals.

2.2. Extraction of L. salmonis SEPs

Following hatching, nauplii were kept for seven days prior to experimentation. At this point, all individuals had molted twice to reach the copepodid stage, as verified through stereo microscopy. To acquire healthy individuals for experimentation while minimizing transfer of waste material and exuviae, a light was directed towards the top of vessels containing lice, eliciting positive phototaxis from healthy copepodids. Responding lice were gently siphoned off into another vessel containing clean, 0.45 μm filtered seawater for ten minutes of rinsing and counting with a Bogorov chamber.

For each replicate, 200 copepodids were then transferred to either 2 mL of 0.45 μm filtered seawater (SW) control or 0.1 mg mL^{-1} isophorone (ISO) solution (also made up in 0.45 μm filtered seawater) using 100 μm plankton mesh. These copepodids were then incubated in darkness at 10°C for three hours to allow for SEPs production. Following this point, a pipette tipped with plankton mesh was used to extract SEPs-containing media while excluding copepodids. This experiment was repeated four times to allow for $n = 4$ replicates for each incubation condition.

Male and female adult lice (strain IoA00) were utilized immediately for experimentation after transportation and had been removed from their hosts for no longer than 24 h at the time of work. Gravid females were excluded from all treatments to mitigate contamination of samples with egg string debris. Adults were collected and rinsed in 0.45 μm filtered seawater at 10°C for ten minutes prior to incubation. These individuals were incubated under the same conditions and duration as the copepodids, but at a density of two females or three males per milliliter of medium. SEPs were carefully pipetted from each adult sample while minimally disturbing the lice. This experiment was repeated twice, providing $n = 2$ replicates per condition for both male and female SEPs to serve as a direct comparison with copepodid samples. Four replicates were performed for copepodids to account for anticipated variation arising from larger batch incubations, whereas two replicates per sex were performed for adults, which have been previously characterized in detail by Hamilton et al. (2018).

SEPs from copepodid and adult samples were then concentrated twice using Amicon 3 kDa centrifugal filters (MilliporeSigma, Darmstadt, Germany). Samples were then divided into 20 μL aliquots, of which one was put aside for downstream sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and quantification using the protein A280 function on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

To remove extraneous solutes (*e.g.*, sodium chloride and isophorone) that might interfere with downstream liquid chromatography-tandem mass spectrometry (LC MS/MS), the samples were further subjected to trichloroacetic acid (TCA) precipitation, in which 100 % TCA solution was added to the samples to yield a 20 % solution. Samples were incubated on ice for one hour, centrifuged to pellet precipitated proteins, and then thoroughly rinsed with ice-cold acetone. Samples were re-centrifuged, and acetone was removed from the protein pellet. All samples were reconstituted in 13 μL of ultrapure water and frozen at –70°C for later use.

2.3. SDS PAGE

Copepodid SEPs were diluted 1:2 with 2X Laemmli buffer solution (Thermo Fisher Scientific, Waltham, USA) and allowed to denature at 100°C for five minutes. The diluted SEPs were then centrifuged at 13,000 x g for another five minutes. 10 μL of the diluted SEPs were then loaded into a 15-well SurePAGE 10 × 8 bis-tris gel (GenScript, Piscataway, USA) submerged in 1X MOPS SDS running buffer along with 1 μL of dual color protein standard (Bio-Rad, Hercules, USA), a positive control comprised of SEPs from adult male *L. salmonis* at a concentration of 0.119 mg mL^{-1} , and negative controls consisting solely of filtered seawater or isophorone solution medium. Gels were subsequently run at

200 V for approximately 40 min. These gels were then rinsed with ultrapure water and fixed overnight in a solution of 10 % acetic acid and 40 % ethanol in ultrapure water. Gels were then subjected to silver staining using a SilverQuest (Thermo Fisher Scientific, Waltham, USA) kit according to the manufacturer's instructions prior to imaging.

Aliquots of each replicate were transferred to Moredun Research Institute in Midlothian, Scotland for LC MS/MS.

2.4. LC MS/MS

LC MS/MS samples were digested using an S-Trap micro column (Protifi) kit, following the manufacturer's instructions. Briefly, 1–2 µg of sample was mixed with the supplied 2X SDS lysis buffer, proteins were reduced with 5 mM Tris (2-carboxyethyl) phosphine (TCEP) and alkylated with 20 mM methyl methanethiosulfonate (MMTS). Proteins were then acidified with 2.5 % phosphoric acid and bound to the S-Trap column in 6 volumes of S-Trap binding/washing buffer (100 mM TEAB in 90 % Methanol). The column was centrifuged (4000 x g for 30 s) and then washed a further 4 times in wash buffer. Proteins were digested using sequencing grade trypsin (in 50 mM TEAB) in a 1:10 ratio to trapped protein for 16 h at 37C. Tryptic peptides were eluted in three steps, firstly, 40 µl 50 mM triethylammonium bicarbonate (TEAB) buffer followed by 40 µl 0.2 % formic acid (FA), and finally 40 µl 50 % acetonitrile (ACN) in water and 0.2 % FA. Elutions were pooled and dried in a vacuum drier. Dried peptides were reconstituted in 20 µl of 0.1 % FA prior to analysis by mass spectrometry.

Liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis was performed using a Vanquish nano-LC system (Thermo Fisher Scientific) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The LC system was equipped with an Acclaim Pepmap nano-trap column (C18 PepMap100, 300 µm × 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) and an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 µm×50 cm EasySpray, Thermo Fisher Scientific). The tryptic peptides were loaded onto the trap column in a volume of 20ul at an isocratic flow of 60 µL/min, peptides were then separated on the Easy Spray analytical column. The elutents used were 0.1 % v/v formic acid (solvent A) in H2O and 80 % v/v CH3CN in 0.1 % v/v formic acid (solvent B). A 110 min gradient was used at 350 nl/min from (i) 0–95 min, 1–35 % B; (ii) 95–115 min, 99 % B.

Data were acquired in positive mode using a data dependent approach, MS1 scans were acquired at 280,000 resolution over a mass range of *m/z* 380–1500 with an AGC of 3e6 and a maximum IT of 50 ms. In each cycle, the 15 most intense ions with charge states of $\geq$ 2 and intensity thresholds of $\geq$ 2.0e4 were selected for MS/MS and subjected to high-energy collision dissociation (HCD) at a normalized collision energy of 30 %. The isolation window was set at 1.2 *m/z*, at a resolution of 17,500, an AGC target of 1e5 and a maximum IT time of 50 ms. The dynamic exclusion time was set to 45 s.

The MS raw data was processed using the Proteome Discoverer platform (version 2.4, Thermo Fisher Scientific) and Sequest HT algorithm. The MS data was searched against the a combined data base comprising the current *L. salmonis* v1.2 annotation and the *Salmo salar* v3.1 annotation. A maximum of two missed tryptic cleavages were allowed. The oxidation of Methionine and protein N-terminal acetylation were set as variable modifications, as was the Methylthio addition to Cysteines. MS and MS/MS ion tolerances were set at 10 ppm and 0.02 Da, respectively. A maximum false discovery rate (FDR) of 1 % at both the peptide and the protein levels was set and only proteins identified with $\geq$ 2 peptides were accepted.

2.5. In silico analysis

To ensure a high level of confidence in the detected proteins, proteomic data for both adult and copepodid samples were filtered to retain only samples containing two or more peptides, high false discovery rate confidence thresholds, a sum posterior error probability of 100 or less, and a Sequest HT score of at least 20. Peptide sequences were then analyzed with SignalP to identify secretory proteins by detecting the presence of signal peptides. Peptide sequences were also run through InterPro to more thoroughly characterize the proteins present and to provide gene ontology (GO) terms for subsequent analysis, including for biological process, cellular component, and molecular function. These GO terms were then used to construct bar charts to allow for functional comparisons between groups of samples. Due to the large quantity of uncharacterized proteins lacking GO annotations (especially for copepodid samples), only the subset of proteins bearing GO terms were considered in these analyses. Statistical differences in the numbers of proteins present per group were assessed using Welch's T-testing.

Nucleic acid sequences for 30 known *L. salmonis* labial gland genes (including possible isoforms) were further subjected to Protein BLAST to acquire their corresponding amino acid sequences. These sequences were then searched against the secretory protein profiles for both adults and copepodids at a threshold of 90 %.

3. Results

3.1. Protein concentrations

Analysis of the protein concentrations of filtered and precipitated SEPs from copepodids, adult males, and adult females treated with either SW or ISO revealed that mean concentrations (± SD) were variable across groups but did not differ significantly between treatments within life stage or sex. For copepodids, mean concentrations were higher in SW-treated samples (854.5 ± 1185.8 µg/mL, n = 4) than in ISO-treated samples (356.3 ± 275.9 µg/mL, n = 4), though this difference was not significant (Welch's *t*-test, p = 0.468). Similarly, no significant differences were observed between SW and ISO-treated adults: males yielded 305.0 ± 254.6 µg/mL in SW and 553.0 ± 602.5 µg/mL in ISO (p = 0.667, n = 2), while females yielded 437.5 ± 3.5 µg/mL in SW and 1067.5 ± 1161.8 µg/mL in ISO (p = 0.584, n = 2). When pooled by stage, protein concentrations in copepodid SEPs (605.4 ± 893.5 µg/mL, n = 8) did not differ significantly from those in adult SEPs (590.8 ± 676.6 µg/mL, n = 8) (p = 0.969).

These data indicate that SEP protein yields may be broadly comparable between SW and ISO inductions, and that adult and copepodid stages may produce similar total concentrations under the tested conditions. Further concentration data may be found in Figure S1.

3.2. Protein profiles

Analysis of SDS PAGE gels of copepodid samples (Figs. 1A, 1B) showed some variation in protein profiles between the SEPs of larvae incubated in SW or ISO. All samples consistently showed banding at ~175 kDa, 60 kDa, 55 kDa, 40 kDa, 21 kDa, and 10 kDa, in addition to showing a pattern suggestive of the presence of low molecular weight proteins (<10 kDa). All samples except for the first seawater induction (SW 1) and the third isophorone induction (ISO 3) exhibited banding at ~70 kDa. ISO 3 showed the most divergent banding pattern, with unique banding at ~130, 120, and 24 kDa. In all non-control samples, the most intense band was found at approximately 60 kDa, with the exception of ISO 3, which showed the most intense banding at approximately 130 and 120 kDa. The positive control sample exhibited banding at ~200, 175, 150, 140, 125, 70, 60, 40, 25, 23, and 15 kDa (along with low molecular weight proteins) and exhibited the most pronounced banding at ~175 kDa. Negative controls featured no banding, except for a faint signal at ~250 kDa.

SEPs from adult female lice incubated in SW and ISO yielded approximately 33 and 31 protein bands, respectively. Protein sizes ranged from 210–10 kDa in SW and 150–10 kDa in ISO. In both treatments, the most prominent bands were low-molecular-weight proteins between 15–10 kDa, with ISO-treated samples also exhibiting distinct bands between 150–75 kDa. In adult males, around 20 bands were

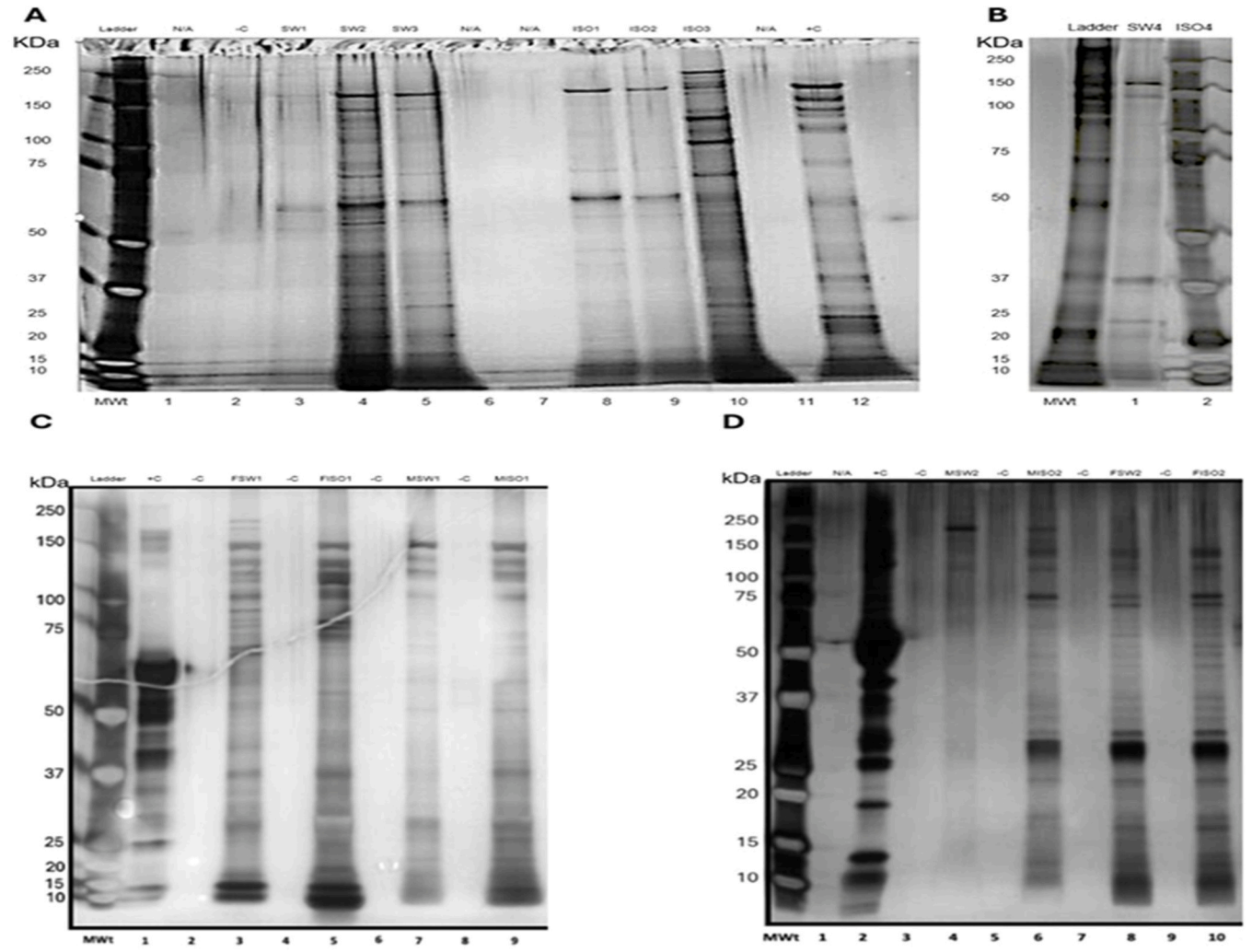

Fig. 1. Protein profiles of L. salmonis SEPs as visualized through silver stained SDS PAGE. Abbreviations: SW = seawater; ISO = isophorone; F = adult female; M = adult male. (A) Protein profiles of copepodid samples SW1, SW2, SW3, ISO1, ISO2, and ISO3. (B) Protein profiles of copepodid samples SW4 and ISO4. (C) Protein profiles of adult samples MSW1, MISO1, FSW1, and FISO1. (D) Protein profiles of adult samples MSW2, MISO2, FSW2, and FISO2.

resolved in SW and 21 in ISO, spanning 150–10 kDa in both cases. The dominant bands in SW-treated males were primarily between 150–100 kDa, while ISO-treated males displayed strong bands in both the 150–100 kDa and 25–10 kDa ranges. No bands were detected in negative control lanes.

3.3. Copepodid SEPs

A total of 433 distinct proteins were detected across all copepodid samples (mean 95.5 ± 146.74 (1 S.D.)), with 381 unique to the SW treatment and 15 unique to the ISO incubation. 36 proteins were shared between the two treatment conditions. Analysis of signal peptides revealed that 164 distinct copepodid proteins were secretory in nature, of which 150 and 3 were unique to the SW and ISO treatments, respectively, and 11 were shared between the treatments.

Cumulatively, copepodid proteins with signal peptides included 18 distinct proteases, including 9 serine type endopeptidases, 5 cysteine-type peptidases, 1 metallocarboxypeptidase, 1 metalloendopeptidase, 1 serine-type endocarboxypeptidase, and 1 dipeptidyl-peptidase. Notably, SW induced copepodid samples also contained 1 gamma-interferon-inducible thiol (GILT) reductase, 3 protease inhibitors (two serine-type endopeptidase inhibitors and an endopeptidase inhibitor), a heme peroxidase, and an antioxidant reductase. Copepodid samples also included proteins implicated in protein binding, carbohydrate binding, phospholipase binding, calcium ion binding, lipid transport, iron binding, isomerase activity, peroxidase activity, alpha-mannosidase activity, signaling receptor activity, RNA endonuclease activity, and dioxygenase activity. An additional 23 uncharacterized proteins were noted among the copepodid proteins with signal peptides.

SW control replicates exhibited a high degree of variability in the numbers of proteins detected (after filtration) across replicates, with a mean of 110.75 and an SD of 171.9, whereas these values stood at 19.25 and 16.32, respectively, for the ISO treatment replicates. Despite this high degree of variability, no significant difference in protein richness was found between SW control and ISO-treated groups (Welch's *t*-test, $p = 0.480$, $n = 4$ per group). The secretory protein profiles of the SW and ISO samples shared just 2 proteases (an aminopeptidase and a trypsin-like protein), 2 proteins potentially involved in oxidative stress mitigation (ascorbate peroxidase and a peroxidasin homolog), 1 protein associated with arthropod immunity (phenoloxidase activating factor 2), 1 possible immunomodulatory protein (pregnancy like-1 associated protein), 1 cystatin domain-containing protease inhibitor, and 1 uncharacterized protein. All three of the proteins unique to the secretory profile of copepodids incubated in ISO were putative vitellogenins.

3.4. Adult SEPs and secretory proteins

A total of 117 distinct proteins (mean 56 ± 12.70) were detected

across all adult samples. Variability in protein number between replicates in each treatment group was far lower than in copepodids, with means and SDs of 44.5 and 0.71 for the SW induction and 39.4 and 2.12 for the ISO induction. When comparing sexes within treatment groups, significantly more unique secretory proteins were detected in samples from ISO-treated females than ISO-treated males (mean ± SD: 42.5 ± 3.5 vs. 37.0 ± 1.4; Welch's *t*-test, $p = 0.0198$, $n = 2$ per sex). No significant sex difference was detected in the SW group ($p = 0.74$). When sexes were pooled within treatments, more unique secretory proteins were detected in SW-treated samples on average than in ISO-treated samples (mean ± SD: 44.5 ± 0.71 vs. 39.5 ± 2.12), but this difference was not statistically significant (Welch's *t*-test, $p = 0.158$, $n = 2$ per group).

A cumulative total of 69 adult proteins were found to contain signal peptides. Of these, 31 secretory proteins were unique to females and 10 to males. A further 28 distinct adult proteins were only detected in samples from the ISO treatment, whereas only 3 were unique to the SW treatment. 17 distinct secretory proteins were found to be shared between both adult treatment conditions.

Adult secretory proteins were rich in proteases, including 14 serine-type endoproteases, 5 metalloendopeptidases, 3 cysteine-type peptidases, and 2 metallocarboxypeptidases. Two protease inhibitors were also present (one cysteine-type endopeptidase inhibitor and one serine-type endopeptidase inhibitor), as well as one fibronectin type II domain-bearing protein implicated in heparin binding. Other detected secretory proteins were involved in protein binding, lysozyme activity, phosphatase activity, lipid transport, protein folding, peroxidase activity, and growth factor activity, along with 9 uncharacterized proteins.

The secretory protein profiles of adults incubated in SW and ISO shared 17 total proteins, including 10 proteases (brachyurins, trypsin-1-like proteins, chymotrypsin B1-like proteins, a transmembrane protease serine-9 like, a choriolytic enzyme-like, and a zingipain-2-like), 5 phenoloxidase activating factor-2-like proteins, 1 fibronectin type II-like domain protein, and 1 protein resembling the *Yersinia pestis* toxin pesticin. Of the remaining 52 total secretory proteins, only three were unique to the SW treatment, of which one resembles epididymal sperm binding protein-1, while the remaining two were uncharacterized.

3.5. Comparison of adult and copepodid secretory proteins

Across both incubation conditions, adults and copepodids were found to share a total of 21 proteins with signal peptides in common. These included 1 vitellogenin, 8 proteases, and 2 protease inhibitors. Two proteins were found to bear similarity to glutathione peroxidase and a thioredoxin domain, which may be involved in the regulation of oxidative stress. Two secretory proteins with resemblance to fibronectins type II and type III were noted, which may be involved in the modulation of host coagulation. A putative member of the takeout superfamily of proteins was also detected. An additional two uncharacterized proteins were also noted among the list of shared proteins.

A total of 48 proteins were detected exclusively in the adult secretory proteins (*i.e.*, they were not among the proteins detected in copepodid samples). 20 of these proteins were predicted to exhibit protease activity, while just 2 were predicted to serve as protease inhibitors. Notably, two proteins were found to bear resemblance to von Willebrand factor type A and fibronectin type II, which may play a role in the modulation of host coagulation. Two proteins with similarity to the cytokines pleiotrophin/midkine were noted, as was pesticin. An additional 6 uncharacterized proteins were also found to be unique to the profile of adult secretory proteins.

A total of 143 secretory proteins were uniquely detected in copepodid samples. These included 4 vitellogenins, 11 proteases, 3 protease inhibitors, and 22 uncharacterized proteins. 9 proteins in total were found to have potential roles in the modulation of louse oxidative stress, including 3 heme peroxidases, 4 thioredoxins, and 1 glutathione peroxidase. Additionally, a 70 kDa heat shock protein (HSP) was detected among the copepodid secretory proteins. Copepodid SEPs were also notable for containing an array of proteins with potential immunological function, including macroglobulins, lectins, immunoglobulins, and 1 gamma interferon inducible lysosomal thiol reductase (GILT) protein. Proteins potentially implicated in host hemostatic modulation include fibronectin types II and III, as well as a von Willebrand factor type C domain.

3.6. Functional comparison of GO terms

Analysis of GO terms for adult and copepodid secretory proteins revealed considerable differences in the cellular components, molecular function, and biological processes between the life stages. In regard to cellular component, of the nine adult proteins with valid GO terms, 67 % were associated with extracellular localization, as opposed to 30.7 % (of the 52 proteins with corresponding GO terms) of the copepodid proteins. Copepodid proteins also exhibited a greater diversity of biological processes than adults, but the profiles of both were dominated by proteins implicated in proteolysis (24.2 % and 69.6 %, respectively). Accordingly, the most frequently occurring molecular function GO term for adult proteins was serine-type endoprotease activity at 39.4 %, with other types of protease activity (i.e., metalloprotease, metalloendopeptidase, and cysteine-peptidase activity) cumulatively constituting an additional 18.2 %. Protease inhibitor activity (for both serine-type and cysteine-type endopeptidases) cumulatively comprised 12.1 %. In contrast, the most frequently occurring molecular function term for copepodid proteins was protein binding at 12.5 %. However, when taken cumulatively, the various types of protease activity (serine-type endopeptidase, cysteine-type peptidase, serine-type carboxypeptidase, and metalloendopeptidase activity) constituted 15.7 % of proteins, whereas the various kinds of protease inhibitor activity (serine-type endopeptidase, endopeptidase, and cysteine-type endopeptidase inhibitor activity) constituted just 7.9 % of proteins. These data are presented graphically in Figs. 2 and 3. Further graphical comparisons of adult and copepodid secretory proteins can be found in Figures S2, S3, and S4.

3.7. Detection of labial gland proteins

In total, three distinct labial gland proteins were detected with 100 % sequence alignment across both adult and copepodid secretory protein profiles. Two of these (LslGA4 and LslGA1) were only detected in adult SEPs, whereas one (LslGPA2) was only detected in copepodid SEPs. Additionally, LslGP3 was potentially detected in adult SEPs at a sequence alignment of 93 %.

4. Discussion

This study demonstrates that, under the tested conditions, there are differences in the secretomes of *L. salmonis* adults and copepodids, as only ~15 % of detected copepodid secretory proteins were shared with adults. While this number is low, it is already well-established that differences in secretory protein profiles and expression levels exist between the different life stages of *L. salmonis*. Hamilton et al. (2018) identified 19 secretory proteins in common between the SEPs of pre-adult and adult female *L. salmonis* (with 8 and 29 secretory proteins unique to those respective life stages), while Øvergård et al. (2022) detected differential expression levels of labial gland protein expression at different life stages. Furthermore, Tadiso et al. (2011) noted differences in the transcription of *S. salar* genes associated with innate immunity related to the transition between attached copepodids and chalimi, potentially suggesting a change in *L. salmonis* immunomodulatory secretions. This variance in secretome across *L. salmonis* life stages may suggest the existence of differential immunomodulatory strategies that promote parasitism based upon the unique biological needs of each molt. In this case, copepodids, unlike later *L. salmonis* molts, lack the physiological characteristics required to anchor themselves to the host

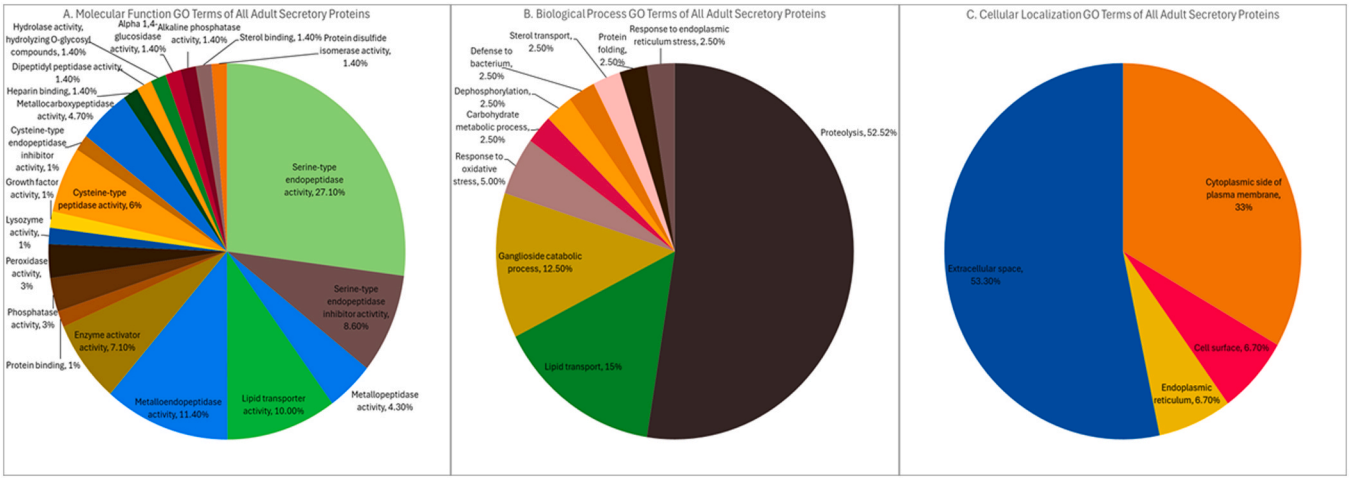

Fig. 2. GO terms for secretory proteins detected in samples from adults. In charts representing over 12 GO terms, the top 12 terms are included in the chart, while the remaining terms are included in the 'other' category. More comparative details of secretory protein profiles between adults and copepodids may be found in Figs. S2, S3, and S4.

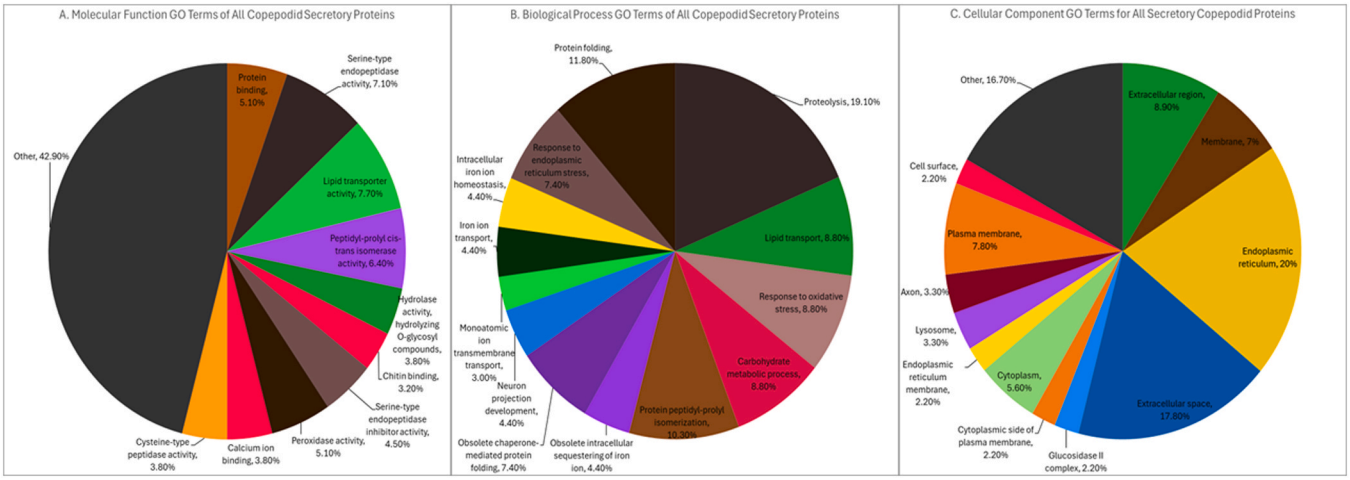

Fig. 3. GO terms for secretory proteins detected in samples from copepodids. In charts representing over 12 GO terms, the top 12 terms are included in the chart, while the remaining terms are included in the 'other' category. More comparative details of secretory protein profiles between adults and copepodids may be found in Figs. S2, S3, and S4.

with either a frontal filament or a suction cup-like cephalothorax. This weak attachment to the host relative to other molts makes copepodids particularly vulnerable to rejection or evasion, thus necessitating a different secretion-driven immunomodulatory strategy from other life stages. Furthermore, salmonid superficial mucus is known to contain a rich array of immune cells, as well as mucins, lysozymes, complement factors, and immunoglobulins, presenting a significant obstacle to the initial infection started by copepodids (Reverter et al., 2018). The unique protease and protease inhibitor profile of copepodids (relative to adults) may thus in part represent a strategy to overcome this early barrier to establish the initial infection. This associated variance in secretome further underscores the importance of tailoring of more targeted anti-louse treatments and vaccines to the distinct secretory profiles of each life stage.

While the analysis of the functional and localization data provided by GO annotations is likely limited in the study of *L. salmonis* due to their origins in human and model organism studies, they nevertheless suggest that *L. salmonis* copepodid and adult SEPs contain a wide range of secretory proteins that may play a role in virulence and survival on the host (du Plessis et al., 2011). The profile of proteins common to both adult and copepodid SEPs was dominated by proteases (especially serine proteases). Proteases are known to be widely employed by terrestrial arthropod ectoparasites (*e.g.*, ticks, mosquitoes, and mites) to both facilitate the digestion of host tissue, as well as to modulate host immunity (Černý and Arora, 2024). For example, the scabies mite (*Sarcoptes scabiei*) employs scabies mite inactivated serine protease paralogs to suppress the host complement system by cleaving C1q (Bergstrom et al., 2009).

Accordingly, some *L. salmonis* proteases have also been established to play a role in virulence and survival on the host. Two of the four labial gland proteins that were detected in adult SEPs (LslGA4 and LslGA1) are all members of the astacin family of metalloendopeptidases. While it has been suggested by Midtbø et al. (2024a, 2024b) that these two proteins do not serve to modulate host immunity, they have been hypothesized to play a role in *L. salmonis'* ability to suppress microbes during parasitism, thus assisting survival on the host. In contrast, the remaining two labial gland proteins, LslGPA2 and LslGP3, are known to be immunomodulatory, with the former suppressing inflammation and adaptive immunity, and the latter inducing apoptosis in host leukocytes and suppressing the adaptive immune response (Midtbø et al., 2024a, 2024b).

The types of protease inhibitors common to copepodid and adult secretory protein profiles (Kunitz domain-containing and cystatin

domain-containing) may also constitute virulence factors, as observed among other ectoparasites. In such ectoparasites, these protease inhibitors exhibit a wide array of functions that affect wound healing and coagulation and can serve to suppress host immunity (Chmelař et al., 2017). For example, *Ixodes scapularis* secretes a class of cystatins called sialostatins, which are known to inhibit proteases like cathepsin S in dendritic cells to suppress antigen presentation, or macrophage casapase-1 to dampen the IL- 1_{β} response (Zavasnik-Bergant, 2008). A zingipain-2-like protein containing a cathepsin propeptide inhibitor domain was also detected in copepodid samples, perhaps serving to regulate the activity of louse proteases, but also potentially modulating host immunity through the inhibition of its cathepsins. Notably, copepodids were also found to produce a leukocyte elastase inhibitor-like protein, a member of the serpin family of protease inhibitors. Multiple species of hard ticks have been found to employ serpins as virulence factors, performing such diverse functions as suppressing coagulation, cytokine production, and leukocyte proliferation (Chmelař et al., 2017). Serpins have also been detected in the SEPs of the fish louse *Argulus foliaceus,* another aquatic ectoparasitic arthropod, raising the possibility of the evolutionary conservation of this putative virulence factor (AmbuAli et al., 2020).

Beyond proteases and protease inhibitors, a number of other potential virulence factors were also detected, some of which had been previously detected in adult and pre-adult SEPs by Hamilton et al. (2018). For example, an array of redox proteins (i.e., peroxidases and thioredoxins) were detected in both adult and copepodid SEPs. While such proteins could have a number of potential roles in maintaining louse oxidative homeostasis, they may also serve to protect them from host reactive oxygen species. Furthermore, ganglioside GM2 activator was also detected in adult SEPs in this study, the expression of which is known to be upregulated during blood feeding in *Ixodes ricinus* (Plchová, 2012). Vitellogenins were also detected in both adult and copepodid samples. While vitellogenins are known to play a role in *L. salmonis* reproduction and egg string development, their detection in non-reproductive copepodids suggests the possibility that certain vitellogenins possess alternative functions in this species (Dalvin et al., 2011). For example, vitellogenins in *Formica fusca* ants are known to be implicated in the regulation of social behavior, while *Apis mellifera* vitellogenins are involved in the regulation of immunity and oxidative stress (Morandin et al., 2019; Salmela and Sundström, 2017). While the specific role of these non-reproductive *L. salmonis* vitellogenins remains unclear, their expression across life stages and possible role in immunity merits deeper characterization and investigation of these proteins.

This study also detected several secretory proteins putatively thought to be implicated in immunity, which may also serve as virulence factors. Notably, a GILT-like protein was detected in copepodid SEPs, which has been observed to play a role in arthropod innate immunity (Izumida et al., 2021). However, in *Anopheles gambiae*, the GILT protein mosGILT is known to be expressed in both mosquito salivary gland and midgut tissue and is upregulated in the latter during blood feeding (Dolan et al., 2025). This *Anopheles* protein is known to both modulate viral transmission and promote *Plasmodium* sporogony and thus introduces the possibility that this *L. salmonis* GILT-like protein may also be implicated in immunomodulation or the transmission of vector-borne disease (Chowdhury et al., 2021; Dolan et al., 2025).

Ultimately, the effectiveness of isophorone (at the concentration used) as an inducer of secretory activity across both adults and copepodids remains inconclusive. Isophorone occurs naturally in host salmonid mucus and is known to serve as a semiochemical and chemoattractant for *L. salmonis* copepodids (Bailey et al., 2006). In this study, although the number of proteins present in copepodid SEPs was dramatically more variable in SW inductions as opposed to the ISO treatments, no significant differences in detected protein numbers were observed between treatments in either life stage. However, the overall profiles of specific proteins within each treatment group (especially among adults) did also vary. Further transcriptomic and quantitative proteomic analysis may be required to more accurately determine the effectiveness of isophorone as an inducer of secretory activity in *L. salmonis*.

5. Conclusions

These data provide a trove of information on the protein composition of *L. salmonis* copepodid SEPs, contextualized through their comparison with adult SEPs. An array of virulence factors, both known and putative, have been elucidated, meriting further functional validation. However, many of the proteins detected in these protein profiles remain wholly uncharacterized, partially uncharacterized, or are poorly understood, warranting further investigation. A comprehensive approach involving *in silico* analysis, *in situ* hybridization, immunological testing, and transcriptomic investigation is required to fully understand the deeper biological significance of these secreted proteins and to better illuminate the interactions between *L. salmonis* and its hosts.

CRediT authorship contribution statement

Kevin McLean: Software, Investigation, Formal analysis, Data curation. **Dorota Androscuk:** Software, Resources, Investigation, Formal analysis, Data curation. **Sean Monaghan:** Writing – review & editing, Supervision, Project administration. **Jay Haywood:** Methodology, Investigation, Formal analysis, Data curation. **Alexander Dindial:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **James Bron:** Writing – review & editing, Supervision. **Kim Thompson:** Supervision. **William Roy:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2025.110608.

Data Availability

Due to the author's ongoing PhD, raw proteomic data will be made available upon thesis publication or upon reasonable request to the corresponding author.

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