Characterization of three salmon louse (*Lepeophtheirus salmonis)* genes with fibronectin II domains expressed by tegumental type 1 glands

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Abstract

The salmon louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae), is currently the most significant pathogen affecting the salmon farming industry in the Northern Hemisphere. Exocrine glands of blood-feeding parasites are believed to be important for the host-parasite interaction, but also in the production of substances for integument lubrication and antifouling. In *L. salmonis*; however, we have limited knowledge about the exocrine glands. The aim of this study was therefore to examine three genes containing fibronectin type II (FNII) domains expressed expressed in *L. salmonis* tegumental type 1 (teg 1) glands, namely LsFNII1, 2 and 3. LsFNII1, 2 and 3 contains four, three, and two FNII domains respectively.

Sequence alignment of LsFNII domains showed conservation of amino acids that may indicate a possible involvement of LsFNII domains in collagen binding. Ontogenetic analysis of *LsFNII1*, *2* and *3* revealed highest expression in pre-adult and adult lice. Localization of *LsFNII1*, *2* and *3* transcripts showed expression in teg 1 glands only, which are the most abundant exocrine gland type in *L. salmonis*. *LsFNII1*, *2* and *3* was successfully knocked-down by RNAi, however, alteration in gland morphology was not detected between the knock-down and control groups. Overall, this study gives first insight into FNII domain containing proteins in *L. salmonis*.

1. Introduction

The salmon louse, *Lepeophtheirus salmonis* is a marine obligate ectoparasite infesting salmonids belonging to the genera *Salmo, Oncorhynchus* and *Salvelinus* (Kabata, 1979). The lifecycle consist of eight developmental stages separated by ecdysis; two planktonic nauplius, one infective copepodid, two attached chalimus, two mobile pre-adult and one adult stage (Hamre et al., 2013; Johnson and Albright, 1991; Schram, 1993). The salmon louse feeds on mucus, skin and blood (Brandal, 1976), and can thereby cause light to severe skin lesions (Jónsdóttir et al., 1992; Wootten et al., 1982). At present, salmon louse is the most severe disease problem in salmon aquaculture.

The fibronectin type II (FNII) domain is one of three types of internal repeats (type I, II and III), found within the multi domain glycoprotein fibronectin. FNII domains are approximately 60 amino acids long, and contain four conserved cysteines that forms disulfide bridges (Skorstengaard et al., 1986). These bridges are essential for the function of the FNII domains that are in fibronectin located in the collagen-binding region. Here two FNII domains together with two flanking FNI domains binds to the α-chains of collagen and gelatin (denatured collagen) (Guidry et al., 1990; Steffensen et al., 2002). FNII domains have also been

identified in other vertebrate proteins that binds collagen such as the matrix metalloproteinases (MMP) 2 and 9, bovine seminal plasma protein PDC-109, blood coagulation factor XII and mannose receptor of macrophages (Collier et al., 1988; McMullen and Fujikawa, 1985; Seidah et al., 1987; Taylor et al., 1990; Wilhelm et al., 1989).While the FNII domains of fibronectin are not capable of binding collagen alone (Steffensen et al., 2002), both MMP-2 and 9, which bind and degrade components of the extracellular matrix, each have three FNII domains that bind collagen/gelatin where one of those is capable of solely bind gelatin (Banyai and Patthy, 1991; Collier et al., 1992). The bovine seminal fluid protein PDC-109 and its homologous also bind to collagen, despite the fact that the ligands of these proteins are phospholipids (Desnoyers and Manjunath, 1992). Moreover, the binding specificity of FNII domains present among the different proteins varies. For instance, the FNII domains present in the collagen binding region of fibronectin binds to native collagen type I and III, while FNII domains present in the mannose receptor binds to native collagen type I, III and IV collagen, while those of MMP-2 bind I, III and V (Napper et al., 2006; Steffensen et al., 1995; Steffensen et al., 2002). Moreover, it has been suggested that the collagen binding property of the mannose receptor could play a role in clearance of collagen fragments or in mediating cell-matrix adhesions (Napper et al., 2006).

Since FNII domains have not been found in model invertebrate genomes as in *Caenorhabditis elegans* and *Drosophila melanogaster* they have been regarded as vertebrate specific (Chalmers and Hoffmann, 2012; Ozhogina et al., 2001). Instead, invertebrates have kringle domains, suggested to be ancestral FNII domains. However, FNII domains have recently been found in the genome of two freshwater planarian species *Dugesia ryukyuensis* and *Schmidtea mediterranea* (non-parasitic turbellarian) (Chalmers and Hoffmann, 2012) and in *L. salmonis* (Øvergard et al., 2016). In the *L. salmonis* genome, more than 200 copies of the FNII domain

within more than 80 genes have been identified (www.licebase.org), making FNII the most expanded protein domain. In comparison, only five genes containing kringle domains have been found in the *L. salmonis* genome. Interestingly, a recent study showed that one gene, LsFNII1 that has four FNII domains, was expressed in tegumental type 1 glands (teg 1), which is the most abundant type of exocrine gland found in salmon louse (Øvergard et al., 2016). Here, tegumental glands can be divided into type 1, 2 and 3 according to when they appear during development. Teg 1 glands can be detected already at the first planktonic larval stage with secretory ducts extending both dorsally and ventrally. As teg 1 glands also produce mucus and express astacin metallopeptidases (Bell, 2001; Øvergard et al., 2016), they have been suggested to lubricate the integument with anti-fouling agents (Bron et al., 2000; Øvergard et al., 2016).

The aim of the present study was primarily to explore the temporal and spatial expression of three genes with FNII domains, and investigate louse phenotype and histological morphology of knock-down animals. Since the genes were found to be expressed in teg 1 glands, cephalic teg 1 gland secretory pores were mapped, as the sites of secretion may give functional information of salmon louse proteins with FNII domains.

2. Material and Methods

2.1 Animals

A laboratory strain of *L. salmonis* was raised on Atlantic salmon (*Salmon salar*) in tanks with salinity of 34 ‰ and temperature of approximately 10 ℃. All experiments were conducted in accordance to Norwegian animal-welfare regulations. Prior to sampling, the fish was either sedated with a mixture of benzocaine (60mg/L) and methomidate (5mg/L) or killed by a blow to the head. Fish infected with dsRNA injected lice were kept in single tanks as described earlier by Hamre and Nilsen (2011).

2.2 Collection of animals for analysis

Eggs were kept in flow-through incubators and cultivated to copepodids stages (Hamre et al., 2009). Copepodids 9 days post hatching were used to infest Atlantic salmon. All developmental stages of *L. salmonis* were collected in five biological replicates. Each replicate contained immature egg strings (light colored, n=1), nauplius I –II and free-living copepodids (n ≈ 100), copepodids 2 and 4 days post infestation (DPI) respectively (n=60), chalimus I (n=30), chalimus II (n=20), pre-adult or adult stages (n=1).

2.3 RNA extraction and cDNA synthesis

All samples for RNA isolation were collected in RNA later (LifeTechnologies), kept at 4 °C overnight and stored at −20 °C. RNA was isolated using 1 ml TRI Reagent (Sigma Aldrich). Homogenization was carried out using 1.4 mm zirconium oxide beads (Precellys 24) for nauplius, copepodids and chalimus and 5 mm stainless steel beads for preadult and adult lice. The sample was homogenized for 2X 2 min at 50 Hz with a tissueLyser LT (Qiagen). Phase separation was accomplished by adding 0.2 ml chloroform to the samples, and centrifuged at 12,000 x g for 15 min at 4 ℃. Samples for ontogenesis were thereafter purified using RNeasy kits with DNase treatment preformed on the column. The water phase was withdrawn and mixed with 1x volume of 70 % ethanol and transferred to an RNeasy spin column. Further, RNA from immature eggs to preadult II stages was isolated using the RNeasy micro kit (Qiagen) while RNA from adult lice were isolated using RNeasy mini kit (Qiagen), according to supplier's instructions. RNA from adult RNAi treated animals was isolated using TRI Reagent (Sigma Aldrich) according to manufacturer's instructions, and DNase treated with DNaseI (Amplification Grade, Invitrogen). The amount and purity of the isolated RNA were measured with a Nanodrop Spectrophotometer (Nanodrop ND-1000). Extracted RNA was either kept at −80 °C until use or cDNA synthesis was performed directly.

cDNA synthesis for standard PCR was performed with the qScript cDNA synthesis kit (Quanta Bioscience) according to supplier's instructions, using 1 µg DNase treated total

RNA. cDNA synthesis for quantitative RT-qPCR was performed using AffinityScript cDNA synthesis kit (Agilent Tecnologies). Each reaction consisted of 1x cDNA synthesis mastermix, 100 ng Oligo dT, 50 ng Random primers, 0.5 U AffinityScript RT enzyme and 200 ng total RNA in a final volume of 10 µl. cDNA was diluted 1:10 in H_2O before storage at -20 ℃.

2.4 PCR, RACE, cloning and sequencing

Candidate FNII genes were obtained from the salmon louse genome (www.licebase.org) based on InterProScan prediction on protein domains. Rapid amplification of 5`and 3` cDNA ends were performed using the SMARTer™ RACE cDNA Amplification Kit (Clonetech). RACE products were cloned using TOPO TA Cloning® Kit for sequencing (Invitrogen). Clones were further used as template in PCR reactions, using 10 µM M13 forward and reverse primer, 2 mM Mg^{2+}, 100 µM dNTP's, 1x Green GoTaq® Flexi Buffer and 1.25 u of Go Taq Flexi DNA Polymerase (Promega), and run according to the suppliers recommendation. PCR products were purified with ExoSAP-it (Affymetrix) prior to sequencing at the sequence lab facility at the University of Bergen using BigDye Terminator 3.1 reagents (Applied Biosystems). To ensure amplification of the entire coding sequence, the three genes of interest were further sequences using LsFNII1_F and LsFNII1_R for the first gene, LsFNII2_F #1 and LsFNII2_R #3 for the second gene, and LsFNII3_F and LsFNII3_R for the third gene.

Sequences were analyzed and assembled using Vector NTI 10 (Invitrogen).

The three genes of interest were further BLASTed against the salmon lice genome in Licebase (www.licebase.org), in order to identify possible paralogs. ORFs were identified using Prediction of Translation Initiation ATG (Nishikawa et al., 2000). Protein domains were identified using InterPro database (Mitchell et al., 2015). Sequence editing and alignment of selected FNII domains were performed using BioEDIT v. 7.2.3 (Hall, 1999). For further

prediction of protein structures the Phyre^2 protein fold recognition server was used (Kelley et al., 2015).

2.5 Quantitative RT-qPCR

The RT- qPCR reaction was performed using 1x PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 2 µl cDNA, 0.5 µM forward and reverse primer (Table 1) in a final volume of 10 µl per reactions. The efficiency for SYBR Green primers were checked by a five-point standard curve of 4-fold dilutions, and calculated by the equation $E\% = (10^{1/slope} - 1) \times 100$ (Radonic et al., 2004). The reaction set up was: initiation 50 ℃ for 2 min, holding 95 ℃ for 2 min, 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 1 min, in addition of a melt curve analysis at 60-90 ℃ to check for primer/dimer formation on an Applied Biosystems 7500 Fast Real-Time PCR system. The RT- qPCR was always performed with two technical replicates. Moreover, a no template control (NTC) were used to monitor possible contamination and primer/dimer formation, and a minus reverse transcription control (–RT) were used to control for possible DNA contamination. The relative expression level was calculated using $2^{-\Delta Ct}*100$. Target genes were normalized using the salmon louse elongation factor 1 alpha (eEF1α) standard gene assay (Frost and Nilsen, 2003). Primers used for real time RT-qPCR are listed in Table 1.

Table 1

Primers used for PCR and RT-qPCR. The T7 promoter extension is shown in parentheses.

Primer name	Sequence	Size (bp)/efficiency (%)
5`Race LsFNII1	GCACACCATGGCACACCACCATTATCAGC	
3`Race LsFNII1	GCTGATAATGGTGGTGTGCCATGGTGTGC	
5`Race LsFNII2	CATATTCGAGGGGTCCACAGCTGTAGCA	
3`Race LsFNII2	ACTCATGGTGTGCTACAGCTGTGGACCC	
5`Race LsFNII3	CGCACCATTTCTTACCTTCGTTATCGGC	
3`Race LsFNII3	ACAAGTGCACAGATGCCGATAACGAAGG	
LsFNII1_F	TATCTGACTGAAGATGAAGCTAATTTGG	
LsFNII1_R	ACATTGGTTGGTGTGCCTTAACA	
LsFNII2_F #1	GGGAAACCCTATTGTATCCTGTCC	
LsFNII2_FT7 #1	(TAATACGACTCACTATAGGG)GGGAAACCCTATTGTATCCTGTCC	
LsFNII2_R #1	CCCGCATATGTACAGCTTGTGTGT	
LsFNII2_RT7 #1	(TAATACGACTCACTATAGGG)CCCGCATATGTACAGCTTGTGTGT	
LsFNII2_F #2	ACCCTCTCAGAGATTAACTGCG	
LsFNII2_FT7 #2	(TAATACGACTCACTATAGGG)ACCCTCTCAGAGATTAACTGCG	
LsFNII2_R #2	CATATGGAACGGCAAACACCA	
LsFNII2_RT7 #2	(TAATACGACTCACTATAGGG)CATATGGAACGGCAAACACCA	

LsFNII2_R #3	TTCGTTGCACACCAAGGAAG	
LsFNII3_F	TACATTTGCGTGCCTTCTCCTC	
LsFNII3_FT7	(TAATACGACTCACTATAGGG)TACATTTGCGTGCCTTCTCCTC	
LsFNII3_R	TGACATTGAGAGCTCATGTTGCAT	
LsFNII3_RT7	(TAATACGACTCACTATAGGG)TGACATTGAGAGCTCATGTTGCAT	
CPY_F	(TAATACGACTCACTATAGGG)ATAGGGCGAATTGGGTACCG	
CPY_R	(TAATACGACTCACTATAGGG)AAAGGGAACAAAAGCTGGAGC	
SYBR_LsEF1α_F	GGTCGACAGACGTACTGGTAAATCC	229 bp/96 %
SYBR_LsEF1α_R	TGCGGCCTTGGTGGTGGTTC	
SYBR_ LsFNII1_F	GCTCCTAAGAATACGCCTAAGGCA	276 bp/101 %
SYBR_ LsFNI1_R	CAGAGCCACAATTTCCGTAAGC	
SYBR_ LsFNII2_F	CCCTCTCAGAGATTAACTGCGTGTTC	122bp/91 %
SYBR_ LsFNII2_R	CCATATTCGAGGGGTCCACAGC	
SYBR_ LsFNII3_F	ACATTTGCGTGCCTTCTCCTCA	273 bp/97 %
SYBR_ LsFNII3_R	CGCATTGATAATTTCCAGTGGTGAT	
SYBR_ LsFNII4_F	GTTGATACCTACGGAGATTGCAATGCTG	210 bp/107 %
SYBR_ LsFNII4_R	TTCGAAATGGTAGGCTTGTTCAGAGTTG	

2.6 In situ hybridization

Adult female and male lice were fixated in phosphate buffered 4 % paraformaldehyde (pH 7.4) over night at 4 °C. Subsequently, specimens were processed in the Histokinette 2000 (Reichert-Jung) and embedded in paraffin wax. Sections, 3.0 µm thick, were cut with a Leica RM 225 microtome (Leica Microsystems). Sense and antisense RNA probes were synthesized from PCR products made by using primer pairs with and without a T7 promoter overhang (Table 1). The length of the PCR products was verified by 1 % agarose gel, and purified using Gene Elute PCR Cleanup kit (Sigma, Aldrich). RNA probes were synthesized and labelled using DIG RNA Labelling kit (Rocher). A spot test was performed to check incorporation of DIG to the RNA probes. *In situ* hybridization was performed as described earlier by Dalvin et al. (2013) with some modifications: xylene was replaced by histoclear (National Diagnostics) in removal of paraffin, digestion by proteinase K was prolonged to 13 minutes, and 100 µl hybridization mix with a probe concentration of 2.5 ng/µl was used for each slide. Hybridization with sense probe was used as a negative control.

2.8 RNA interference

The RNAi trial was conducted as earlier described by Dalvin et al. (2009). Briefly, dsRNA was synthetized using MEGAscript® RNAi kit (Ambion) according to suppliers' instructions using primers listed in Table 1. The dsRNA fragments were diluted to 600 ng/µl prior to injection, and 1 µl of bromphenol blue was added to 50 µl of the dsRNA solution to visualize successful injection. Pre-adult II female lice and adult male lice were removed from Atlantic

salmon, and dsRNA were immediately injected dorsally into the haemocoel of the cephalothorax with approximately 1 µl target gene dsRNA solution or cod trypsin CPY dsRNA (control) (Table 2). After injection, the lice were incubated in seawater for approximately 3 hours, and equal numbers of female and male lice were placed back on the fish in single fish tanks. The experiments were terminated after the adult female lice had produced the second egg strings, approximately 40 days post injection. Egg strings were harvested and put on incubators until hatching, as previously described by Hamre et al. (2009). The recovered lice were photographed and placed in RNAlater™ (Ambion Inc.) for RT-qPCR analysis or fixed in Karnovsky's fixative for histological examination.

An unpaired student T-test was performed to check for significant knock-down. P values below 0.05 were considered significant.

Table 2
Overview of conducted RNAi experiments.

Experiment	RNAi treatment	Number injected	Recovered lice
1	Control	29	15
	LsFNII1	30	14
2	Control	29	16
	LsFNII2#1	30	13
	LsFNII2#2	30	15
3	Control	30	8
	LsFNII2#2	30	8
4	Control	30	14
	LsFNII3	30	17

2.9 Histology

Salmon lice used for histological examination were fixed in Karnovsky's fixative overnight and then washed twice in PBS, dehydrated with ethanol solutions (50%, 70% and 96%), pre-infiltrated with Technovit/ethanol solution (50/50) for four hours (Technovit 7100, Heraeus Kulzer Technique) followed by Tecnovite infiltrating and hardening overnight before embedding. Sections 2 µm were cut using a microtome (Leica RM 2165). Sections were stained for 1 minute with toluidine blue (1% in 2% borax), washed and mounted with Mountex (Histolab Products).

2.10 Scanning electron microscopy (SEM)

For SEM, adult female specimens were fixed by immersion in a mixture of 10 ml 10% formaldehyde (fresh from paraformaldehyde), 10 ml 25% glutaraldehyde, 20 ml 0.2 M cacodylate buffer and 60 ml PBS, and the pH adjusted to 7.35. Whole lice were rinsed in PBS and postfixed in 1% OsO_4. Thereafter they were dehydrated in an acetone series, dried to critical point using a CPD 030 Bal-Tec (Bal-Tec Union Ltd., Balzers, Liechtenstein), mounted on stubs with carbon conductive tape and coated with gold-palladium using an Emitech K550X sputterer (Emitech, Ashford, England). The specimens were further studied in a Zeiss Supra 55VP field emission SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at the laboratory of Electron Microscopy, University of Bergen.

For localization of external pores, specimens were washed in 1x PBS with 0.5 % Tween-20 and given a short sonication of 10 sec before fixation in order to remove bacteria and other microorganisms. Moreover, some specimens were given a transversal cut after fixation, to be able to mount the specimens on the stub in such a way that the lateral and anterior pores would be visible. Additionally, cephalic appendages were removed from one specimen in order to localize ventral pores.

3. Results

3.1 Sequence analysis

LsFNII1 previously found to be expressed by tegumental type 1 glands has not been fully sequenced (Øvergård et al, 2016). Hence, RACE was performed identifying 1048 base pairs (bp) of the LsFNII1 sequence (KU821104). LsFNII1 was found to contain an open reading frame (ORF) of 912 bp, that translates into a putative protein of 303 amino acids (aa). A search for conserved protein domains in the Interpro database (Mitchell et al., 2015) revealed a signal peptide in the N-terminal region followed by four FNII domains (Fig. 1A). No other domains could be identified.

Furthermore, two additional LsFNII domain-containing proteins were sequenced and named LsFNII2 and LsFNII3. The LsFNII2 sequence (submitted to Genbank) was found to be 1190 bp containing an ORF of 1014 bp translating into a putative protein of 337 aa. Further analysis of conserved domains in InterPro database (Mitchell et al., 2015) revealed a signal peptide in the N-terminal region, followed by three FNII domains (Fig. 1A). The LsFNII3 sequence (submitted to Genbank) consists of 531 bp and an ORF of 432 bp. The ORF translated into a putative protein of 143 aa, where further analysis of conserved domains revealed a signal peptide followed by two FNII domains (Fig. 1A). Blast searches in the salmon louse genome (www.licebase.org) revealed that LsFNII3 contain sequence highly similar to another LsFNII containing gene, EMLSAG00000006557. This gene was named LsFNII4.

Multiple sequence alignment of FNII domains from LsFNII1, 2 and 3 with FNII domains from other species (Fig. 1B) demonstrated that FNII domains from *L. salmonis* show relatively high sequence conservation. All LsFNII domains analyzed possess the four conserved cysteines that are important in disulfide binding, as well as some of the conserved residues that are predicted to be involved in collagen binding (Banyai et al., 1994).

3.2 Ontogenetic analysis of gene expression.

The expression levels of *LsFNII1*, *2* and 3 were investigated throughout the *L. salmonis* lifecycle by RT-qPCR (Fig. 2). Due to the high level of similarity with LsFNII3, expression analysis of *LsFNII4* was also conducted.

LsFNII1 transcripts were not detectable before the chalimus II stage (Fig. 2A). An elevated expression was detected from pre-adult to adult stages, with the highest expression level seen in mature adult males. Similar to *LsFNII1*, the expression level of *LsFNII2* was lowest in

immature egg strings and the planktonic larval stages (Fig. 2 B). The expression level increase during the parasitic stages. A sharp increase in expression was seen from chalimus I stage to preadult I female and male. The highest expression level of *LsFNII2* was found in adult males. The expression level of *LsFNII2* was decreased during maturation of adult females and in older males. The *LsFNII3* expression levels show similar expression as *LsFNII1*and *2* (Fig. 2 C). Lower expression levels of *LsFNII3* were seen in the stages from immature egg strings to chalimus I, with an elevated expression seen from chalimus II. During the pre-adult stages, *LsFNII3* expression is rather constant, though mature adult males show a higher relative expression. In contrast, the relative expression level of *LsFNII4* was low in all developmental stages except of the copepodids 2 and 4 dpi (Fig. 2D). High standard deviation levels are seen between biological replicates on mature adult males for *LsFNII1 and 3*, and on preadult I and II female and male, and adult male for *LsFNII2*.

3.3 In situ hybridization

The localization of the *LsFNII2* and *3* transcripts were determined in adult female and male lice respectively by in situ hybridization. In the adult stages, the expression of LsFNII4 was insignificant, and is not expected to be localized by the LsFNII3 RNA probe.

LsFNII2 and *3* transcripts were localized to teg 1 glands only (Fig. 3). Positive staining of teg 1 glands within the sub-epidermal tissue in the cephalothorax, thoracic legs and genital segment was seen, while no hybridization was detected using the sense probe as a negative control (results not shown).

3.4 RNA interference

To investigate the functional role of LsFNII1, 2 and 3, RNAi trials were conducted on maturing female lice from pre-adult II to adult females. Knock-down of *LsFNII1, 2* and *3* was confirmed by RT-qPCR (Fig. 4). In total, four RNAi trials were conducted (Table 2).

All three transcripts, *LsFNII1, 2* and *3* were downregulated (87-99%) compared to the control. Due to sequence similarity, *LsFNII3* dsRNA fragment could potentially also affect transcription levels of LsFNII4. Therefore, the relative expression of LsFNII4 in LsFNII3 dsRNA injected lice was checked. No down regulation of *LsFNII4* was observed (data not shown). Even though all three transcripts were successfully knocked down, no difference in survival, reproduction or morphology was observed between the treated group and the control group.

3.5 Localization and environment surrounding tegumental type 1 gland pores

In order to examine the possible function of teg 1 gland secretions, the external pores and the immediate surrounding of the *L. salmonis* integument were investigated using scanning electron microscopy. Initial experiments revealed that glandular pores were difficult to detect as the louse were covered with bacteria and other microorganisms. The bacteria adhering to the salmon louse integument were mainly found to be rod-shaped bacteria. Interestingly, less bacterial growth was observed in close proximity to pores (Fig. 5C).

Subsequently, washing with sonication were performed prior to fixation in order to remove microorganisms, and pores could thereby be localized on the ventral and dorsal side of the cephalothorax. Most pores were identified dorsally, where at least 53 pores were detected (Fig. 5 A). Two of these small pores were situated anterolaterally on each side of the cephalic margin prior to the extension of the marginal membrane (Fig. 5 B). Ventrally, pores were seen in the extremities and two large pores were seen adjacent to the postantennary process (Fig. 5D), as previously identified by Øvergard et al. (2016).

4. Discussion

To our knowledge, the presence of FNII domains in invertebrates have only been reported twice, in two freshwater non-parasitic planarian species (Chalmers and Hoffmann, 2012), and

312 in *L. salmonis (*Øvergard et al., 2016). The present study is; however, the first functional
313 study of invertebrate genes containing FNII domains.
314
315 Many vertebrate FNII domains are found to bind collagen (Banyai et al., 1994; Steffensen et
316 al., 1995; Steffensen et al., 2002). Sequence alignment of selected FNII domains (Fig. 1 B)
317 shows that many of the highly conserved amino acids predicted to be important for collagen
318 binding are present in LsFNII1, 2 and 3., for example the four cysteines that form disulfide
319 bridges. Moreover, it has been demonstrated in MMP-2 that three FNII domains confer
320 stronger affinity than a single domain (Banyai et al., 1994). All LsFNII proteins described
321 here contain from two to four FNII domains, all possessing the four conserved cysteines.
322 However, between the second and third cysteine the consensus sequence G-R-X-D-G-X-X-W
323 (where X is any amino acid), important for collagen binding in MMP-2 (Briknarova et al.,
324 1999; Tordai and Patthy, 1999), is rather poorly conserved as in bovine seminal plasma
325 protein PDC-109, which nevertheless is capable of collagen binding (Banyai et al., 1990).
326 Moreover, an N-terminal extension of approximately 15 residues, prior to the first cysteine, is
327 missing in the second, third and fourth FNII domain of LsFNII1, and all FNII domains of
328 LsFNII3; the equivalent also being the case in PDC-109, in addition to the two flatworm FNII
329 domain-containing proteins (Chalmers and Hoffmann, 2012). Most of the FNII domains of
330 LsFNII1, 2 and 3 possess the N-terminal extension, though they lack the N-terminal
331 consensus sequence T–X–X–G–N–X–X–G where the first three residues are predicted to
332 contribute to a β-sheet (Banyai et al., 1996). Nevertheless, the conservation of the four
333 important cysteines and other important residues involved in collagen binding suggests that
334 LsFNII1, 2 and 3 have the ability to bind collagen, or maybe collagen-like proteins as
335 previously suggested (Øvergard et al., 2016). On the other hand, it cannot be excluded that
336 these proteins bind other unknown ligands.

During the life cycle of *L. salmonis*, the expression profiles of *LsFNII1, 2 and 3* were similar, with no or low expression in free-living stages, followed by a steady increase during the early parasitic stages. The highest level of expression was detected in pre-adults and adults, which may indicate an involvement in the host-parasitic interaction. However, the low expression of *LsFNII2* and *3* in the early parasitic stages suggest that they are not. Moreover, LsFNII1 transcripts have previously been detected exclusively in teg 1 glands (Øvergard et al., 2016), which, as shown in the present study, is also seen for *LsFNII2* and *3*. The high number of teg 1 secretory ducts extending out on the dorsal surface of the integument, away from the fish host, also argues against a role of these proteins in the host parasite interaction. Teg 1 glands are already present at the nauplius I stage, but in low numbers, and the increase in relative expression level of *LsFNII1, 2* and *3* during development coincides with the increase in number of teg 1 glands in latter stages. Additionally, the expression level of LsFNII1, 2 and 3 was highest in males, despite the fact that the number of teg 1 glands have been estimated to be the same for males and females (Øvergard et al., 2016) although adult females have a larger chephalothorax (mm), and a long genital segment, consisting mainly of developing oocytes and large cement glands (Ritchie et al., 1996). Thus, the higher levels of *LsFNII1, 2* and *3* in adult males, relative to females, may be explained by differences in body size in relation to the abundance of teg1 gland-tissue.

To analyze the functional role of FNII-domain-containing proteins secreted by the *L. salmonis* teg 1 glands, *LsFNII1, 2* and *3* were knocked-down by RNAi. However, even though an efficient knock-down of all tree LsFNII transcripts were obtained, no visible alteration in lice gross morphology or histological appearance of gland tissue was observed. Hence, this indicates that the gene products do not have essential functions during the timeline of the

362 experimental period, or that their functions are compensated by one or more of the many FNII
363 domain-containing proteins identified within the *L. salmonis* genome. Further analysis of
364 protein stability, functional redundancy between FNII domain-containing proteins and
365 ultrastructural studies of knock-down animals could elucidate this.
366
367 Tegumental glands in crustaceans have been suggested to be involved in secretion of
368 epicuticle, tanning of the tegument, mucus production for feeding and lubrication, and
369 production of a bacteriostatic and antifouling agent (Alexander, 1989; Boxshall, 1982; Brunet
370 et al., 1991; Yonge, 1932). Ultrastructural studies of the *L. salmonis* integument have revealed
371 that the epicuticle is covered by a mucoid layer named the fuzzy coat (Bron et al., 2000),
372 which is also seen on the surface of other copepods, including free living and parasitic species
373 (Bresciani, 1986; Briggs, 1978). In addition, the *L. salmonis* teg 1 glands secret neutral
374 and/or acidic mucus (Bell, 2001). Moreover, their presence in planktonic stages, and
375 abundance in mature lice, in addition to the fact that their secretory ducts extends out on both
376 the dorsal and ventral surface of the lice (Øvergard et al., 2016), strongly indicates that they
377 are responsible for producing the fuzzy coat. Moreover, a key role of the teg 1 gland in
378 maintenance of the fuzzy coat is supported by the observation of a high number of teg 1
379 secretory pores on the dorsal side of the louse (Fig. 5A), where the fuzzy coat is thickest
380 (Bron et al., 2000). At the ventral side, most teg 1 exit pores were in appendages, such as the
381 maxilla, maxilliped and the thoracic leg 1 and 2. These are constantly moved when the lousee
382 is sitting on its host (personal observation), possibly lubricating the ventral integument of the
383 cephalothorax. As the predicted protein sequences of LsFNII1, 2 and 3 all possess a signal
384 peptide, they are most probably secreted by the teg 1 gland, and might constitute components
385 of the fuzzy coat.
386

387 During SEM, we observed that the integument of *L. salmonis* was covered with bacteria and
388 other microorganisms (Fig. 5). Growth on the *L. salmonis* integument by a variety of
389 organisms such as bacteria, fungi, algae, ciliated protozoa and parasites has been reported
390 earlier (Barker et al., 2009; Freeman, 2002). Moreover, the growth of microorganisms seems
391 to be more prominent on older lice (personal observation), as does the expression of *LsFNII1*,
392 *2* and *3*. Interestingly, less bacterial growth was observed in close proximity to teg 1 gland
393 exit pores. Collagen-like proteins have been shown to be expressed by many bacteria
394 (Rasmussen et al., 2003). As eukaryote collagens, these bacterial collagen-like proteins
395 consist of Gly-X-Y repeats, and, despite the lack of hydroxyproline, form a highly stable
396 triple helix necessary for collagen function (Rasmussen et al., 2003). Many of them are
397 anchored to the bacterial cell wall, and have been shown to be important for bacterial
398 colonization (Yu et al., 2014). An example of this is the collagen like protein Slc1 from
399 *Streptococcus pyogenes* that mimics collagen by the binding of collagen receptors facilitating
400 host cell adherence and activates extracellular signaling (Caswell et al., 2008; Lukomski et al.,
401 2017). Interestingly, on the dorsal integument of the louse, the adhering bacteria were
402 anchored via fibrils (Fig. 5B), possibly made up of prokaryotic collagen. As LsFNII1, 2 and 3
403 proteins are predicted to be secreted, and possibly bind collagen-like proteins, potentially
404 secreted by bacteria that colonize the integument, an antifouling function inhibiting bacterial
405 adherence may be suggested. As mentioned by Øvergard et al. (2016), an extensive growth of
406 bacteria and other microorganisms on the salmon louse integument may cause a significant
407 increase in drag and lead to host detachment.
408
409 In summary, we have identified two additional *L. salmonis* genes containing FNII domains,
410 with a structure highly similar to FNII domains of vertebrates that may suggest a function
411 related to collagen binding. However, further studies are needed to confirm collagen binding

properties. In addition, the investigated genes are active in teg 1 glands and expression of *LsFNII1, 2* and *3* increases through the *L. salmonis* life cycle. In knock-down animals, no alteration in phenotype was observed, and thus details on function were not obtained. Since teg 1 glands have secretory ducts that terminate in pores on the surface of the integument, it is probable that the proteins derived from the studied genes, exert their function on the surface of the louse. Currently we have limited information on the secretory products of the glands of *L. salmonis*, and what function they may have.

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Figure captions

Figure 1: Sequence analysis of LsFNII1, 2 and 3. (A) Schematic presentation of LsFNII1-iuiLsFNII3 and conserved protein domains. B) Sequence alignment of selected FNII domains from the first and second FNII domains in fibronectin (FN #1 and FN #2), three FN II domains form matrix metalloproteinases 2 and 9 (MMP-2 and 9), FN II domain from mannose receptor (MMR), two FNII domains from PDC-109 (PDC-109a/b), FNII domain from endo-180 (Endo-180), four FNII domains form LsFNII1, three FNII domains from LsFNII2, two FNII domains form LsFNII3, two FNII domains from *Dugesia ryukyuensis* (DrVal9 and 12), and two FNII domains from *Schmidtea mediterranea* (SmdVal4 and 8). The four conserved cysteines that are involved in disulfide bonding are highlighted in yellow, while residues that are predicted to be involved in collagen binding are highlighted in grey.

Figure 2: Expression of LsFNII1-4 in *Lepeophtheirus salmonis* throughout the lifecycle. Relative expression of LsFNII1 (A), LsFNII2 (B), LsFNII3 (C), and LsFNII4 (D). Columns show mean relative transcription in different *L. salmonis* developmental stages, and error bars are showing the standard deviation. Note the difference in scale of the Y-aksis. N=5 for each stage.

Figure 3: Localization of LsFNII2 and LsFNII3 mRNA in adult female and male lice, respectively. (A) Overview picture of results obtained with LsFNII2 antisense probe in cephalothorax. Strong hybridization is seen in teg 1 glands located in sub epidermal tissue and thoracic legs (*). (B) Magnification of marked area form picture A. (C) Overview picture of results obtained with LsFNII3 antisense probe in cephalothorax. Strong hybridization is seen in teg 1 glands located in sub epidermal tissue and thoracic legs (*). (D) Magnification of marked area from picture (A) from a parallel section.

Fig 4: Gene expression analysis in *LsFNII1, 2* and *3* knockdown lice and control lice. Columns show mean relative expression levels. Bars show standard deviation. (A) Relative expression of LsFNII1 in control animals and in LsFNII1 injected animals. (B) Relative expression of LsFNII2 in control animals and LsFNII2 injected animals. LsFNII#1 and LsFNII#2 indicates fragment 1 and 2, respectively. (C) Relative expression of LsFNII3 in control animals and LsFNII3 injected animals. Asterisk (*) indicate statistical significant P-values (<0.05). (D) Histological section of LsFNII2 knocked down animal stained with toluidine blue showing teg 1 glands with normal morphology.

Fig 5: Tegumental pores of *L. salmonis* visualized by SEM. (A) Dorsal side of an adult female cephalothorax with black dots indicating positions of secretory pores. (B) A small secretory pore located at the anterolateral edge where the marginal membrane extends laterally. Rod-shaped bacteria (arrowhead) are seen adhering to the integument via fibril-like structures. (C) A secretory pore positioned anteriorly. Note, growth of rod-shaped bacteria which is less prominent in close proximity to the pore. (D) Two large teg 1 pores at the base of the postantennary process of an adult female louse. (E) Ventral view of the third leg rami showing growth of bacteria. Fewer bacteria are seen in close proximity to the secretory pore (arrowhead).

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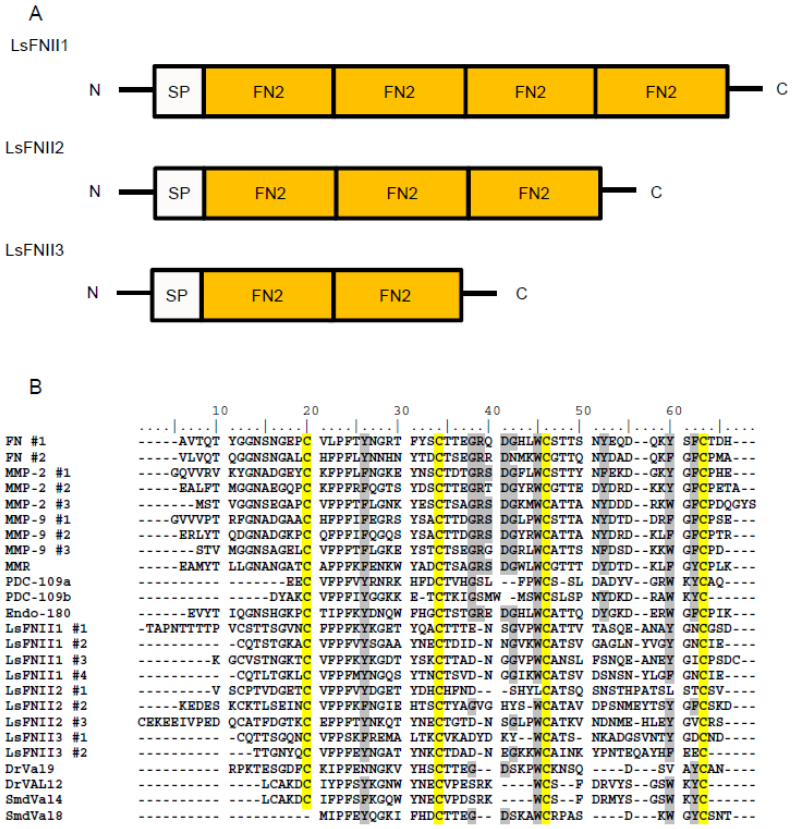
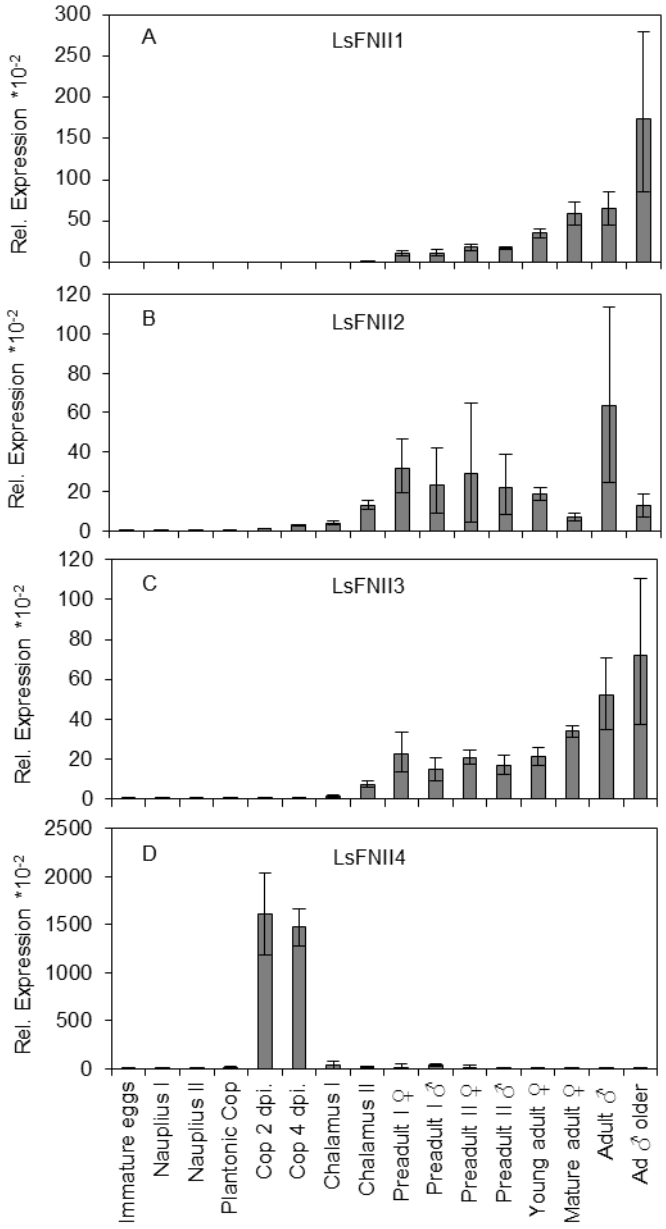

A
LsFNII1
N
SP
FN2
FN2
FN2
FN2
C
LsFNII2
N
SP
FN2
FN2
FN2
C
LsFNII3
N
SP
FN2
FN2
C
B
10
20
30
40
50
60
FN #1 -----AVTQT YGGNSNGEPC VLPFTYNGRT FYSCTTEGRQ DGHLWCSTTS NYEQD--QKY SFCTDH---
FN #2 -----VLVQT QGGNSNGALC HFPFLYNNHN YTDCTSEGRR DNMKWCGTTQ NYDAD--QKF GFCPMA---
MMP-2 #1 ----GQVVRV KYGNADGEYC KFPFLFNGKE YNSCTDTGRS DGFLWCSTTY NFEKD--QKY GFCPHE---
MMP-2 #2 -----EALPT MGGNAEGQPC KFPFRFQGTS YDSCTTEGRT DGYRWCGTTE DYDRD--KKY GFCPETA--
MMP-2 #3 -------MST VGGNSEGAPC VFPFTFLGNK YESCTSAGRS DGKMWCATTA NYDDD--RKW GFCPDQGYS
MMP-9 #1 ----GVVVPT RPGNADGAAC HFPFIFEGRS YSACTTDGRS DGLPWCSTTA NYDTD--DRF GFCPSE---
MMP-9 #2 -----ERLYT QDGNADGKPC QFPFIFQGQS YSACTTDGRS DGYRWCATTA NYDRD--KLF GFCPTR---
MMP-9 #3 -------STV MGGNSAGELC VFPFTFLGKE YSTCTSEGRG DGRLWCATTS NFDSD--KKW GFCPD----
MMR -----EAMYT LLGNANGATC AFPFKFENKW YADCTSAGRS DGWLWCGTTT DYDTD--KLF GYCPLK---
PDC-109a ---------- -------EEC VFPFVYRNRK HFDCTVHGSL --FPWCS-SL DADYV--GRW KYCAQ----
PDC-109b ---------- -----DYAKC VFPFIYGGKK E-TCTKIGSMW -MSWCSLSP NYDKD--RAW KYC------
Endo-180 ------EVYT IQGNSHGKPC TIPFKYDNQW FHGCTSTGRE DGHLWCATTQ DYGKD--ERW GFCPIK---
LsFNII1 #1 -TAPNTTTTP VCSTTSGVNC FPFPKYKGET YQACTTTE-N SGVPWCATTV TASQE-ANAY GNCGSD---
LsFNII1 #2 ---------- -CQTSTGKAC VFPFVYSGAA YNECTDID-N NGVKWCATSV GAGLN-YVGY GNCIE----
LsFNII1 #3 ---------K GCVSTNGKTC VFPFKYKGDT YSKCTTAD-N GGVPWCANSL FSNQE-ANEY GICPSDC--
LsFNII1 #4 ---------- -CQTLTGKLC VFPFMYNGQS YTNCTSVD-N GGIKWCATSV DSNSN-YLGF GNCIE----
LsFNII2 #1 ---------V SCPTVDGETC VFPFVYDGET YDHCHFND-- -SHYLCATSQ SNSTHPATSL STCSV----
LsFNII2 #2 -----KEDES KCKTLSEINC VFPFKFNGIE HTSCTYAGVG HYS-WCATAV DPSNMEYTSY GFCSKD---
LsFNII2 #3 CEKEEIVPED QCATFDGTKC EFPFTYNKQT YNECTGTD-N SGLPWCATKV NDNME-HLEY GVCRS----
LsFNII3 #1 ---------- -CQTTSGQNC VFPSKFREMA LTKCVKADYD KY--WCATS- NKADGSVNTY GDCND----
LsFNII3 #2 ---------- ---TTGNYQC VFPFEYNGAT YNKCTDAD-N EGKKWCAINK YPNTEQAYHF EEC------
DrVal9 ---------- RPKTESGDFC KIPFENNGKV YHSCTTEG-- DSKPWCKNSQ ----D---SV AYCAN----
DrVAL12 ---------- ----LCAKDC IYPFSYKGNW YNECVPESRK ----WCS--F DRVYS--GSW KYC------
SmdVal4 ---------- ----LCAKDC IFPFSFKGQW YNECVPDSRK ----WCS--F DRMYS--GSW KYC------
SmdVal8 ---------- ---------- MIPFEYQGKI FHDCTTEG-- DSKAWCRPAS ----D---KW GYCSNT---

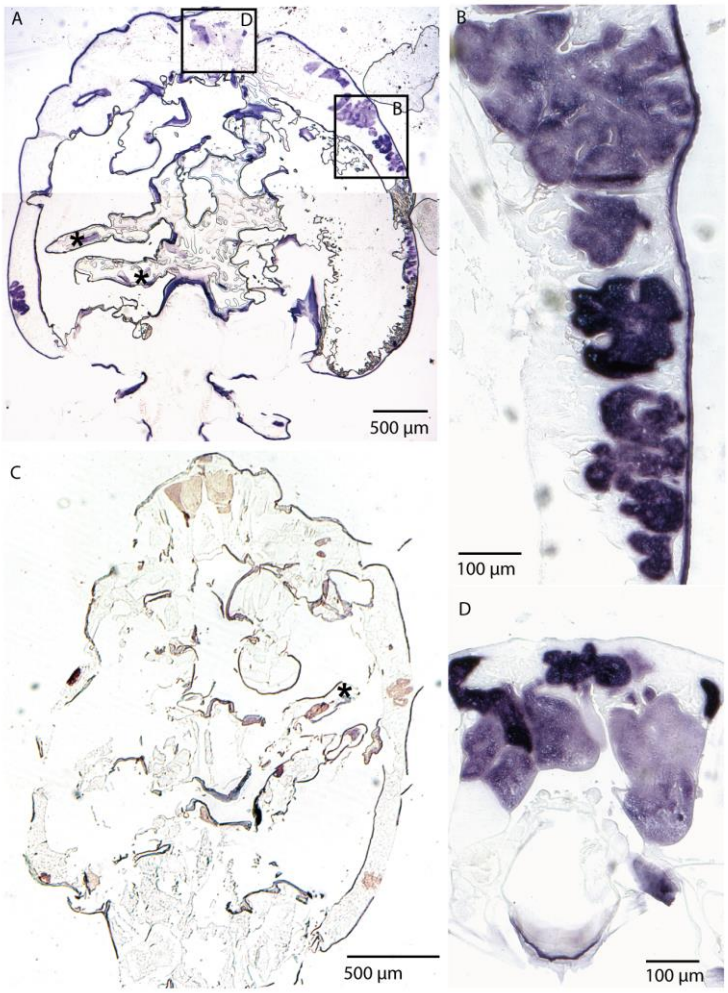
628

Fig1

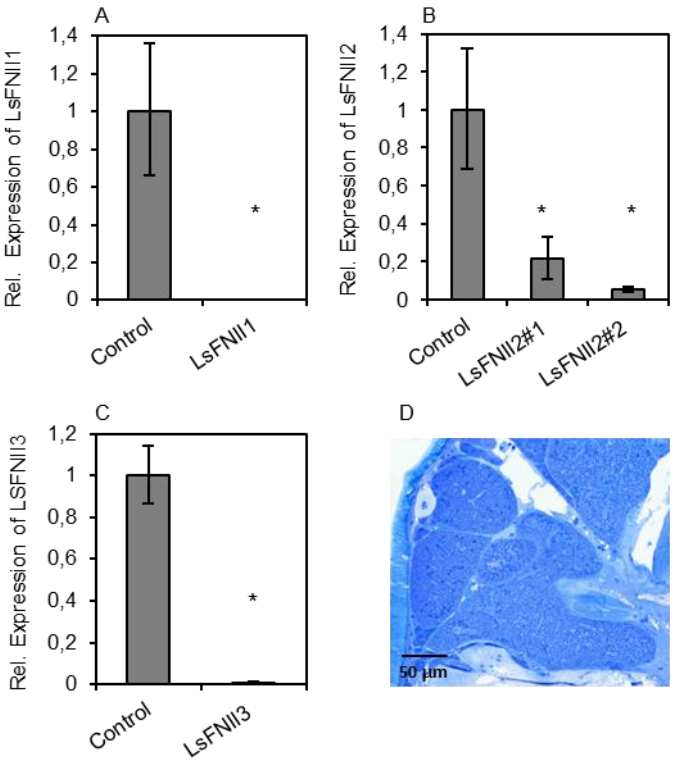

A
LsFNII1
B
LsFNII2
C
LsFNII3
D
LsFNII4
Rel. Expression *10-2
Immature eggs
Nauplius I
Nauplius II
Plantonic Cop
Cop 2 dpi.
Cop 4 dpi.
Chalamus I
Chalamus II
Preadult I ♀
Preadult I ♂
Preadult II ♀
Preadult II ♂
Young adult ♀
Mature adult ♀
Adult ♂
Ad ♂ older

629

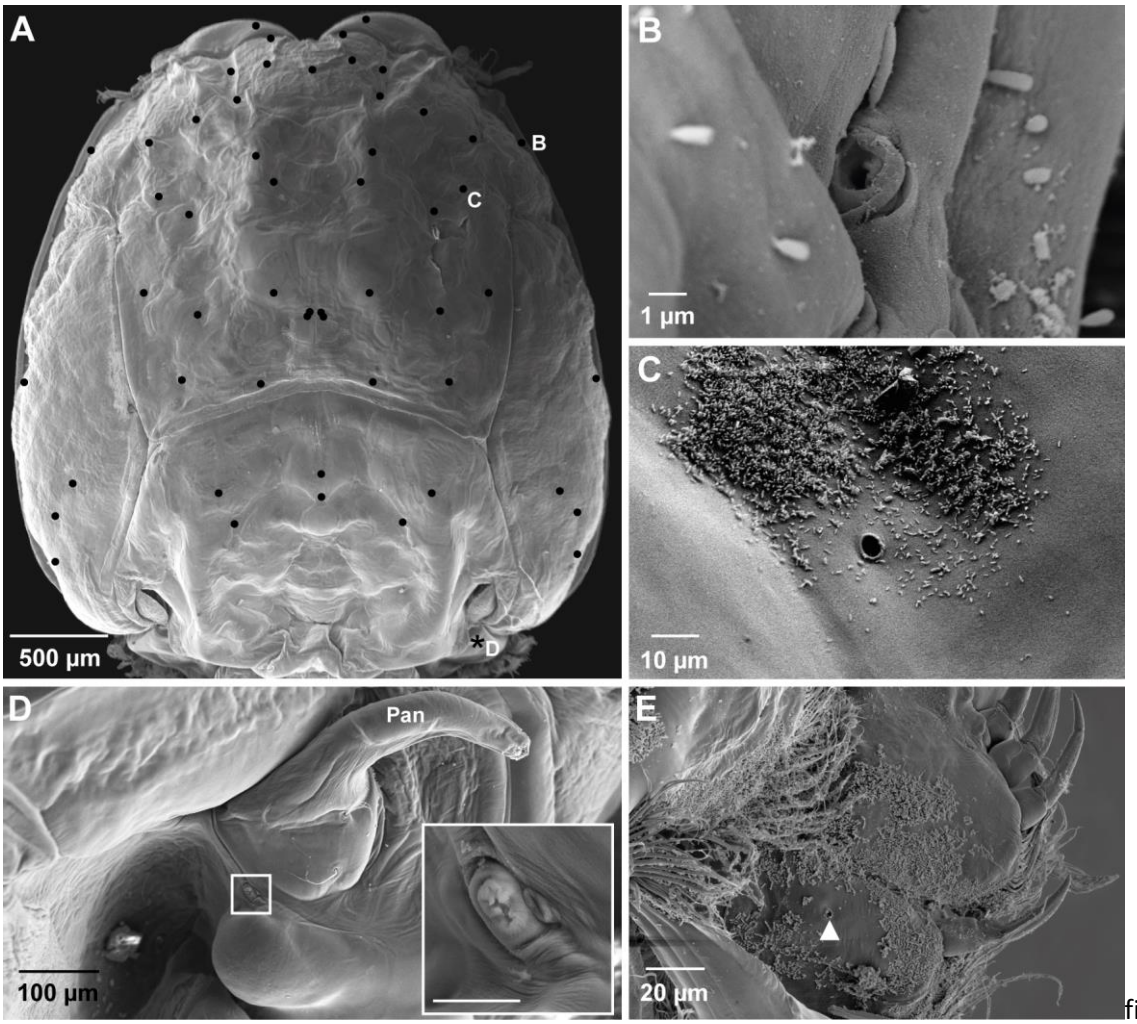
Fig2



630
631 Fig3


A
Rel. Expression of LsFNII1
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
LsFNII1
B
Rel. Expression of LsFNII2
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Control
LsFNII2#1
LsFNII2#2
C
Rel. Expression of LSFNII3
1,2
1
0,8
0,6
0,4
0,2
0
*
Control
LsFNII3
D
50 µm

632 **Fig 4**



633 fig 5