# Knock down of LsKDELR and LsCOPB2 by RNA interference inhibits digestion and reproduction in the parasitic copepod *Lepeophtheirus salmonis*

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**Abstract**

Retrograde transport of proteins from the endoplasmic reticulum to the Golgi is an essential part of the secretory pathway that all newly synthesised secreted and membrane proteins in eukaryotic cells undergo. The aim of this study was to characterise two components of the retrograde transport pathway in the parasitic copepod *Lepeophtheirus salmonis* (salmon louse) on a molecular and functional level. LsKDELR and LsCOPB2 were confirmed to be the salmon louse homologues of the chosen target proteins by sequence similarity. Ontogenetic analysis by qRT-PCR revealed the highest expression levels of both genes in adult females and the earliest larval stage. LsKDELR and *LsCOPB2* localisation in adult females was detected by immunofluorescence and *in situ* hybridisation, respectively. Both LsKDELR and *LsCOPB2* were found in the ovaries, the oocytes and the gut. LsKDELR and LsCOPB2 were knocked down by RNA interference in preadult females, which was confirmed by qRT-PCR. LsCOPB2 knock down lice had a significantly higher mortality and failed to develop normally, while both LsCOPB2 and LsKDELR knock down caused disturbed digestion and the absence of egg strings. This shows the potential of LsKDELR and LsCOPB2 as suitable target candidates for new pest control methods.

**Keywords**

sea lice, ERPR, COPI beta prime, RNAi, ectoparasite, blood digestion, retrograde transport

## 1. Introduction

During protein synthesis in eukaryotic cells, membrane proteins and proteins destined for secretion have to pass through the secretory pathway. Their mRNAs are targeted to the endoplasmic reticulum (ER), where protein synthesis, folding and modification occur. They are then transported to the Golgi apparatus in membrane vesicles encased by coat protein (COP) complexes, and further processed. From the Golgi, mature proteins are transported to their final destination. Three types of protein coated vesicular carrier systems exist: clathrin coated, COPI coated and COPII coated vesicles. Soluble ER resident proteins like enzymes and chaperones are constantly leaking from the ER to the Golgi, being swept along with the cargo in COPII coated transport vesicles following the secretory pathway. Retention of these proteins in the ER is accomplished by retrograde transport from the Golgi to the ER in COPI coated vesicles. Perturbances in retrograde transport cause ER stress, which triggers a signalling cascade called the unfolded protein response (UPR). The UPR leads to either altered expression of numerous proteins in order to maintain ER homeostasis, or - if the attempt fails - apoptosis (Walter and Ron, 2011). COPI transport vesicles are coated by heptameric protein complexes called coatomers. These COPI coats consist of a trimeric outer layer made of the subunits α, β' and ε, and a tetrameric inner layer made of the subunits γ, δ, ζ and β (Fiedler et al., 1996). Resident ER proteins interact directly with the COPI subunits, by interaction with a Vps74p linker, or by binding to the KDEL receptor (KDELR) (reviewed by Beck et al., 2009; Bethune et al., 2006; Szul and Sztul, 2011). Proteins with a C-terminal KDEL motif (HDEL in yeast) are recognized by KDELR, also known as the ER lumen retaining protein receptor (ERPR). KDELR is encoded in yeast by the ERD2 gene and in human by the erd21, 22 and 23 genes. It is a seven transmembrane receptor predicted to fold like a G-protein coupled receptor (GPCR) (Capitani and Sallese, 2009). Recently, KDELR was also shown to regulate protein transport in the Golgi (Pulvirenti et al., 2008), and to act like a GPCR, activating Golgi $G\alpha_{q/11}$ G-proteins that initiate a signalling pathway to regulate secretory traffic (Giannotta et al., 2012). Thus, KDELR plays a dual role, firstly sorting and binding of proteins with a KDEL sequence and secondly by initiating regulatory pathways of the vesicular transport system. Although the secretory pathway is employed in nearly all cell types of an organism,

experimental disturbances of COPI subunits and the KDEL receptor in diverse animals give rise to surprisingly specific phenotypes in specialized tissues: Neurological problems and changes in coloration could be observed in mice with a mutated δ COPI subunit (Xu et al., 2010). In Drosophila, deletion of the COPI γ subunit results in embryonic mortality characterized by changes in epidermis and tracheal tubes (Grieder et al., 2008). Knock down experiments by injection of dsRNA against COPI components have demonstrated high mortality rates in yellow fever mosquito (*Aedes aegypti*) (Isoe et al., 2011) and western corn rootworm (*Diabrotica virgifera*) (Baum et al., 2007). In mosquito, mortality was induced only upon blood feeding. This mortality appeared to be caused by changes in the epithelial cells of the midgut. All COPI subunits except for ε yielded similar phenotypes, reflecting the equally important role in formation of the COPI coat structure. Further knock down studies showed that the lack of alpha COPI protein was not associated with the early expressed serine protease (AaET), but expression of late proteases (AaSPVI, II and AaLT) (Zhou et al., 2011). In mice, a mutation of the KDEL receptor led to accumulation of misfolded proteins in the ER. The mice became sensitive to ER stress and developed heart failures (Hamada et al., 2004).

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod living on salmonid hosts. Intensive farming of Atlantic salmon (*Salmo salar*) offers ideal living conditions for salmon lice with high host densities available all year round. Feeding on host blood, skin and mucous, salmon lice cause physiological changes, morbidity and even mortality, dependent on the number of lice on the fish and their developmental stage. Salmon lice infections pose a serious threat for the salmon farming industry, but also for wild salmonid populations (reviewed by Torrissen et al., 2013). During their lifecycle, salmon lice undergo 8 developmental stages separated by a moult: First three planktonic stages (nauplius I and II, copepodid), after which the copepodids attach to a host and adopt a parasitic life style. Following are two larval stages (chalimus I and II) and two preadult stages (preadult I and II), before the adult stage is reached (Hamre et al., 2013; Johnson and Albright, 1991; Schram, 1993). After the final moult, the adult female has to mature further before egg production is initiated. This process involves growth of the distal parts of the animal - the genital segment and abdomen - while the size of the frontal cephalothorax remains unchanged (Eichner et al., 2008). The mature adult females extrude disc-like eggs that are tightly packed to form a long string of eggs. These egg strings are

extruded pairwise, and one female can produce at least 10 pairs of egg strings under experimental conditions (Heuch et al., 2000; Mustafa, 2000).

To date, retrograde transport of proteins has not been studied in salmon lice. In the present work, we aimed to characterise two key components of the retrograde transport between Golgi and ER in the salmon louse, LsKDELR and LsCOPB2 (COPI subunit β'). To test the suitability of these targets as tools in pest control, we also investigated the effects of gene knock down by RNA interference (RNAi) on egg production and blood digestion in adult lice.

## 2. Materials and Methods

### 2.1 Animals

A laboratory strain of salmon lice (Hamre et al., 2009) was maintained on farmed Atlantic salmon (*Salmo salar*) in tanks with sea water (salinity 34.5 ppt and temperature 10°C). Host fish weighed between 400 g and 500 g. The fish were daily hand fed a commercial diet and maintained according to Norwegian animal welfare regulations. The fish carrying knock down lice were kept in single-fish tanks as described by Hamre and Nilsen (2011).

### 2.2 PCR, RACE and sequencing

Rapid amplification of cDNA ends (RACE) was carried out using the SMARTer RACE cDNA Amplifictation Kit (Clontech). cDNA was synthesised using the qScript cDNA Synthesis Kit (Quanta Biosciences) according to manufacturer's instructions. PCR on cDNA templates was performed using GoTaq Flexi DNA Polymerase (Promega) according to the manufacturer's instructions. The primer sequences used were as follows (T7 promoter extension in parentheses): LsKDELR_for (TAATACGACTCACTATAGGG)AAACTTCATCTCCATTTATAACACGGCCAT, LsKDELR_rev (TAATACGACTCACTATAGGG)TTTTGCCTTTAAGCACTTTGGTGATGTA, LsCOPB2_for (TAATACGACTCACTATAGGG)ATCGTGTCAAGTCCACAGACCTG, LsCOPB2_rev (TAATACGACTCACTATAGGG)CGAGATGAGGTAGGGCTTGTCC. PCR and RACE products were sequenced at the University of Bergen's sequencing facility using BigDye Terminator v3.1

reagents (Applied Biosystems). The new sequences have been submitted to GenBank and can be found with the following accession numbers: *LsCOPB2* **KF683301**, *LsKDELR* **KF683300**, *LsCalr* **KF683299**.

### 2.3 Immunofluorescence

Adult female salmon lice were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours before paraffin embedding. Sections, three µm thick, were incubated for 30 min at 65°C, dewaxed in xylene, rehydrated through a graded ethanol series and brought to distilled water. After blocking in 5 % BSA in Tris-buffered saline (TBS) for one hour, slides were incubated with primary antibody, KDELR1 (ABIN452265, antibodies-online.com) diluted 1:1000 in 2.5 % BSA in TBS overnight. Alexa Fluor 488 conjugated anti-mouse antibody (cat. No. 4408, Cell Signaling Technology) diluted 1:2000 was used as a secondary antibody. Samples were mounted using ProLong Gold (Invitrogen). No signals were observed on slides treated with secondary antibody only.

### 2.4 *In situ* hybridisation

Adult female salmon lice were incubated in 4 % paraformaldehyde in PBS for 24 hours and then kept in 70 % ethanol at 4°C for at least 24 hours before paraffin embedding. Digoxigenin (DIG) labelled antisense and sense RNA probes of 461 bp (LsKDELR) and 566 bp (LsCOPB2) length were prepared by *in vitro* transcription using the DIG RNA Labelling Kit (Roche), with purified PCR products including T7 promoters as templates (see 2.2 for primer sequences). *In situ* hybridisation was carried out as previously described by Dalvin et al. (2013), with the following modifications: Xylene was replaced by Histo-Clear (National Diagnostics) in the dewaxing step. The probe concentration in the hybridisation mix was between 1 and 10 ng/µl and approximately 100 µl hybridisation mix were used per section. Chromogenesis was carried out using nitroblue tetrazolium (NBT, Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche). Hybridisations with sense probes were carried out as negative controls, while hybridisations with a known set of probes detecting LsTryp1 (Kvamme et al., 2004b) served as a positive control.

**2.5 RNA interference**

The RNAi experiment was carried out as previously described by Dalvin et al. (2009). Briefly, dsRNA fragments with a length of 461 bp (LsKDELR) and 566 bp (LsCOPB2) were synthesised using the MEGAscript RNAi Kit (Ambion), with purified PCR products including T7 promoters as templates. The fragments were diluted to a concentration of 600 ng/µl and bromophenol blue was added to visualize successful injections. Needles for injection were drawn from borosilicate glass capillaries with an outer diameter of 1 mm and an inner diameter of 0.5 mm (Stutter). On the day of injection, preadult II female lice were removed from the fish, injected with dsRNA solution using pneumatic control, incubated in sea water for 4 hours and then placed back on fish together with an equal number of adult males. For each dsRNA fragment, three fish - each bearing 13 dsRNA-injected females and 13 males - were used. RNAi experiments with fragments targeting *LsKDELR* and *LsCOPB2* were performed separately, and a cod trypsin dsRNA fragment without any sequence similarity to known salmon louse transcripts was used as a negative control in each experiment. The experiments were terminated after 30 to 32 days, when all females in the control group had developed into fully mature adults and extruded at least their second pair of egg strings. Recovery rates in such RNAi experiments in our laboratory are usually around 50 % in the control group. Loss of lice is likely to be caused by handling of the lice during the experimental procedures and fish movement in the tank. At termination, all recovered lice were subjected to a gross morphological inspection and photos were taken before lice were sampled for RNA extraction or sectioning. Present egg strings were removed from the animals and incubated in sea water until hatching. Emerging offspring was inspected for deformities and counted 9 to 14 days after hatching, when normally developing lice would have reached the copepodid stage. In addition to sampling at the termination date, lice were sampled for histology 15 days after injection of dsRNA.

**2.6 Histology**

Salmon lice were fixed in Karnovsky's fixative, washed twice in PBS, dehydrated in a graded ethanol series, pre-infiltrated with Technovit/ethanol (50/50) for four hours (Technovit 7100, Heraeus Kulzer Technique) and infiltrated with Technovit and hardener overnight before embedding. The

polymerisation time was approximately four hours. Two µm thick sections were produced using a microtome (Leica RM 2165) and stained with toluidine blue (1 % in 2 % borax) for one minute. The stained sections were mounted using Mountex (Histolab Products).

**2.7 RNA isolation and qRT-PCR**

Animals destined for RNA isolation were incubated in RNAlater at 4°C for one day before storage at -20°C. Total RNA was isolated using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. Animals in early developmental stages were pooled to yield enough RNA: about 150 of nauplii I+II and planktonic copepodids; 10 of parasitic copepodids, chalimus I and chalimus II. Sample homogenisation was carried out using 5 mm stainless steel beads and a TissueLyser LT (Qiagen) for 5 minutes at 50 Hz. Genomic DNA was eliminated from the samples by DNase treatment using the Turbo DNA-free kit (Ambion). The amount and purity of the isolated RNA was measured with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). The isolated RNA was stored at -80 °C. SYBR Green qRT-PCR assays were designed using Vector NTI Suite 9 (Invitrogen). Taqman qRT-PCR assays were custom-made. Two-step quantitative real-time polymerase chain reaction (qRT-PCR) was performed as follows: DNase-treated total RNA was reverse transcribed in duplicates using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene). qRT-PCR was run on Applied Biosystems 7500 Real-Time PCR System using either SYBR Green PCR Master Mix or the TaqMan Universal PCR Master Mix (both Applied Biosystems) according to the manufacturer's instructions. Baseline and threshold values were set automatically by the 7500 software and the threshold values were corrected manually to be equal in all runs. The ΔΔCt method was applied to calculate fold changes relative to a calibrator as described by Livak and Schmittgen (2001). LsEF1α, which has previously been validated as a suited reference gene in different life stages of the salmon louse (Frost and Nilsen, 2003), was used to normalise target gene Ct values. The mean of the control samples' Ct values or an arbitrary number was used as the calibrator to centre the mean fold change of expression in the control group around one. The primer sequences for the SYBR Green assays were as follows: LsEF1α_for GGTCGACAGACGTACTGGTAAATCC, LsEF1α_rev TGCGGCCTTGGTGGTGGTTC, LsKDELR_for TTTTCCTGGCCTCAAGTCTCGGAA,

LsKDELR_rev GGCGAGTATGATTACGGGAAGCAA, LsCOPB2_for GCCTCTTTGGACCGCACCAT, LsCOPB2_rev CGAGATGAGGTAGGGCTTGTCC, LsCalr _for GCTTCGACAAGGGTGATTTGG, LsCalr_rev GCATCTTGGGAAGTTTGGATCC. The primer and probe sequences for the Taqman assays were as follows: LsEF1α_for CATCGCCTGCAAGTTTAACCAAATT, LsEF1α_rev CCGGCATCACCAGACTTGA, LsEF1α_reporter ACGTACTGGTAAATCCAC, LsVit1_for ACATCGACTACAAAGGAACTCAGAAC, LsVit1_rev GGAAGCATGTAACGAATGAACTCA, LsVit1_reporter AGATTTTCTTTAGCTTCTGGATACAAACCTGCTCCA, LsVit2_for AATGAGCAATTTAGTTGAGAAAACTTGT, LsVit2_rev CAATCTCGCTTTGAGCATCACA, LsVit2_reporter TGGATAAATCACGTCAAGTTACTTACCCTACCGC, LsYAP_for GGCTTTAGGCTCTGATGTAAGCA, LsYAP_rev TGTTCACAAGTAAAAGCAGTGTCATTT, LsYAP_reporter TCAATCACGATGGACCATTCACTGTTTTTG, LsTryp1_for CACCTTCTCCAGTTCTTAAAGCTGTT, LsTryp1_rev AGATCATGGTCTCATCAATAGATCCA, LsTryp1_reporter AGTTGTTTCTGATGAAGACTGTAGCGATGCCT. Five animals or pools of animals from each developmental stage/RNAi treatment were tested, except for the parasitic copepodid stage where three biological replicates were used.

**2.8 Bioinformatics and statistical analysis**

Nucleotide and protein sequences were tested for sequence similarity to other species using the Blast algorithm (Altschul et al., 1990). The Conserved Domain Database (CDD) (Marchler-Bauer et al., 2013) was searched for conserved motives in the deduced translated protein sequences. Transmembrane helices were predicted using the TMHMM Server, v. 2.0 (Krogh et al., 2001). The ContigExpress module in the Vector NTI Suite 9 (Invitrogen) was used for multiple sequence alignments. Phenotypic differences between knock down lice and control lice were tested by chi-square test and differences in gene expression levels assessed by qRT-PCR were tested by Mann-Whitney test using Statistica 12 (StatSoft).

## 3. Results

### 3.1 Sequence analysis

Nucleotide sequences of homologues of KDELR and the COPI β' subunit were retrieved from EST databases containing sequences from both sexes and all life stages of *L. salmonis* and named *LsKDELR* and *LsCOPB2*, respectively. Full length sequences were obtained by RACE. The deduced amino acid sequence of *LsKDELR* consisted of 212 aa residues. When blasted against the UniProtKB/Swiss-Prot database, it showed high similarity to KDEL receptors in various other species like fruit fly (80 %), human (77 %), rat (76 %), zebrafish (76 %) and African clawed frog (75 %), confirming that LsKDELR is a KDEL receptor homologue. A search in the Conserved Domain Database (CDD) revealed high similarity to the ER lumen protein retaining receptor conserved domain (pfam00810). As expected, LsKDELR was predicted to contain seven transmembrane domains using the TMHMM Server. For *LsCOPB2*, the deduced amino acid sequence consisted of 894 aa residues and showed high similarity to COPI β' subunits in species like mouse (71 %), cattle (71 %), human (71 %), fruit fly (68 %) and nematode (63 %). Searches in the CCD revealed seven WD40 repeats in the N-terminal WD40 domain (cd00200) and the coatomer WD associated region (pfam04053) in the C-terminal end. Both are typical for COPI β' subunits, thus confirming the homology of LsCOPB2.

### 3.2 Ontogenetic analysis of gene expression

The mRNA expression levels of *LsKDELR* and *LsCOPB2* were quantified by qRT-PCR at selected time points in the salmon louse lifecycle. Currently, there is no reliable method to determine the sex of salmon lice before they reach the preadult I stage and thus we did not discriminate between sexes before this stage. Samples of the following developmental stages were analysed: nauplius I+II (2 days old), planktonic copepodids (14 days old), parasitic copepodids (19 days old), chalimus I (21 days old), chalimus II (28 days old), preadult I males (36 to 40 days old), preadult I females (36 to 43 days old), preadult II males (36 to 43 days old), preadult II females (43 days old), adult males (68 days old) and adult females (68 days old). Expression levels of both genes were relatively constant in the

various inspected developmental stages. However, the highest levels of expression were observed in nauplii and adult females when compared to the other stages (Fig. 1).

### 3.3 Localisation of LsKDELR and *LsCOPB2*

To further characterize the two genes, localisation was determined in adult females. LsKDELR was localised using an antibody detecting KDELR in a wide range of species. LsKDELR protein was detected in the ovaries, situated in the anterior of the cephalothorax of the salmon louse, in the oviduct, bridging over to the genital segment, and in the oocytes that fill up the genital segment tightly packed in the long, coiled egg string structure typical for the salmon louse. The subcuticular tissue, which is the tissue localised below the cuticle in the cephalothorax (Dalvin et al., 2009), and the epithelium of the gut, a place of active secretion of digestive enzymes, were also positive (Fig. 2). We did not detect signals in other parts of the lice. A suitable antibody targeting LsCOPB2 was unavailable; hence the localisation of *LsCOPB2* transcripts was explored by *in situ* hybridisation carried out with DIG-labelled RNA probes. Like LsKDELR, *LsCOPB2* transcripts were detected in the ovaries and in vitellogenic oocytes of the animals. A smaller amount of LsCOPB2 transcript could also be detected in the epithelium of the gut (Fig. 3). *LsCOPB2* transcripts were not detected in the subcuticular tissue.

### 3.4 Functional analysis of LsKDELR and LsCOPB2

To learn more about the functions of LsKDELR and LsCOPB2 and the processes they are involved in, an RNAi approach was used to knock down the expression of these genes in females during the maturation from preadult to adult. The knock down of *LsKDELR* and *LsCOPB2* transcripts was confirmed by qRT-PCR (Fig. 4A). Both *LsKDELR* and *LsCOPB2* transcript levels were significantly reduced when compared to the control group. *LsKDELR* transcript levels were reduced by 40 % in LsKDELR knock down lice, and *LsCOPB2* transcript levels by 65 % in LsCOPB2 knock down lice. LsCOPB2 knock down lice also had significantly lower expression of *LsKDELR*, but not vice versa. Representative individuals at the termination time point are shown in Fig. 5 and the outcome of the experiment is summarised in Table 1. Significant mortality was observed in the group injected with the LsCOPB2 fragment, where only 13 % of the lice were recovered, compared to 59 % of the control

lice and 56 % of the LsKDELR knock down lice. While the control animals developed and reproduced normally (Fig 5A), both *LsKDELR* (Fig. 5B) and *LsCOPB2* (Fig. 5C) dsRNA injection caused distinctive phenotypes. Based on gross morphological inspection, normal development to a reproductive adult female appeared to have taken place in the LsKDELR knock down group. Despite this, none of the LsKDELR knock down lice produced egg strings. In addition, visual inspection of the gut indicated reduced gut content in 55 % of the recovered individuals, indicating a disturbance of the digestive process. The phenotype of the five recovered LsCOPB2 knock down lice was even more dramatic. They exhibited delayed development, with shorter genital segments and abdomen than in the control group. The post-moulting growth of adult salmon lice has been described by (Eichner et al., 2008) and is categorised as six distinct states named T1 to T6. According to this system, the surviving LsCOPB2 knock down lice were classified T2 to T4, whereas controls and LsKDELR knock down animals were all classified as T6. None of the LsCOPB2 knock down lice had extruded egg strings. Furthermore, all LsCOPB2 knock down lice had very little blood in the gut.

To obtain further insight into changes caused by gene knock down, animals were sectioned and stained with toluidine blue. LsKDELR knock down lice displayed radical changes in the oocytes at termination of the RNAi experiment (Fig. 6A). A number of normally developing oocytes packed in the coiled egg string in the genital segment were followed by a region of destroyed egg cells. Fig. 6B shows a corresponding section of a control louse with intact vitellogenic oocytes. There were also changes in the subcuticular tissue where yolk proteins are produced (Dalvin et al., 2009) (Fig. 6C). The integrity of the tissue seemed to be disturbed compared to tissue from a control (Fig. 6D), with more gaps between the subcuticular cells and small regions with dense granules which might be derived from dead cells. Since the morphological changes and mortality in LsCOPB2 knock down lice were tremendous at the chosen termination time point, we sampled lice for histological analysis already 15 days after injection to see early effects of the knock down. Similar to the LsKDELR knock down lice, we found destroyed oocytes just adjacent to intact egg cells (Fig. 6E), while oocytes in control lice had developed normally (Fig. 6F). Slight changes in the subcuticular tissue could also be observed in LsCOPB2 knock down lice (Fig. 6G), compared to control lice (Fig. 6H). Small gaps between the cells were found, similar to our observations in LsKDELR knock down lice (Fig. 6C).

### 3.5 Downstream effects of knock down of *LsKDELR* and *LsCOPB2*

RNAi against *LsKDELR* and *LsCOPB2* caused phenotypes with lice unable to produce eggs and with disturbed digestion. To further investigate these effects on a molecular basis, the mRNA expression levels of the salmon louse egg proteins vitellogenin 1 (LsVit1), vitellogenin 2 (LsVit2) (Dalvin et al., 2011) and yolk associated protein (LsYAP) (Dalvin et al., 2009), as well as the digestive enzyme trypsin 1 (LsTryp1) (Kvamme et al., 2004a), were measured by qRT-PCR. As could be expected from the morphological analysis of the RNAi experiment, expression of yolk and yolk associated proteins; LsVit1, LsVit2 and LsYAP was disturbed in both LsKDELR and LsCOPB2 knock down lice (Fig. 4B). Expression levels were reduced by 49 % for LsVit1, 93 % for LsVit2 and 47 % for LsYAP in LsKDELR knock down lice and respectively 94 %, 99 % and 92 % in LsCOPB2 knock down lice, when compared to the control group. Similarly, LsTryp1 expression levels were lower in LsKDELR (54 % reduction) and LsCOPB2 (83 % reduction) knock down lice compared to the control group (Fig. 4C).

Both KDELR and COPB2 play important roles in the recycling of ER resident proteins. Disturbing this process by gene knock down might trigger ER stress and thus induce the UPR. One of the proteins known to be upregulated by the UPR is the ER chaperone calreticulin (Yoshida et al., 1998). To check if the ER stress system is activated by the knock down of LsKDELR and LsCOPB2, the mRNA expression levels of the salmon louse homologue of calreticulin (LsCalr) were assessed by qRT-PCR (Fig. 4C). Interestingly, *LsCalr* expression levels were increased by 94 % in the LsKDELR knock down lice, but decreased by 37 % in the LsCOPB2 knock down lice, when compared to the control group.

## 4. Discussion

Retrograde transport of ER resident proteins is important to ensure the normal execution of the secretory pathway and thus for homeostasis in eukaryotic cells. The aim of this study was to investigate two proteins in the retrograde pathway in salmon lice: LsKDELR, which binds ER resident proteins recognising their KDEL signal, and LsCOPB2, the β'subunit of the COPI coat that covers transport vesicles containing KDELR and its ligand.

The respective sequences of LsKDELR and LsCOPB2 were identified by their similarity to proteins from other species. As expected, both proteins are highly conserved and contain the expected domains confirming the identity of the genes. Quantitative ontogenetic analysis of expression by qRT-PCR revealed that both genes were expressed in a similar fashion, with a relatively stable expression rate throughout lice development (Fig. 1). Indeed, this would be expected for genes involved in a ubiquitous process like the secretory pathway. The highest expression levels were found during the nauplius stage and in adult females. We observed a high variation between the five nauplius samples. This variation might have been caused by regulation within the nauplius stages, since the samples used in this study contained both nauplius I and II animals. The increased use of the secretory pathway during yolk protein production might explain the higher expression levels in adult females.

Localisation of LsKDELR and *LsCOPB2* was determined using immunofluorescence and *in situ* hybridisation (Fig. 2, 3). Positive signals showing the localisation of LsKDELR were found in the ovaries and in oocytes in the genital segment, in the gut and in the subcuticular tissue. A similar pattern was found using antisense probe against *LsCOPB2*, but *LsCOPB2* was not detected in in the subcuticular tissue. Likewise, in another blood-feeding arthropod, the yellow fever mosquito *Aedes aegypti*, the γ subunit of COPI was detected in the ovaries, midgut and fat body (Isoe et al., 2011). The localisation of LsKDELR and *LsCOPB2* in the ovaries and oocytes is in accordance with the results obtained in the RNAi experiment discussed below, where all reproduction is abolished.

RNAi studies were performed to elucidate the functionality of LsKDELR and LsCOPB2. LsCOPB2 knock down lice exhibited high mortality and severe developmental delay. To our knowledge, this is the first time that significant mortality was reported in an RNAi experiment with *Lepeophtheirus*

*salmonis*. The few surviving animals showed delayed post-moulting growth and failed to produce egg strings (Fig. 5C). LsKDELR knock down lice grew to full size, but did not produce egg strings (Fig. 5 B). Histological inspection showed similar alterations in the oocytes and subcuticular tissue for both LsKDELR and LsCOPB2 knock down; however the changes were apparent in LsCOPB2 knock down lice at an earlier time point (Fig. 6). The more severe phenotype observed in the LsCOPB2 knock down lice compared to LsKDELR knock down lice indicates a more essential or broader role of LsCOPB2. This is in accordance with the hypothesis that the role of KDELR is to bind and transport ER resident proteins bearing the KDEL motif back to the ER, while the COPI coat is also involved in other types of retrograde transport (Szul and Sztul, 2011).

Similar to our findings, knock down of a COPI subunit in the yellow fever mosquito resulted in inhibited follicular development and blocked egg shell secretion (Isoe et al., 2011). The same group observed also blood feeding-induced mortality in COPI knock down mosquitos. They found that blood leaked into the abdomen through a disrupted layer of midgut epithelial cells. In the present study, although we observed reduced gut content, no changes in the integrity of the gut of LsKDELR and LsCOPB2 knock down lice were detected in tissue sections or by visual inspection. Little information about the effects of loss of KDELR function in organisms closely related to the salmon louse exists, but in KDELR mutant mice ER stress was observed (Hamada et al., 2004).

Given the phenotypic changes observed in the knock down lice, we wanted to quantify changes in the transcription of genes involved in egg production and digestion. During vitellogenesis, yolk proteins are incorporated into the oocytes (Dalvin et al., 2009; Dalvin et al., 2011; Eichner et al., 2008). We found the expression of the yolk and yolk associated proteins LsVit1, LsVit2 and LsYAP to be decreased in both LsKDELR and LsCOPB2 knock down lice when compared to the control group (Fig. 4B). This decrease was most prominent in LsCOPB2 knock down lice. These results support the findings of our histological analysis and might indicate the existence of a feedback regulation mechanism, arresting the production of egg proteins due to a malfunction of the secretory pathway. Also the expression of the salmon louse midgut protease LsTryp1 was decreased in both LsKDELR and LsCOPB2 knock down lice when compared to the control group (Fig. 4C). This corresponds to the disturbed digestive process we observed in LsKDELR and LsCOPB2 knock down lice (Fig. 5 and

Table 1) and to the findings of Isoe et al. (2011) and Zhou et al. (2011), who detected reduced expression levels of late midgut serine proteases in COPI knock down mosquitos. Hence, reproductive failure could also be the consequence of less available energy due to reduced digestion.

We also wanted to get an indication of whether the knock down animals would attempt to compensate for the loss of ER resident proteins associated with the lack of LsCOPB2 and LsKDELR. Firstly, we looked at transcription of *LsKDELR* in LsCOPB2 knock down animals and vice versa (Fig. 4A). We observed a decrease in *LsKDELR* transcripts in LsCOPB2 knock down animals, indicating a downregulation of the retrograde pathway. On the other hand, this could not be shown in LsKDELR knock down lice where *LsCOPB2* transcripts were unaltered. We then looked at the expression levels of the ER resident chaperone LsCalr (Fig. 4C), which is known to be induced by ER stress (Yoshida et al., 1998). Interestingly, *LsCalr* expression levels were increased in the LsKDELR knock down lice, but decreased in the LsCOPB2 knock down lice, when compared to the control group. Together these experiments might indicate a graded response, where the ER stress caused by LsKDELR knock down induces the UPR, causing the upregulation of a number of transcripts involved in the secretory pathway, while LsCOPB2 knock down lice might have exceeded a limit for compensatory mechanisms (Walter and Ron, 2011).

Lack of reproduction caused by the disruption of the secretory pathway makes LsKDELR and LsCOPB2 potential candidates to be utilised in pest control. The use of chemical inhibitors of the COPI vesicle transport system has been explored in the prevention of pests such as *Aedes aegypti* and *Anopheles stephensi* (Mack et al., 2012). In the case of salmon lice, however, the usefulness of such drugs will be highly dependent on the toxicity for the host fish and the aquatic ecosystem, and how the fish metabolises the drug, as synthesis of certain metabolites may render the fish useless for human consumption. Also the possibilities for effective delivery of the drug to the lice in an aquatic environment will be crucial.

It can be concluded that knock down of LsKDELR and LsCOPB2 in salmon lice causes similar phenotypes with disturbed digestion and lack of the ability to reproduce. However, the exploitation of these findings in the design of new pest control methods is dependent on further investigation.

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## Table and figure captions

Table 1. Effects of gene knock down by RNAi on survival, body development, reproduction and digestion in salmon lice. Shown are percentages of the total number of injected animals in the first column (n=39 for both control, LsKDELR and LsCOPB2), and in the second to fourth column percentages of the number of recovered animals at the termination of the experiment (control n=23, LsKDELR n=22, LsCOPB2 n=5). An asterisk (*) denotes significant difference to the control group shown by chi-square test.

| RNAi treatment | Recovery rate | Normal development | Normal egg strings | Reduced gut content |
|---|---|---|---|---|
| Control | 59 % | 100 % | 74 % | 9 % |
| LsKDELR | 56 % | 100 % | 0% * | 55% * |
| LsCOPB2 | 13% * | 20% * | 0% * | 100% * |

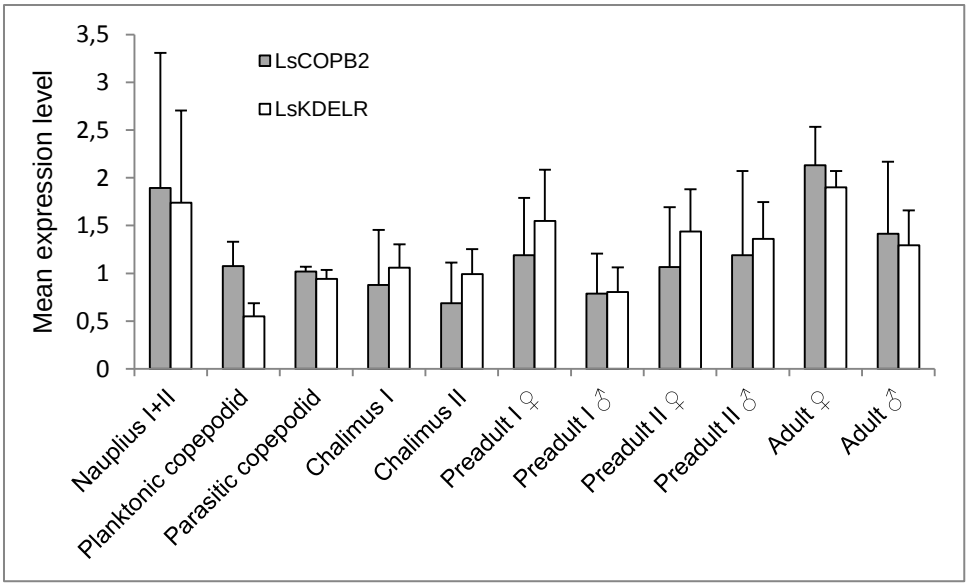


Figure 1. Expression of *LsKDELR* and *LsCOPB2* transcripts in different developmental stages of the salmon louse. Transcript levels were quantified by qRT-PCR using total RNA extracted from the specified developmental stages. Columns show mean expression levels and error bars show the standard deviation. N=5 for each stage (n=3 for parasitic copepodids).

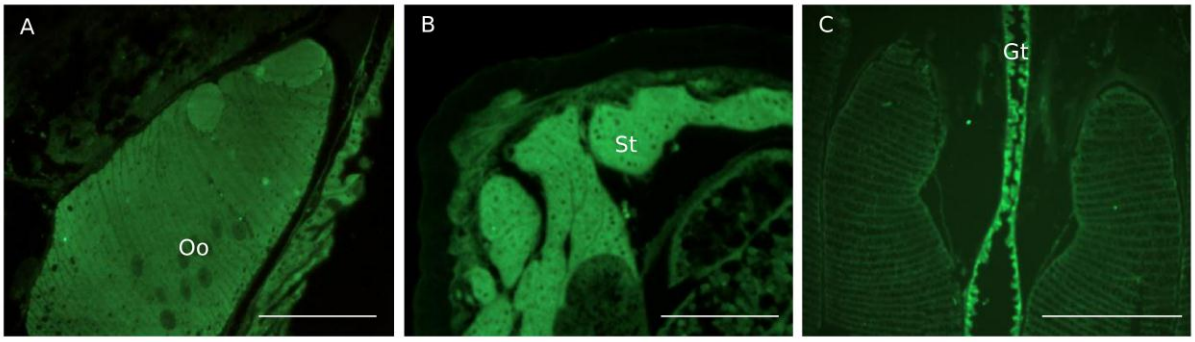


Figure 2. Localisation of LsKDELR protein. LsKDELR was localised by immunofluorescence performed on sections of adult females. Staining indicating presence of LsKDELR protein was detected in (A) Immature (non-vitellogenised) oocytes (Oo) in the genital segment, (B) Subcuticular tissue (St), and (C) the gut (Gt). The scale bar indicates 100 µm (A, B) and 500 µm (C).

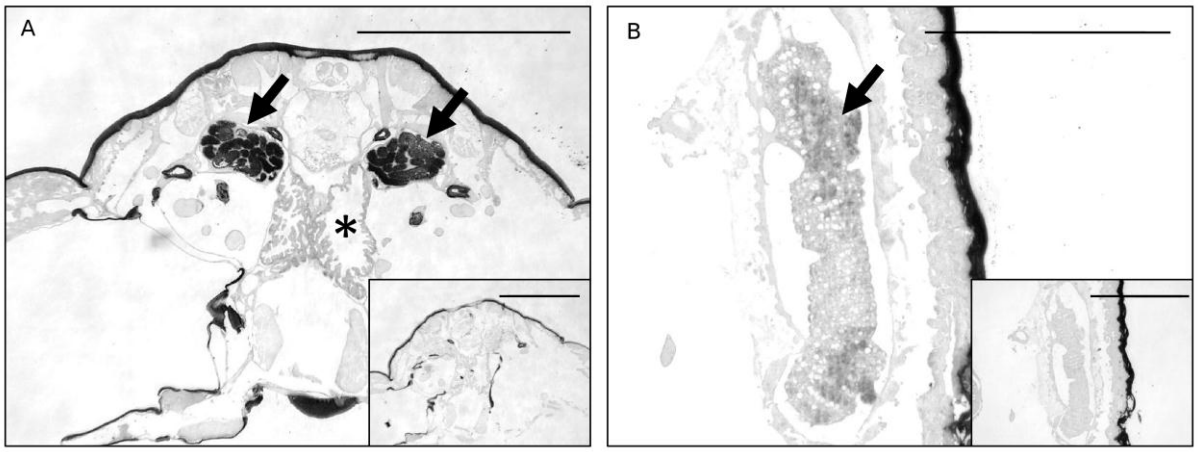


Figure 3. Localisation of *LsCOPB2* transcripts. Transcripts were localised by *in situ* hybridisation performed on sections of adult females. Large pictures show results obtained with antisense probes, whereas small inserts show a parallel slide incubated with sense probe (negative control). (A) Anterior part of the cephalothorax. A strong staining was seen in the ovaries (→) and a weak staining was found in the gut (*). (B) Vitellogenised oocytes in the genital segment stained positive (→). An unspecific staining of the cuticle was observed with both antisense and sense probes. The scale bar indicates 1 mm (A) and 500 µm (B).

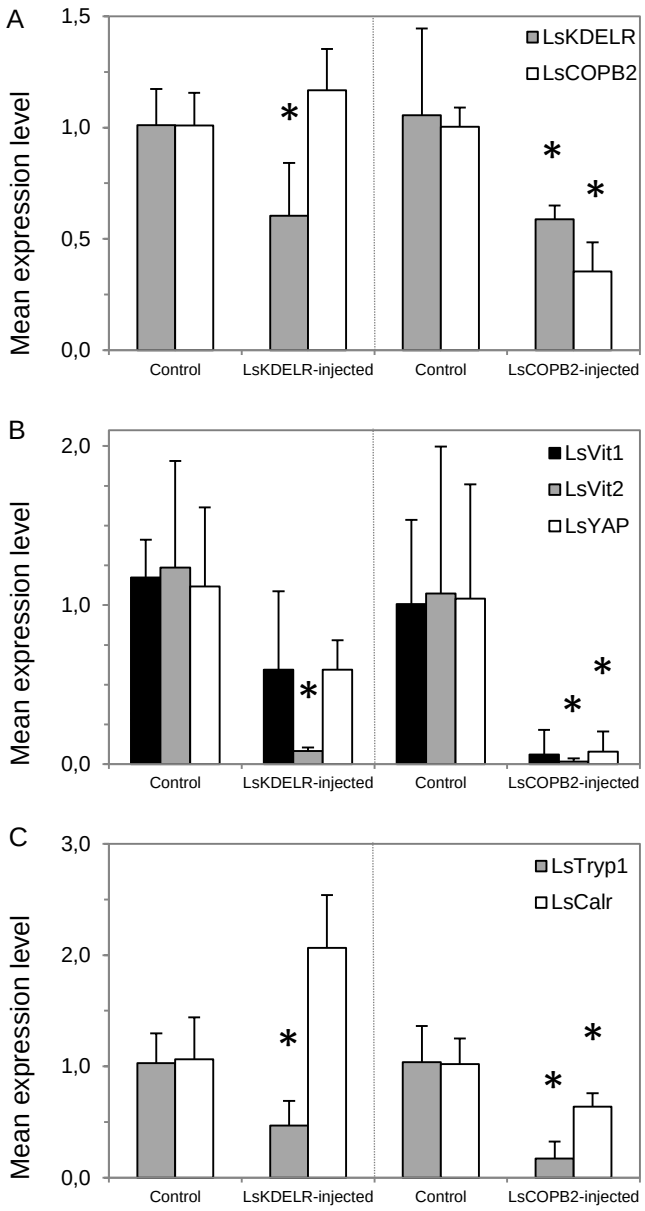


Figure 4. Gene expression analysis in LsKDELR and LsCOPB2 knock down lice and control animals. Transcript levels were quantified by qRT-PCR using RNA extracted from adult females injected with dsRNA against LsKDELR, LsCOPB2 or cod trypsin (control). Columns show mean expression levels and error bars show the standard deviation for each treatment group. N=5 for each group. An asterisk (*) denotes significant difference to the respective control group shown by Mann-Whitney test. (A) Expression of *LsKDELR* and *LsCOPB2*. (B) Expression of the yolk protein transcripts *LsVit1*, *LsVit2* and *LsYAP*. (C) Expression of the digestive enzyme *LsTryp1* and the ER resident protein *LsCalr*.

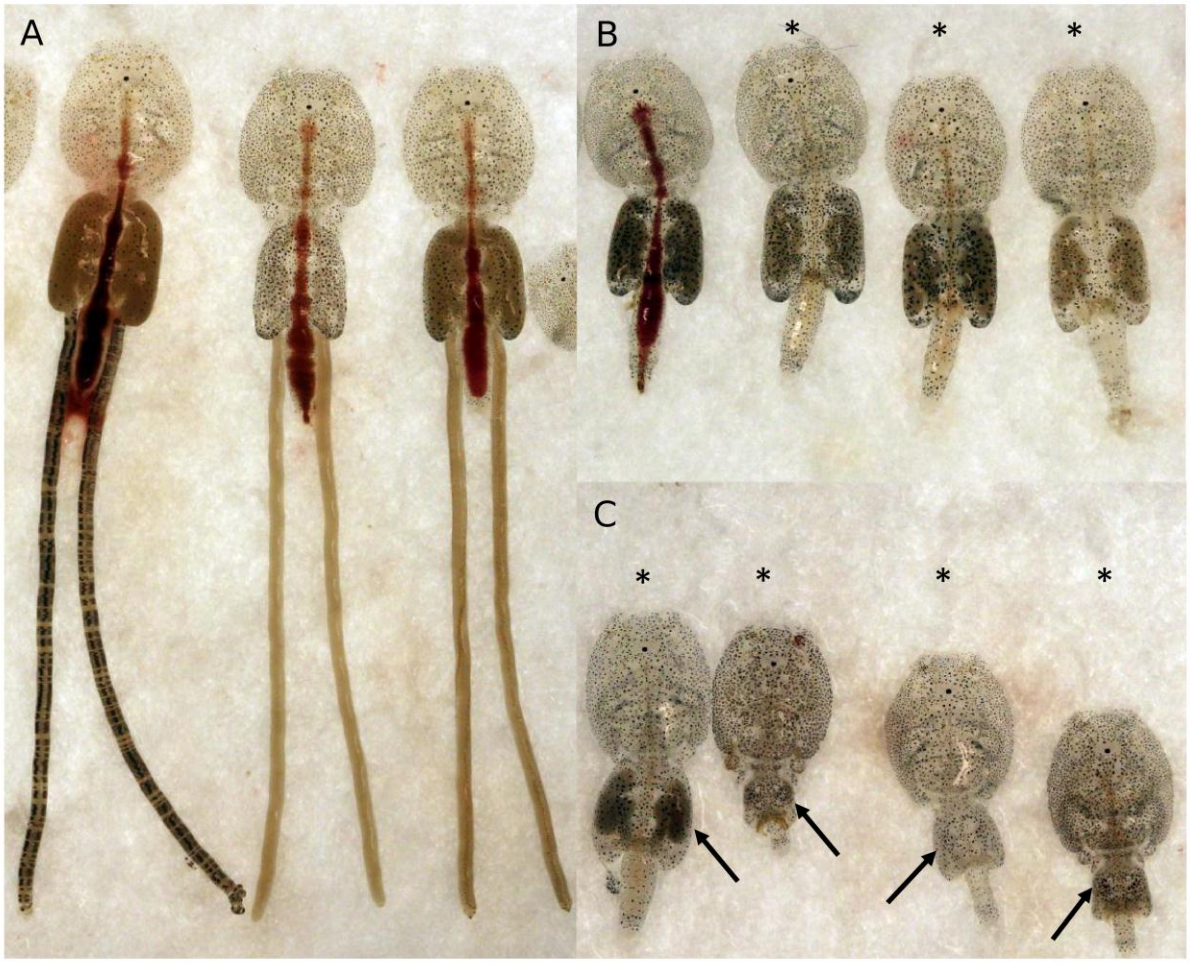


Figure 5. Morphology of RNAi treated animals at termination. (A) Normally developed control animals with egg strings. (B) LsKDELR knock down animals. No animals produced egg strings, and reduced gut content (*) could be observed in some individuals. (C) LsCOPB2 knock down animals. No animals produced egg strings, and reduced gut content (*) could be observed in all individuals. Stretching of the genital segment had not been completed (→).

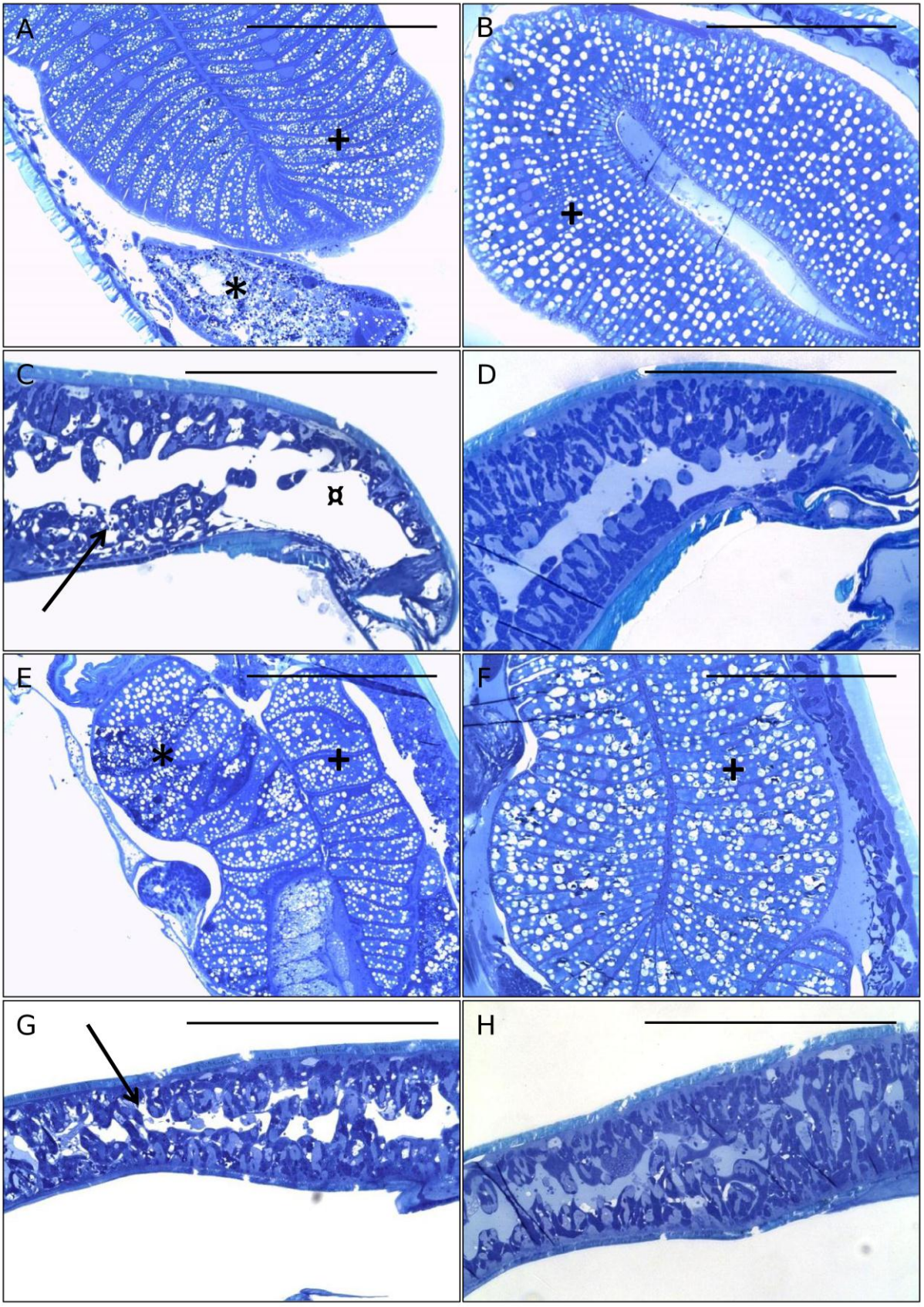


Figure 6. Sections of RNAi treated animals stained with toluidine blue. (A, C) LsKDELR knock down, (B, D) control animal, all terminated 30 days after injection. (E, G) LsCOPB2 knock down, (F, H) control animal, all

terminated 15 days after injection. (A-B) Sections of the genital segment, showing parts of the coils of maturing eggs filling this part of the animal. Eggs undergoing vitellogenesis with highly abnormal morphology (*) and normally developed vitellogenised eggs (+) are visible. (C-D) Sections of the cephalothorax, showing subcuticular tissue lining the cuticula on the upper and lower side of the animal. The subcuticular tissue consists of several cell types facing the haemolymph on the inside of the animal. In the LsKDELR knock down animal (C), the subcuticular tissue is thinned with large areas without cells (¤) and unstained patches in the tissue with dot-like structures (→). (E-F) Sections of the genital segment, showing parts of the coils of maturing eggs filling this part of the animal. Vitellogenised eggs with highly abnormal morphology (*) and normally developed vitellogenised eggs (+) are visible. (G-H) Sections of the cephalothorax, showing subcuticular tissue lining the cuticula on the upper and lower side of the animal. The subcuticular tissue consists of several cell types facing the haemolymph on the inside of the animal. In the LsCOPB2 knock down animal (G), small unstained patches in the tissue (→) are visible. The scale bar indicates 500 µm.