Vol. 94: 211–224, 2011
doi: 10.3354/dao02331

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 9

OPEN ACCESS

Characterisation of two vitellogenins in the salmon louse *Lepeophtheirus salmonis*: molecular, functional and evolutional analysis

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ABSTRACT: The salmon louse *Lepeophtheirus salmonis* Krøyer affects a variety of wild salmonoid hosts, but is also an important pest in aquaculture, which is a globally important and rapidly growing industry. Salmon lice have large reproductive outputs, and knowledge of reproductive processes may be crucial for the control of this parasite. Here, we report on the characterisation of 2 vitellogenins (LsVit1 and LsVit2), which are the precursors of salmon-louse egg-yolk glycoprotein. The structure of LsVit1 and LsVit2 was examined and compared to that in other oviparous animals. Phylogenetic analysis of LsVit1 and LsVit2 confirmed the view that crustaceans are a polyphyletic group. Transcriptional and translational analysis demonstrated production of LsVit1 and LsVit2 in the subcuticular tissue of the adult female lice. LsVit1 and LsVit2 could also be found in maturing oocytes and developing embryos and early larval stages. LsVit2 was found to be processed into 2 smaller fragments, whereas LsVit1 was found to be full length when deposited into the oocytes. Degradation of LsVit1 and LsVit2 was characterised through embryogenesis and the early non-feeding larval stages. Finally, protein content and the level of free amino acids were analysed in embryos and larval stages and their role in nutrition and osmoregulation discussed. In conclusion, our results confirm the role of vitellogenins in reproduction as providers of embryonic and larval nutrition.

KEY WORDS: Vitellogenin · Copepoda · Reproduction · Vitellin · Ectoparasite · Aquaculture · Sea lice · Nauplii

INTRODUCTION

The salmon louse *Lepeophtheirus salmonis* is an ectoparasitic copepod with a lifestyle adapted to the low population density of their salmonid hosts. Female lice are long-lived, store sperm in spermatophores, have large reproductive output and produce larvae with considerable dispersal capacity. Aquaculture in coastal areas of Norway, Scotland, Ireland and Canada has dramatically increased the number of available hosts. The resulting increase in salmon lice abundance causes major economic losses in aquaculture and also represents a threat to wild salmon populations (Bjorn et al. 2001). The reproductive system of salmon louse has been described morphologically (Ritchie et al. 1996), but few molecular studies of reproduction in salmon louse and the associated copepod group have been published (Todd et al. 2005, Lee et al. 2008, Dalvin et al. 2009, Hwang et al. 2009).

In oviparous animals, eggs are supplied with sufficient nutrients to ensure proper development and growth until external food can be ingested and utilised

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autonomously. In lecithotrophic embryos of some marine invertebrates, however, dissolved nutrients, including amino acids (AA), may be absorbed from the surrounding water, thereby representing an additional supply of organic compounds to the developing embryos (Manahan et al. 1982, Vavra & Manahan 1999). AA are important substrates for synthesising body proteins and act as precursors for a range of biomolecules, while they are also used as a source of energy by the developing embryos. Vitellogenins are the major yolk proteins in most invertebrate and vertebrate egg-laying animals. Several different vitellogenins typically give rise to vitellin present in mature eggs (Sappington & Raikhel 1998); however, in some animals, such as insects, the role of multiple vitellogenin genes is unknown (Tufail & Takeda 2008). Vitellogenins and proteins with domain structure similar to vitellogenins are also involved in other developmental processes, such as regulation of osmolarity in pelagic fish eggs (Kristoffersen et al. 2009), immunity (Liu et al. 2009) and clotting (Hall et al. 1999).

In vertebrates, vitellogenins are produced in the liver, and tissues with similar function have been reported to produce vitellogenins in invertebrates. In insects, vitellogenins are produced in the fat body, structurally modified and processed into smaller fragments (Tufail & Takeda 2008) and thereafter incorporated into the developing eggs by receptor-mediated endocytosis (Sappington & Raikhel 1998). After incorporation into the oocyte, vitellogenins are stored in a crystalline form, named vitellin.

In crustaceans, the vitellogenins are primarily produced in the hepatopancreas, but minor production has also been observed in ovaries (Eastman-Reks & Fingerman 1985, Chen et al. 1999, Zmora et al. 2007, Tiu et al. 2009). As in insects, crustacean vitellogenins are commonly processed into smaller fragments before incorporation into vitellins (Okuno et al. 2002). Despite the crucial role of vitellin degradation for nourishment of the developing and growing embryo and also larval survival, few molecular studies (Walker et al. 2006, Garcia et al. 2008) have investigated the degradation of vitellins during ontogeney.

Similar to most other marine copepods, the adult salmon louse is an osmoconforming regulator in seawater (Hahnenkamp & Fyhn 1985). In such osmo-conforming animals, free amino acids (FAA) serve important roles in cell volume regulation and are therefore present in high intracellular concentrations. During degradation of vitellins in developing lice, there is likely to be a high production of AA with large implications for the turnover in the FAA pool. Due to the multiple roles served by FAA in early development, it is important for the embryo to balance the strategy for osmoregulation with the demand for substrates for body proteins, other biomolecules and energy. No studies have thus far investigated these issues in salmon louse.

A transcriptomic study of post-moulting growth in adult female salmon louse (Eichner et al. 2008) revealed several putative egg-yolk protein transcripts, including 2 female-specific vitellogenin transcripts. The corresponding genes were named *LsVit1* and *LsVit2* (*Lepeophtheirus salmonis* vitellogenin 1 and 2) and were expressed in adult females only.

The main objective of the present study was to characterise these 2 vitellogenins. Sequencing and phylogenetic analysis of LsVit1 and LsVit2 shows that copepod and hexapod vitellogenins have the same domain structure and are closely related in the phylogenetic tree. By means of *in situ* hybridisation and immunohistochemistry, the localisations of transcription and translation and vitellogenesis were identified. The issue of processing of LsVit1 and LsVit2 was also addressed when our data showed that they are differentially processed. Finally, the function of LsVit1 and LsVit2 was addressed by investigating the degradation of the proteins during embryonic and larval development at both the protein and AA level.

MATERIALS AND METHODS

Animal culture. Salmon lice eggs were hatched in incubators with flowing seawater and used to infect salmon (Hamre et al. 2009). Salmon lice were kept in culture on Atlantic salmon *Salmo salar* in tanks with seawater (salinity 34.5, 20 µM filtered). Fish were anaesthetised with a combination of metomidate and benzocaine. All experiments were conducted in accordance with Norwegian animal-welfare regulations.

Peptide sequencing, purification of LsVit1 and LsVit2 and antibody production. Adult female lice were collected with forceps from anaesthetised fish. Unfertilised eggs were harvested by puncturing the genital segment. Water-soluble proteins from eggs were extracted from 50 lice in 2.5 ml cold sonication buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl and 1 mM EDTA). Eggs were disrupted by sonication using a micro-ultrasonic cell disrupter. The sonicated extract was clarified by centrifugation (13 000 × *g* for 20 min at 4°C) and pellet and lipids were discarded. The total protein content in the supernatant was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Protein bands were excised from the gel and internal AA sequence analysis performed by either EuroSequence (Groningen, Netherlands) or Probe (Proteomic Unit, University of Bergen, Norway). This involved *in situ* tryptic digestion of the protein band, extraction of the

peptides and reverse phase high performance liquid chromatography (RP-HPLC) separation of the fragments generated. The phenylthiohydantoin AAs (Hewick et al. 1980) released step-wise during degradation of the purified fragments were identified using a Model 494 Procise Sequencing system (Applied Biosystems), on-line connected to an RP-HPLC unit. The internal peptide sequences were back-translated and used to identify expressed sequence tag (EST) sequences corresponding to the protein bands. LsVit1 and the 2 LsVit2 fragments were purified by preparative SDS-PAGE using a Prep Cell model 491 (BioRad) according to the manufacturer's instructions. Purified LsVit1 and LsVit2 fragments were analysed by SDS-PAGE and Coomassie staining, and quantified relative to bovine serum albumin (BSA). One rabbit was immunised with the purified protein (500 µg ml^{-1}) by Eurogentec at their facility using their immunisation protocol (boosters on Days 14, 28 and 56).

***In situ* hybridisation.** The localisation of *LsVit1* and *LsVit2* mRNA in adult female salmon lice was determined by *in situ* hybridisation as described previously (Kvamme et al. 2004) using a PCR product generated from a cDNA clone as template for digoxigenin (DIG)-labelled RNA probe synthesis. Labelled probes (LsVit1: 563 bases; LsVit2: 282 bases) were visualised using anti-DIG fragment antigen binding conjugated to alkaline phosphatase (Roche) and a chromogen substrate containing levamisol (Sigma-Aldrich), nitroblue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Hybridisations were performed with antisense probes to show localisation of transcripts and with sense probes as a negative control. Slides treated with sense probes were completely negative except for occasional edge effects along the cuticle also observed in the antisense-treated samples.

Immunohistochemistry. Adult female lice were fixed at 4°C overnight and nauplii were fixed for 2 h in phosphate-buffered 4% paraformaldehyde (pH 7.4). Extruded egg strings were placed in 7.5% nitric acid, 30% EtOH, H_2O for 4 h at 4°C (McClendon 1907). Subsequently all samples were washed in PBS, dehydrated through a graded ethanol series and embedded in paraffin wax. Sections, 3.0 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 20 min, slides were incubated with the primary LsVit1 and LsVit2 (fragments C and D, see Fig. 1) antiserums diluted 1:500 in 2.5% BSA in TBS. After 3 washes in TBS, secondary antibody, biotinylated goat anti-rabbit antibody diluted 1:300, was added for 30 min. After washing, streptavidin alkaline phosphatase complex diluted 1:1000 was added at room temperature for 30 min followed by washing and developed with New Fuchsin Chromogen (DAKO) with 1 mM levamisole (Sigma). Slides were mounted and photographs taken.

Confocal immunofluorescence. Salmon lice and sections were prepared as for immunohistochemistry. Sections were permeabilised in 1% Tween dissolved in PBS and blocked in 3% BSA, and the primary LsVit1 and LsVit2 antiserum fragments C and D (see Fig. 1) were diluted 1:500 and incubated overnight. After 3 washes in PBS, Alexa Fluor 594 conjugated goat anti-rabbit antibody (Invitrogen) diluted 1:200 was used as a secondary antibody. All samples were mounted using ProLong Gold or ProLong Gold with DAPI (Invitrogen), and confocal imaging was performed with a Leica TCS SP2 AOBS microscope at the Molecular Imaging Centre (FUGE, Norwegian Research Council, University of Bergen). No signals were observed on slides treated with secondary antibody only. Images of whole lice were collected with the transmission detector.

Protein extraction and Western blotting. Adult lice (female and male) were collected with forceps from anaesthetised fish. Cephalothorax samples were obtained from adult females by cutting away the genital and abdominal segment from lice with a razor blade. Unfertilised egg samples were obtained by puncturing the genital segment of adult females and pulling out the eggs. Extruded egg samples consisted of whole egg strings removed from the genital segment of the female louse. Samples of Nauplius stages I and II and copepodites were harvested directly from hatching incubators. For the utilisation experiment (see 'Results: Utilisation of LsVit1 and LsVit2 during embryogenesis and larval development'), mature unfertilised eggs were sampled from females bearing external fertilised egg strings due to hatch within 24 h. Hatching was monitored (Day 0) and the remaining samples were collected on Day 1 (Nauplius I), Day 2 (early Nauplius II), Day 4 (late Nauplius II), Day 5 (early copepodid) and Day 10 (late copepodid). Protein extracts were prepared by grinding samples in liquid nitrogen followed by addition of lysis buffer: 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X with 0.25 M phenylmethanesulphonylfluoride and protease inhibitors (complete protease inhibitor cocktail; Roche) added immediately before use. Samples were left on ice for 15 min and centrifuged at 16 100 × *g* for 15 min at 4°C. The protein content of the supernatant was measured and equal amounts of protein were loaded on an SDS-PAGE gel (NuPAGE® Novex; Invitrogen). Alternatively, for the analysis of developmental degradation, protein gels were loaded with extracts corresponding to 6 lice in each lane. Western blotting was performed using a

WesternBreeze® chromogenic kit (Invitrogen) with LsVit1 and the 2 LsVit2 antiserums as a primary antibody (dilution 1:2000). Blocking was performed overnight. A BenchMark™ Protein Ladder (Invitrogen) was used for determination of molecular weights, and a SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) was used to easily visualise molecular weight ranges during electrophoresis and quickly evaluate Western transfer efficiency. Total protein stain was performed using Novex Reversible Membrane Protein Stain (Invitrogen).

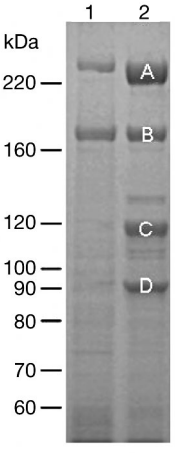
AA and protein analyses. Complete egg strings (n = 4) were carefully removed from different salmon lice and transferred to small incubators and reared under optimised conditions until the Nauplius II stage. No feed was offered to the developing eggs and larvae. At regular intervals (0, 2, 4, 6, 8 and 11 d post fertilisation [dpf]), groups of eggs (n = 51 to 112) were cut off from each egg string and transferred into tubes containing 250 µl 6% trichloroacetic acid (TCA). After precipitation and centrifugation (10 000 × *g*), FAA from the supernatant were quantified by RP-HPLC with a Gilson HPLC using fluorometric detection (orthophtal-dialdehyde and 9-fluorenyl-methoxy-carbonyl reagents) as described by Kristoffersen & Finn (2008). Protein content of the precipitate was analysed by the Lowry technique as described by Rønnestad et al. (1998).

Bioinformatics. Sequence assemblies, handling, editing and alignments were performed using Vector NTI 9 (Invitrogen). Various basic local alignment search tool (BLAST) searches were performed in GenBank. Signal peptides were predicted by the SignalP server (www.cbs.dtu.dk/services/SignalP/) using neural networks and hidden Markov models trained on eukaryotic signal peptides (Bendtsen et al. 2004). The alignment used in the phylogenetic analysis was performed using ClustalW and the resulting alignment was manually edited in GeneDoc (Nicholas et al. 1997) afterwards to ensure that the different common domains were properly aligned. Lineage-specific insertions (domains) were removed in order to remove the number of gaps in the final alignment. Phylogenetic analysis was performed using PHYLIP (Protml), MrBayes and TreePuzzle. The Protml analysis was performed with the Jones-Taylor-Thornton model of AA change. The slow and accurate settings with gamma distribution and $\alpha = 3.61$ (estimated in TreePuzzle) was used. The Bayesian inference was conducted using a GTR + I + G model, run with 4 simultaneous chains and 655 000 generations. The TreePuzzle analysis was performed applying rate variation and all the parameters were estimated from the data set. A total of 10 000 puzzle steps were performed. The GenBank accession numbers for the included sequences are AAL12620, ACJ65208, BAD51933, ABQ58114, XP_970210, XP_002415017, BAG12081, XP_971398, Q16927, XP_001843136, XP_001607388, XP_002599686, ACJ12892, NP_001023276, BAD74020, XP_001634649, NP_001152753, AAB03336, AAV31928, AAT48601, ABD83654, BAD05137, BAE94324, BAA22791, ABU41135, ABU41134, XP_002431396, ACU00433, ABZ91537, XP_313104, BAC22716, BAF98238, XP_002634040, Q94637, ABO09863, AAG17936 and Q05808.

RESULTS

Protein analysis of unfertilised eggs

SDS-PAGE analysis of proteins from adult males and adult gravid females revealed 4 prominent proteins in females in sizes ranging from approximately 95 to 220 kDa (Proteins A to D; Fig. 1). One additional prominent protein found in egg, LsYAP with a molecular weight of 35 kDa, has previously been described (Dalvin et al. 2009). The 4 proteins were digested with trypsin and then subjected to N-terminal sequencing, giving peptide sequences for all proteins. Sequence analysis using the peptide sequences gave a significant match with 3 previously identified cDNAs (Eichner et al. 2008). Proteins A, C and D are transcribed in adult female lice only (Eichner et al. 2008). The present study focuses on 3 of these yolk proteins, Proteins A, C and D, whereas the characterisation of Protein B will be presented elsewhere (R. Skern-Mauritzen, S. Dalvin & F. Nilsen unpubl. data).



Protein	Size	cDNA/protein sequence
A	220 kDa	LsVit 1: cDNA 6093 bp Protein: 1965 AA Predicted MW: 220.5 kDa
B	175 kDa	B: cDNA 4747 bp Protein: 1521 AA Predicted MW: 173.1 kDa
C	120 kDa	LsVit 2: cDNA 5948 bp Protein: 1903 AA Predicted MW: 213.2 kDa
D	95 kDa	LsVit 2: same as above

Fig. 1. *Lepeophtheirus salmonis*. Identification of yolk proteins. Left panel: total water-soluble protein obtained from adult males (Lane 1) and females (Lane 2) analysed by SDS-PAGE. Right panel: the major yolk proteins (Proteins A to D), the predicted size of their transcripts, and predicted size of the translational products in number of amino acids (AA) and molecular weight (MW). Protein sizes were estimated using the BenchMark™ protein marker

Sequence analysis of 2 salmon-louse vitellogenins

The obtained peptide sequence data from Proteins A, C and D unambiguously linked these proteins to 2 previously obtained cDNA sequences from the salmon louse. Protein A is encoded by *LsVit1*, whereas Proteins C and D are both encoded by *LsVit2* (Eichner et al. 2008). LsVit1 is unprocessed and is present in mature eggs as a single polypeptide. Conversely, the peptide sequencing showed that the LsVit2 protein is cleaved into 2 peptides, an N-terminal fragment (Protein C) and a C-terminal fragment (Protein D).

The structural features of the cDNA sequences and the translated protein sequences were compared to vitellogenins from other organisms (Table 1). The putative protein sequences obtained from LsVit1 and LsVit2 consist of an N-terminal vitellogenin N domain (pfam1347) and a C-terminal von Willebrand domain (vWD), a domain structure typically found in vitellogenins. In addition, both LsVit1 and LsVit2 contain a domain of unknown function (DUF) 1943 (pfam09172) as well as a signal peptide predicted with high probability using SignalP. The identified protein domains are longer in LsVit1 than in LsVit2.

Despite the shared domain structure between LsVit1 and LsVit2, the proteins exhibit a mere 23% identity at AA level. LsVit1 and LsVit2 are more similar to corresponding vitellogenin paralogues from other copepods than to each other. The sequence similarity of LsVit1 to vitellogenin 1 in *Tigriopus japonicus* and *Paracyclopina nana* is 37% and 33% respectively. The sequence similarity of LsVit2 to vitellogenin 2 in *T. japonicus* and *P. nana* is 33% and 31% respectively. LsVit1 and LsVit2 together with vitellogenin from *T. japonicus* and *P. nana* have a domain structure similar to insect vitellogenins, but different from other crustaceans and vertebrates (Fig. 2). The core vitellogenin domain structure consists of 3 different domains present in all species, and the insect-type vitellogenins (here represented by salmon louse) possess only these 3 domains. Non-insect species commonly contain additional domains, although not necessarily the same type. Vitellogenin from water flea *Daphnia magna* (Cladocera) contains an N-terminal superoxide dismutase domain (pfam00080) whereas the lobster *Homarus americanus* (Decapoda) vitellogenin contains an additional DUF (pfam06448). Vertebrate vitellogenin, here represented by rainbow trout *Oncorhynchus mykiss*, also has an additional DUF (pfam09175). Both the total length and size of the common domains vary between vitellogenin from different species (Table 1). The salmon-louse vitellogenins have an intermediate length relative to the selected species in Table 1, where the decapod (represented by lobster) vitellogenin is the longest.

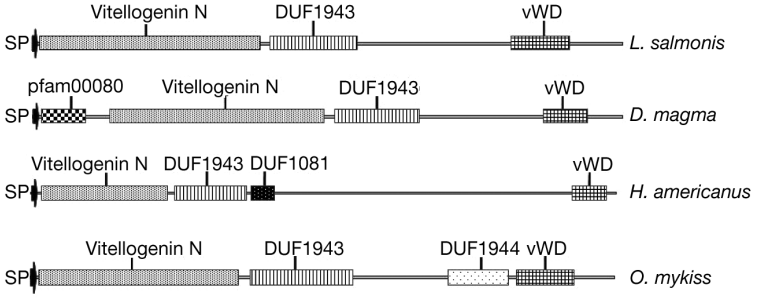


Fig. 2. Domain structure of vitellogenins found in salmon louse *Lepeophtheirus salmonis*, water flea *Daphnia magna*, lobster *Homarus americanus* and rainbow trout *Oncorhynchus mykiss*. A signal peptide (SP), a vitellogenin N domain (pfam01347), a domain of unknown function (DUF) 1943 (pfam09172) and a von Willebrand domain (vWD) (pfam00094) are found in all 4 species. Additional domains vary between species

Table 1. Overview of the protein domains found in salmon louse *Lepeophtheirus salmonis* vitellogenins, and comparison to water flea *Daphnia magna* (GenBank no. BAE94324), yellow fever mosquito *Aedes aegypti* (GenBank no. Q16927), lobster *Homarus americanus* (GenBank no. ABO09863), and rainbow trout *Oncorhynchus mykiss* (GenBank no. Q92093) vitellogenins. The length of the transcript, the open reading frame (ORF) and the 5′ and the 3′ untranslated region (UTR) are given for LsVit1 and LsVit2. A signal peptide (SignalP), a vitellogenin N (Vit N) domain (pfam01347), a domain of unknown function (DUF) 1943 (pfam09172) and a von Willebrand domain (vWD) (pfam00094) are found in all 6 vitellogenins. AA = amino acid, nd = not determined

Vitellogenin in the species	Transcript length (bp)	ORF (bp)	5′ UTR (bp)	3′ UTR (bp)	No. of AAs	Domain size (no. of AAs)			
						SignalP	Vit N	DUF1943	vWD
Vit1 *L. salmonis*	6093	5895	63	115	1965	16	727	288	194
Vit2 *L. salmonis*	5948	5709	53	171	1903	15	692	218	160
Vit *D. magna*	nd	nd	nd	nd	2002	17	709	278	146
VitA1 *A. aegypti*	nd	nd	nd	nd	2148	16	824	185	188
Vit *H. americanus*	nd	nd	nd	nd	2583	18	547	310	148
Vit *O. mykiss*	nd	nd	nd	nd	1659	15	568	290	165

In order to explore the relationship of the salmon-louse vitellogenins to other invertebrate vitellogenins, we conducted a phylogenetic analysis with a wide diversity of sequences (Fig. 3). The phylogenetic analysis grouped LsVit1 and LsVit2 together with 2 vitellogenins from *Tigriopus japonicus* and with the water flea *Daphnia magna* (Brachiopoda) vitellogenins as a sister group. The closest relatives to the Copepoda/ Brachiopoda clade are the Hexapoda, which is well supported in the present analysis of the vitellogenins. Our phylogenetic analysis indicates that crustaceans are not monophyletic, as the Malacostraca did not group together with the other arthropods included. The phylogenetic analysis also points to an ancient vitellogenin gene duplication in the copepod since the 2 salmon-louse paralogues are more closely related to the corresponding paralogue in *T. japonicus* than to each other.

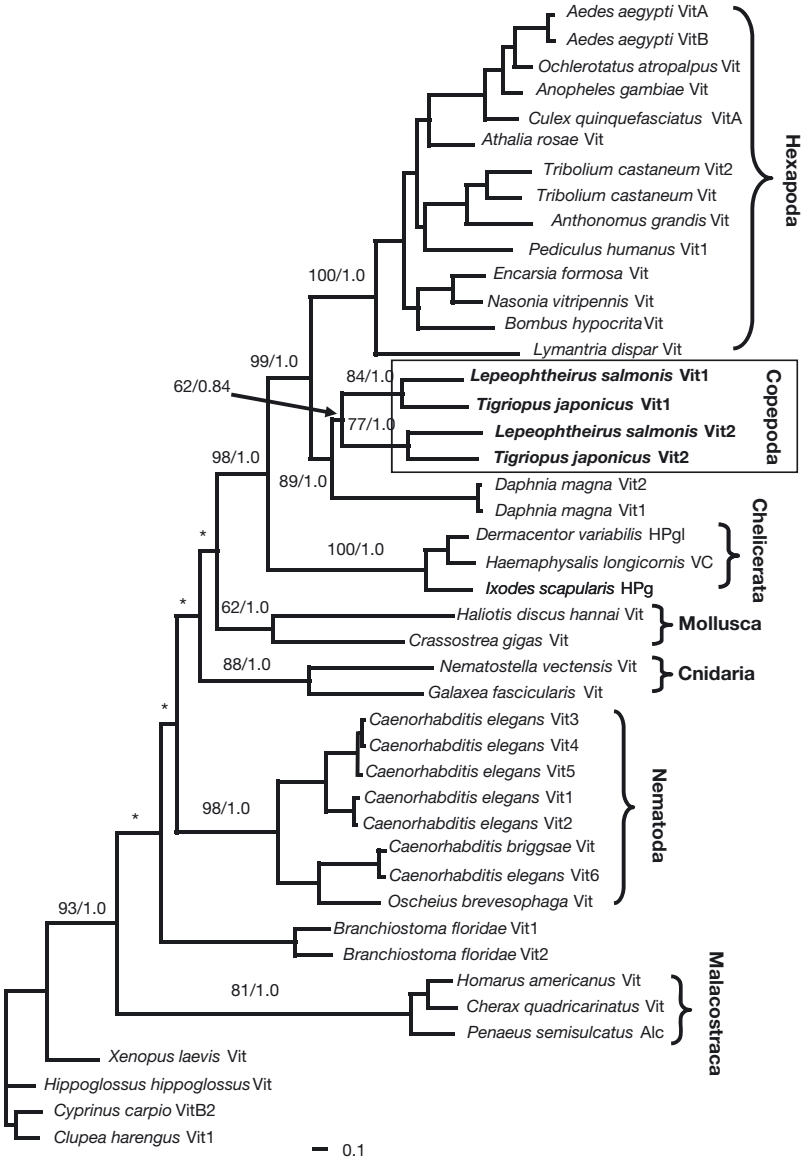


Fig. 3. Maximum-likelihood phylogenetic tree showing relationships between invertebrate vitellogenins. The tree was generated in PHYLIP (Protml); values on the nodes are support values obtained with MrBayes and TreePuzzle. For clarity, only selected values are shown. *Key nodes without >50% support

Production of vitellogenins in subcuticular tissue

In contrast to decapods, the salmon louse has no hepatopancreas. Transcription and production of digestive enzymes takes place in the intestine (Kvamme et al. 2004), whereas transcripts of the previously described yolk protein LsYAP are localised to cells beneath the cuticle, designated as the subcuticular tissue (Dalvin et al. 2009). This tissue has not been characterised, but inspection of various histological preparations (data not shown) reveals a tissue type consisting of variably shaped cells organised in an irregular pattern. It is difficult to distinguish individual cells and they appear to be multinucleate. The cells are the most commonly found cell type in this tissue. In addition to these cells, the tissue below the cuticle consists of several other cell types, including muscles attached to and sometimes lining the cuticle, very large multinucleate cells and a smaller cell type of unknown character lining the cuticle in some areas.

An ontogenetic (i.e. Nauplius I, copepodids, Chalimus III, male and female pre-adult and adult male and female lice) quantitative PCR analysis of the 2 LsVit revealed significant transcription in adult females only (data not shown). Further studies of LsVit production were therefore performed in mature adult females (Fig. 4A). Analysis of transcriptional activity of *LsVit1* and *LsVit2* by *in situ* hybridisation demonstrated that transcription of vitellogenins also takes place in the subcuticular tissue, but only in the cell type described in the previous paragraph as the main type of cells found in this tissue type (Fig. 4B,C). Transcripts of both *LsVit1* and *LsVit2* were found throughout and exclusively in this tissue.

Antibodies were raised against LsVit1 and the N-terminal and C-terminal fragment of LsVit2. Immunoflu-

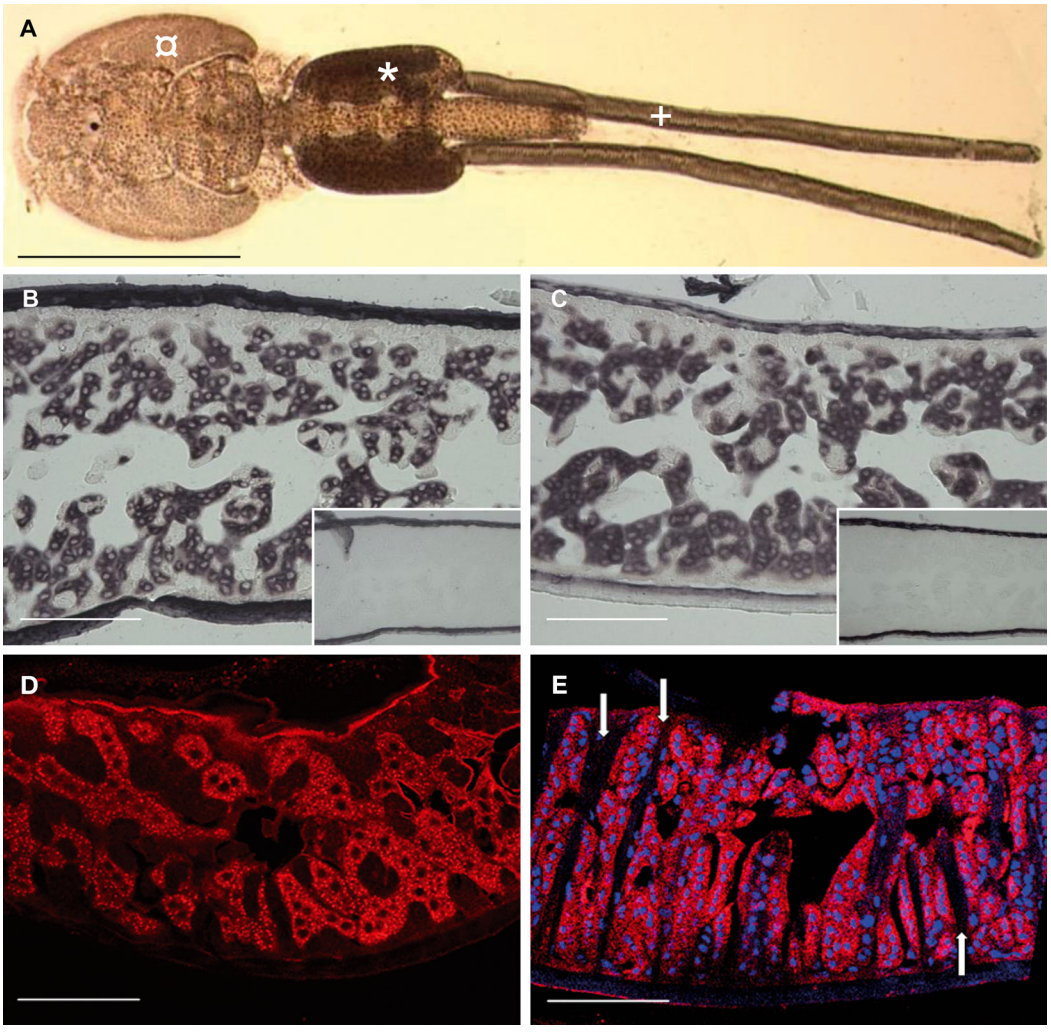


Fig. 4. *Lepeophtheirus salmonis*. Production of LsVit1 and LsVit2. (A) Light-microscopy top view of adult female with egg strings attached. Genital segment (*) and embryos (+) in egg strings and area where subcuticular tissue is found (¤) are indicated. (B–E) Cross-sections of adult female subcuticular tissue as indicated with (¤) in (A). *In situ* hybridisation using (B) *LsVit1*- and (C) *LsVit2*-specific antisense probes. Both transcripts are localised to the subcuticular cells located between the layers of cuticle. Sections depict an area of the cephalothorax where the subcuticular cells form a double layer between the ventral and dorsal cuticle. Insets illustrate the use of a corresponding sense probe on parallel sections. (D) Immunofluorescence using the LsVit1 antibody. The protein shown in red can be observed in the same type of cells as the *LsVit1* transcript (cf. Fig. 4B). (E) Immunofluorescence using the C-terminal part of the LsVit2 antibody and DAPI nuclear stain (in blue). The protein shown in red can be observed in the same type of cells as the *LsVit2* transcripts (cf. Fig. 4C). Scale bars = (A) 5 mm, (B–E) 200 µm

orescence studies of subcuticular tissue established that LsVit1 and LsVit2 proteins also localise to the same cell type as the *LsVit1* and *LsVit2* transcripts and were often observed in a vesicle-like pattern, giving rise to a dotted appearance of the cells (Fig. 4D,E). No differences between the localisation of LsVit1 and the 2 fragments of LsVit2 were observed (Fig. 4E, LsVit2 C-terminal; LsVit2 N-terminal not shown). Haematoxylin-eosin staining of a parallel section (not shown) revealed that the vertical stripes of cells not positive for LsVit2 (C-terminal) (Fig. 4E, white arrows) are muscle cells. The amount of LsVit1 and LsVit2 protein varied through the reproductive cycle of the louse. Larger amounts of protein appeared to be present in animals with a genital segment containing ova (data not shown) compared to those with vitellogenic oocytes.

LsVit1 and LsVit2 appear to be secreted into the haemolymph in the haemocoel that bathes the subcuticular tissue and then transported by the haemolymph to the genital segment, where they are found in the oocytes. This hypothesis is supported by the observation of vitellogenin in the subcuticular tissue, haemocoel (results not shown) and ova.

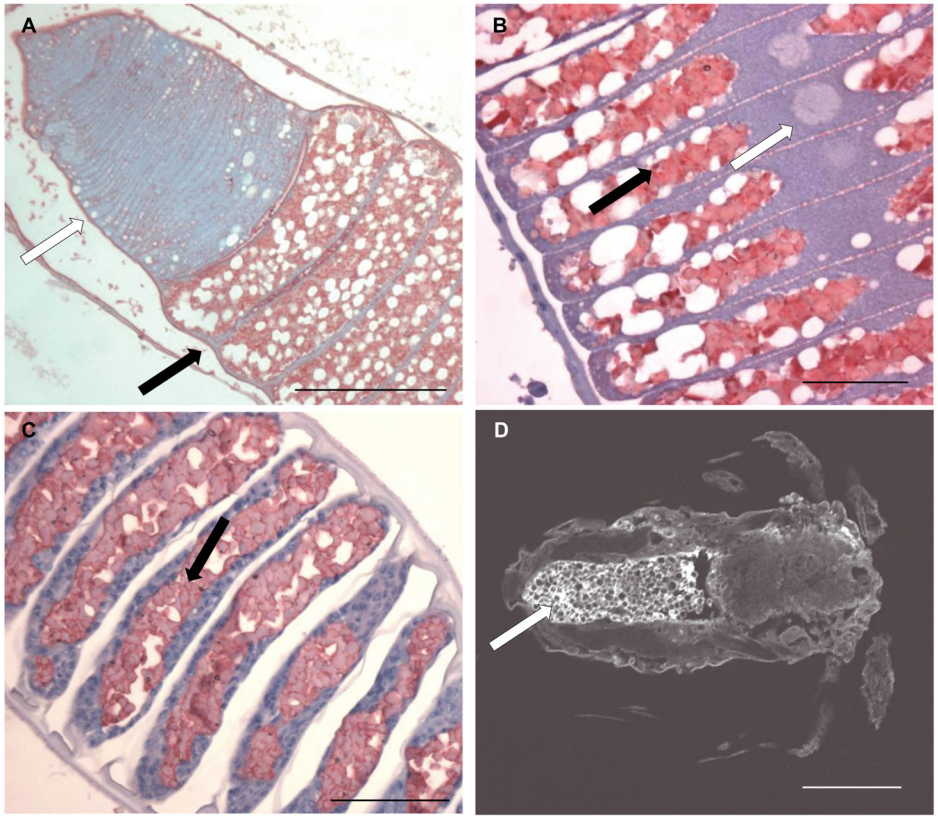


Fig. 5. *Lepeophtheirus salmonis.* Localisation of LsVit1 and LsVit2 in eggs, embryos and nauplius. (A) Immunohistochemistry of eggs in the genital segment (marked with * in Fig. 4A) stained with LsVit2 (C-terminal part) antibodies (red) and counterstained with haematoxylin (blue). This cross-section is taken from an adult female fixed during the brief period (approximately 30 min) in which extrusion of eggs takes place. Both very immature oocytes (white arrow) that have just entered the genital segment and ova (black arrow) being transported for fertilisation are present. (B) Cross-section of genital segment of female (marked with * in Fig. 4A) containing immature, not fully vitellogenised unfertilised eggs. LsVit2 (N-terminal part) protein (red) localises to vitellin crystals (black arrow). The counterstain haematoxylin colours the cytoplasmic part of the egg. The nucleus is visible in some eggs (white arrow). (C) Cross-section of egg strings approximately 2 d after fertilisation. Localisation of LsVit2 (C-terminal part) protein (red) in developing embryos (black arrow). Egg strings with developing embryos indicated (+) in Fig. 4A. Cells of developing embryos are visible in blue (counterstained with haematoxylin). (D) Immunofluorescence showing localisation of LsVit1 in a Nauplius I larva (white arrow). The fluorescent image has been combined with a transmission image to visualise LsVit1-positive vitellin (white) in the context of the whole animal (grey tones). Large amounts of vitellin are present in the developing gut in the distal part of the animal. Scale bars = (A) 500 µm, (B) 50 µm, (C) 200 µm and (D) 100 µm

Vitellogenesis in salmon louse

The salmon louse oviducts run from the posterior part of the ovarium into the gonad segment. The size of the oocytes in the oviduct increases gradually from anterior to posterior. About halfway down the oviduct, a few small acidophilic vacuoles appear, but they did not stain positive in immunohistochemistry using LsVit1 and LsVit2 antibodies (data not shown). The most anterior oocytes in the genital segment were also negative for vitellogenins, indicating that they are not competent for vitellogenin acquisition, but they contained an increased number of vacuoles compared to the oocytes in the oviduct (Fig. 5A).

In oocytes that are undergoing vitellogenesis, LsVit1 and LsVit2 (N- and C-terminal fragments) are co-located between lipid vacuoles (Fig. 5B, only LsVit2 N-terminal is shown). All 3 proteins can also be identified as vitellin components in the developing embryos in the external egg strings (Fig. 5C, only LsVit2 C-terminal is shown) and in nauplii (Fig. 5D, only LsVit1 is shown). No differences between the localisation of LsVit1 and LsVit2 (N- and C-terminal fragments) were observed in ova, embryos and nauplii.

Vitellogenin processing

As peptide sequencing revealed that the *LsVit2* transcripts gave rise to 2 proteins in unfertilised ova, vitellogenin processing was analysed by SDS-PAGE and Western blotting using the same antibodies as used for immunohistochemistry in samples extracted from

cephalothorax and unfertilised eggs (Fig. 6). Cephalothorax samples contain 2 sources of vitellogenins: subcuticular cells (representing production) and haemolymph (representing transport to the ova and possibly storage). Unfertilised eggs samples contain only eggs that have been removed from the genital segment. The samples represented vitellins and other proteins deposited in mature but unfertilised eggs.

Total protein staining of extracts from the cephalothorax revealed 2 major large bands of approximately 225 and 160 kDa (Fig. 6A, Lane 1). The 225 kDa large protein band corresponds to the predicted size of both the LsVit1 and the LsVit2 protein. Staining of samples from unfertilised eggs undergoing vitellogenisation (Fig. 6A, Lane 2) revealed 4 major bands, of which the 3 largest (Fig. 6A, white arrows) were identified as LsVit1 and 2 fragments of LsVit2 (see 'Protein analysis of unfertilised eggs' above).

Western blotting of the same protein extracts using the LsVit1 antibody (Fig. 6B) identified 1 band in cephalothorax samples and a band of corresponding size in unfertilised eggs (Fig. 6B, white arrow). The size of the large band corresponds to the size of the protein identified as LsVit1 by peptide sequencing.

Western blotting using the LsVit2 C-terminal antibody (Fig. 6C) identified 2 bands in the cephalothorax samples. These 2 bands correspond to the size of the protein identified as full-length LsVit2 (Fig. 6C, grey arrow) and processed LsVit2 (C-terminal) (Fig. 6C, white arrow) protein by peptide sequencing. Both full-length and processed LsVit2 (C-terminal) protein were also identified in the unfertilised egg samples, but the amount of full LsVit2 protein (Fig. 6C, grey arrow) was relatively small compared to the amount of the processed LsVit2 (C-terminal) protein (Fig. 6C, Lane 2, white arrow).

Similarly, Western blotting using the LsVit2 N-terminal antibody (Fig. 6D) identified 2 bands in the cephalothorax samples. These 2 bands correspond to the size of the protein identified as full-length LsVit2 (Fig. 6D, grey arrow) and processed LsVit2 (N-terminal) (Fig. 6D, white arrow) protein by peptide sequencing. Both full-length and processed LsVit2 (N-terminal) protein were also identified in the unfertilised egg samples, but the amount of full LsVit2 protein (Fig. 6D, grey arrow) was less than the amount of the processed LsVit2 (C-terminal) protein (Fig. 6D, Lane 2, white arrow).

Female lice released a new pair of egg strings at regular intervals, and at 10°C a new pair of egg strings was released every 10 d (authors' unpubl. data). Considering the large biomass of vitellogenins dedicated to egg production, a cyclical pattern in the production of these proteins could be expected. Despite this, total protein stain and Western blotting of LsVit1 and LsVit2 proteins of females from different stages of the reproductive cycle failed to identify clear patterns (data not shown).

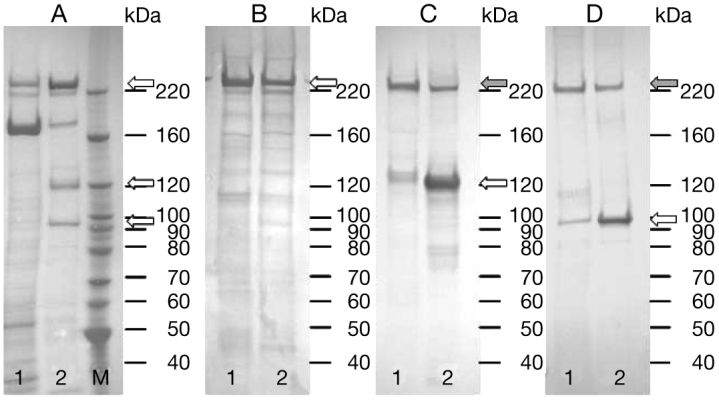


Fig. 6. *Lepeophtheirus salmonis*. Production and processing of LsVit1 and LsVit2. (A) SDS-PAGE of protein extracted from cephalothorax (Lane 1) and unfertilised eggs undergoing vitellogenisation (Lane 2) stained for total protein (Lane M) with the BenchMark™ Protein Ladder. Three major bands (white arrows) of approx. 220, 120 and 95 kDa can be observed in egg extracts. (B) Western blot of cephalothorax (Lane 1) and eggs (Lane 2) using the LsVit1 antiserum. Full-length LsVit1 protein was detected in both samples (white arrow). (C) Western blot of cephalothorax (Lane 1) and eggs (Lane 2) using the LsVit2 C-terminal antiserum. Full-length LsVit2 protein (grey arrow) and processed LsVit2 protein (white arrow) are present in both samples. (D) Western blot of cephalothorax (Lane 1) and eggs (Lane 2) using the LsVit2 N-terminal antiserum. Full-length LsVit2 protein (grey arrow) and processed LsVit2 protein (white arrow) are present in both samples

Utilisation of LsVit1 and LsVit2 during embryogenesis and larval development

The main biological role of vitellins is to ensure nutrition and protein supply for development from the fertilised egg until the first feeding life stage. In salmon louse, this takes around 14 d (at 10°C) and involves hatching of the eggs and 2 moults before the copepodid is able to infect the host fish and feeding can take place. Degradation of LsVit1 and the 2 processed fragments of LsVit2 were investigated by SDS-PAGE and Western blotting in samples from unfertilised eggs and through development over nauplii stages to copepodids (Fig. 7A). As expected, overall protein content decreased significantly in a gradual time-dependent fashion, as seen by total protein staining (Fig. 7B), but was also confirmed by quantitative assessment (see 'Quantification of protein and amino acids during embryogenesis and larval development' below). Western blotting using the antibodies against LsVit1

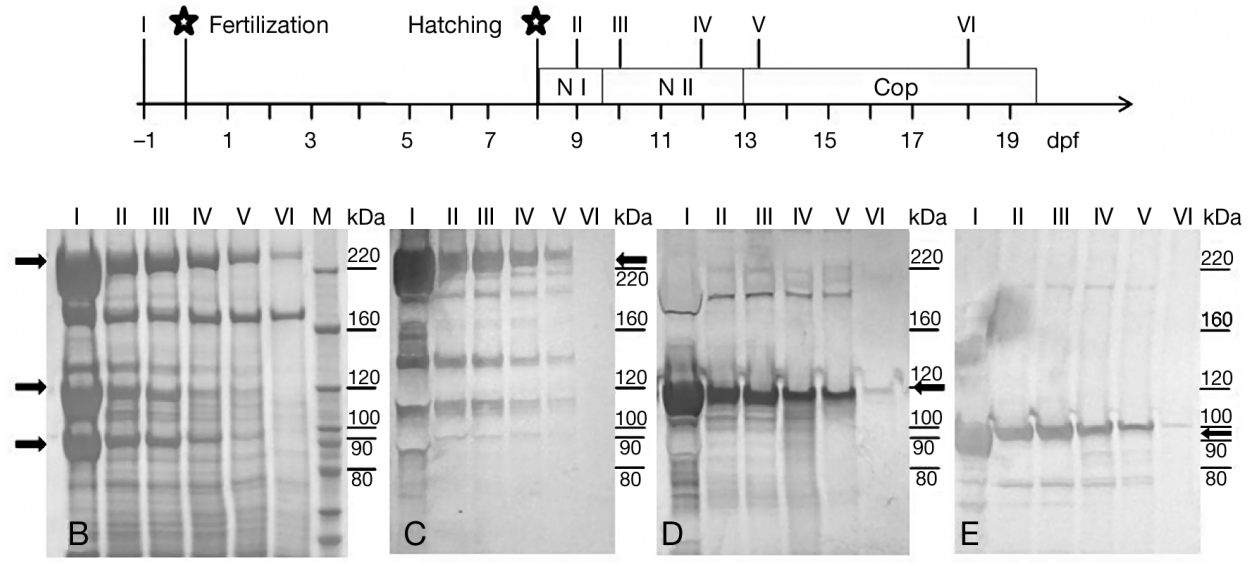


Fig. 7. *Lepeophtheirus salmonis*. Utilisation of LsVit1 and LsVit2 in early life stages. (A) Overview of sample collection during larval development (N I = Nauplius I, N II = Nauplius II and Cop = copepodid). Numbers on the lower part of the scale indicate when sampling took place in relation to development days post fertilisation (dpf) and corresponds to samples for SDS-PAGE and Western blotting (B–E). Sample size (n = 6) is the same in each lane. Developmental stages (same as lane labels): I = unfertilised eggs, II = Nauplius I, III = early Nauplius II, IV = late Nauplius II, V = early copepodid and VI = late copepodid. (B) SDS-PAGE of protein stained for total protein (Lane M is the BenchMark™ Protein Ladder). Black arrows correspond to vitellogenin positions. (C) Western blot using the LsVit1 antibody. LsVit1 protein is indicated (black arrow). (D) Western blot using the LsVit2 (C-terminal) antibody. LsVit2 C-terminal protein is indicated (black arrow). (E) Western blot using the LsVit2 (N-terminal) antibody. LsVit2 N-terminal protein is indicated (black arrow)

(Fig. 7C, black arrow) and the C-terminal (Fig. 7D, black arrow) and the N-terminal part of LsVit2 (Fig. 7E, black arrow) confirmed the identity of the individual bands. The largest decrease in amounts of all 3 proteins could be seen from unfertilised eggs to newly hatched nauplii (Fig. 7B, Lanes I and II), which also represents the longest developmental time between the samples. The degradation of the N- and C-terminal LsVit2 proteins appeared to occur slightly faster than the degradation of LsVit1: by the early copepodid stage, only small amounts of the processed LsVit2 proteins could be detected, whereas a significant amount of LsVit1 was still present (Fig. 7A, Lane V). In 10 d old copepodids, the amount of LsVit2 fragments was below the detection level, and only relative small amounts of LsVit1 were left (Fig. 7A, Lane VI).

Quantification of protein and amino acids during embryogenesis and larval development

To further investigate the degradation of vitellins, total protein and the content of individual AA was quantified throughout embryonic and larval stages. At fertilisation, eggs contained a mean (±SEM) of 2.97 ± 0.21 µg total protein ind.$^{-1}$, while the FAA pool represented 0.42 ± 0.02 µg ind.$^{-1}$ (Fig. 8, top left). There was a general decline in the protein content with development, except for a slight increase from 0 to 2 dpf. At 11 dpf, the protein content in nauplii was 1.91 ± 0.29 µg ind.$^{-1}$. The total content of FAA was constant during development, and at 11 dpf there was 0.51 ± 0.03 µg ind.$^{-1}$.

The composition of the FAA pool changed dramatically with development due to large changes in the content of individual FAA during ontogeny (Fig. 8). For some AA, there was a decline in content with development (e.g. tryptophan, arginine, lysine and tyrosine), while for other AA there was an increase (e.g. proline, glycine and glutamine). A third group of AA remained more or less constant, e.g. threonine and histidine, and also for taurine there were only small changes in content with development.

There was no apparent relationship between the AA composition of the vitellogenin proteins that degraded during development and the changes that were observed in the FAA pool during the same period (data not shown). Proline and glycine, which showed the largest increase with development and represented ca. 33 and 21 mol% of the total FAA pool respectively at 11 dpf (Fig. 8), were not similarly represented in LsVit1 and LsVit2, where they only comprised approximately 6 and 5 mol% of the AA.

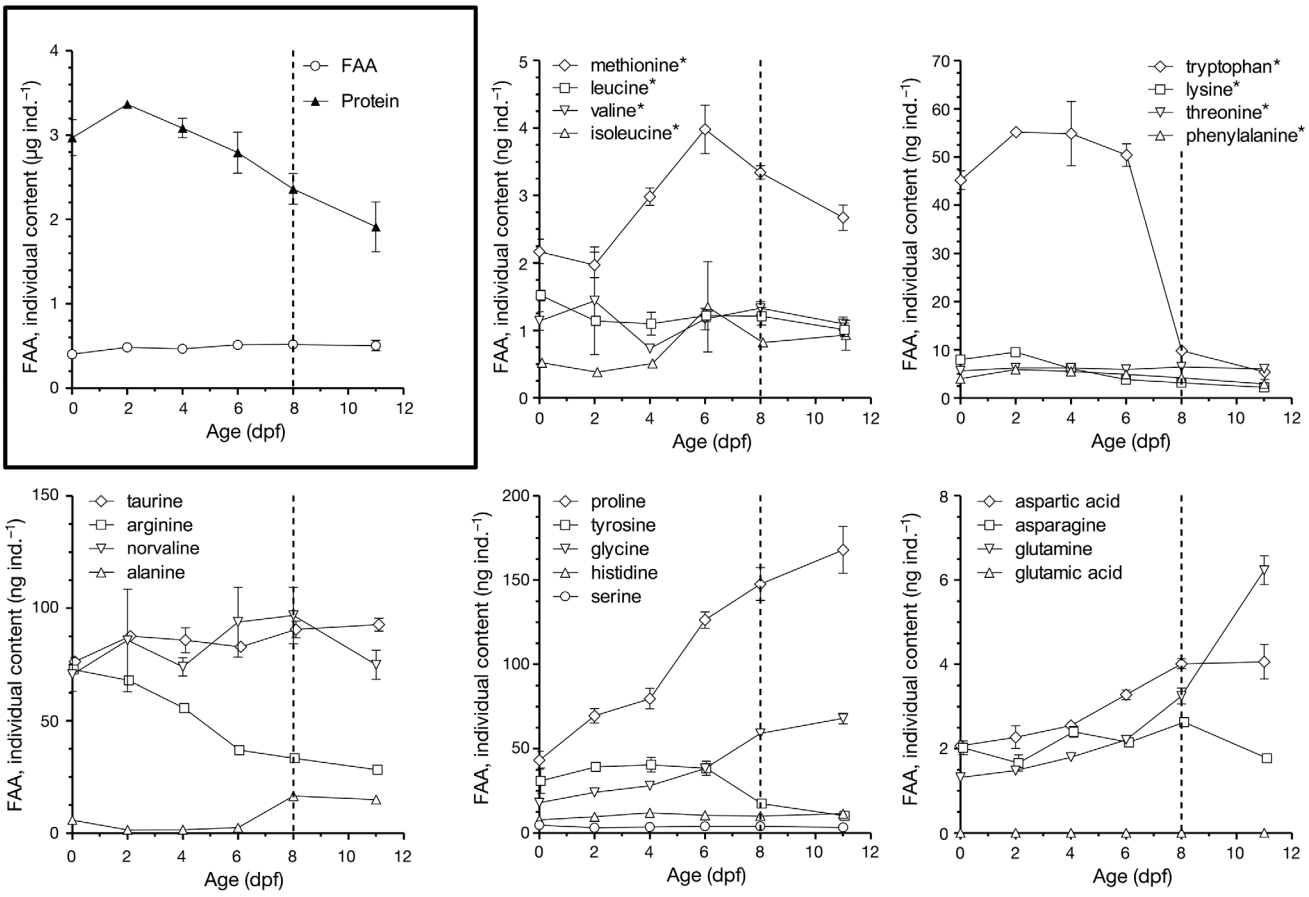


Fig. 8. *Lepeophtheirus salmonis*. Protein and free amino acids (FAA) content during endogenous feeding. Age is indicated in days post fertilisation (dpf). Vertical dashed line at 8 dpf: hatching day. Individual FAA are sorted according to whether they are essential (*) or non-essential in vertebrates. Inset (upper left): contents of total protein and sum of all individual FAA. Data are mean ± SEM (n = 4)

DISCUSSION

The present study describes 2 vitellogenins from salmon louse: LsVit1 and LsVit2. These 2 proteins constitute a major part of the protein pool present in vitellogenised salmon-louse eggs. Earlier results (Eichner et al. 2008) demonstrated that LsVit1 and LsVit2 are only transcribed in adult females. This is as expected of a vitellogenin, which is deposited in eggs to act as nutritional storage for embryos. Expression of *LsVit1* and *LsVit2* is initiated during the post-moulting growth of the genital segment, which is observed in females that have undergone the final moult to the adult stage. High expression levels are observed from the T3 stage (see Eichner et al. 2008).

Translation and subsequent sequence analysis of these 2 genes revealed identical structure of the 2 proteins and the same domain pattern as insect vitellogenins. The closest relatives to the Copepoda/Brachiopoda clade are the Hexapoda, which is well supported in the present phylogenetic analysis of the vitellogenins. Four deep branches were not well supported in any of our phylogenetic analyses ('*' in Fig. 3), and one of these branches led to the Malacostraca. Based on the present analysis using vitellogenins, there are indications that the included crustaceans are polyphyletic. In addition, the domain structure of the malacostracan and copepod vitellogenins is also different, and points to a long evolutionary distance between these groups. This is in accordance with several other studies (e.g. Aleshin et al. 2009, Mallatt et al. 2010) that have also noted the paraphyletic nature of Crustacea. However, Regier et al. (2010) obtained a monophyletic grouping of Copepoda and Malacostraca by using a large data set of 62 nuclear protein-coding genes. There could be several explanations for these differences, and the inclusion of different species and differences in molecular markers might account for a significant proportion of the discrepancy.

The identity between the 2 salmon-louse vitellogenins was 23% at the AA level; they are more similar to the corresponding vitellogenin paralogues in *Tigriopus japonicus* than to each other. This observation is supported by the phylogenetic analysis. This clearly points to an early copepod-specific gene duplication that precedes the split between *Tigriopus* (Harpactoida) and *Lepeophtheirus* (Siphonostomatoida). When sequence data was available, we included vitellogenin paralogues from other species in our analysis, and with the exception of nematodes, all paralogues seemed to be species (or genus)-specific duplications and not an early duplication event as seen in the copepods. Future studies should explore whether this early gene duplication has resulted in any functional differences in the 2 proteins.

Transcription and translation of *LsVit1* and *LsVit2* was shown to take place in the most commonly found type of cells in the subcuticular tissue. Although ovarian expression of vitellogenins has been reported in some crustaceans, we did not observe any transcription in ovaries. We have not determined the mode of transport of the vitellogenins from the subcuticular tissue to the site of incorporation into the eggs, but we did occasionally observe the presence of these proteins in the haemolymph. This observation is in agreement with previous reports from studies in insects, where vitellogenins are transported to the oocytes in haemolymph and taken up in a receptor-mediated process (Osir & Law 1986, Cho & Raikhel 2001). Our results clearly demonstrate that vitellogenesis in salmon louse takes place in the genital segment only. Clear morphological changes take places in the oocytes during transport through the oviduct (authors' pers. obs.). These vacuoles probably contain lipids and non-vitellogenin proteins. Ritchie et al. (1996) reported that salmon-louse oocytes increase in size during transport from the ovary to the genital complex, and that this increase is suggested to be due to vitellogenesis (incorporation of vitellogenin proteins into the eggs). Although we also observed a modest increase in the size of oocytes moving through the oviduct, our results indicate that the increase in size is not due to vitellogenesis.

Further studies investigating the maturation and incorporation of maternal RNAs and proteins taking place before the eggs reach the genital segment are warranted.

Post-translational processing, a feature commonly reported in vitellogenins (Sappington & Raikhel 1998), was evident in the LsVit2 protein. LsVit2 is translated as a single protein, as demonstrated by the single large protein band found in the cephalothorax samples. In samples from eggs, it is evident that processing of LsVit2 took place, resulting in 2 protein bands at 95 and 120 kDa, corresponding to the original identified Proteins D and C, respectively (Fig. 6C,D). The LsVit2 protein was purely detected as 2 smaller processed fragments whereas the larger full-length band was absent. The LsVit1 protein on the other hand appeared to be unprocessed, as we detected the approximately 220 kDa protein band in both the cephalothorax and egg samples. Western blotting for LsVit1 (Fig. 6B) in unfertilised eggs also showed reactivity towards smaller fragments. We are currently unable to determine whether these are degradation products of LsVit1 or due to binding of the antibody to other yolk proteins. The antibody was produced from a gel-purified protein band, and it is possible that trace amounts of proteins other than LsVit1 were injected into the rabbit during the immunisation protocol. Alternatively, part of the LsVit1 pool of proteins may already have been modified into shorter fragments in vitellogenised eggs.

Together with the LsYAP protein (Dalvin et al. 2009), the 2 vitellogenins LsVit1 and LsVit2 are the most abundant yolk proteins in salmon-louse ova. During development from egg to nauplii before the lice attach to their hosts, the embryo and larvae rely on endogenous reserves provided by the mother as substrates for energy and synthesis of new body tissues. Utilisation of LsVit1 and LsVit2 was monitored and vitellogenesis had taken place until the offspring had developed into copepodids, the first feeding life stage. The decrease in the protein amount was gradual with development. Breakdown of LsVit2 appeared slightly faster than LsVit1, but common to both LsVit1 and LsVit2, only a tiny fraction of the proteins was detected in the late nauplius and copepodid stage. This may in fact explain the observation in the laboratory (not shown) that copepodids die within 2 to 4 d after this time point if they have not found and attached to a host, probably due to lack of nutrition. Data on degradation of LsVit1 and LsVit2 presented here correlate well with the overall reduction in proteins during embryonic development and until late in the copepodid phase. The constant level of FAA supports the assumption that LsVit1 and LsVit2 are used in metabolism. The decline in protein content from 0 to 11 dpf (Fig. 8) thus mainly represents an energetic cost for sustaining life in the developing larval tissues. It should however be noted that the total metabolic cost most likely is higher, since neither lipids nor carbohydrates were quantified in the present study. In other crustaceans, such as European lobster *Homarus gammarus* and the prawn *Macrobrachium rosenbergii*, lipids are important energy substrates (Rosa et al. 2005, Yao et al. 2006). It is not known at present if salmon lice can actively absorb organic matter from seawater, as can many other marine invertebrates (Manahan et al. 1982), but any such uptake would also contribute to the energy budget.

The protein content determined at fertilisation represents mainly vitellogenins, since the cytoplasm is a minor compartment at that time. As the larvae develop, yolk proteins are used to synthesise body tissue proteins, and the protein content at 11 dpf thus mainly represents body protein. On a pooled basis, only a very small fraction of the protein utilised was retained in the FAA pool at 11 dpf.

The relatively constant FAA pool indicates that total FAA, including taurine and norvaline, is tightly regulated in developing louse larvae. The adult salmon louse is an osmoconforming copepod, and FAA serve important roles in cell volume regulation (Hahnenkamp & Fyhn 1985). A relatively constant total FAA content with development indicates that a similar strategy for osmoregulation is present in the early larval stages.

The results show that while the overall FAA content remains relatively stable, the composition of individual FAA changes dramatically from fertilisation to the Nauplius II stage. For example, at fertilisation, the pool contains a large fraction of tryptophan, which is steadily depleted over the hatching period (Fig. 8). Tryptophan serves as the precursor for important biomolecules such as serotonin, melatonin and niacin. The extent to which the significant decline of tryptophan is correlated with the synthesis of any of these compounds is at present unknown.

During development, there is an increase in proline and glycine in particular, and in the Nauplius II stage, the FAA pool is dominated (on a molar basis) by proline, taurine, norvaline and glycine. No comparable data for developing eggs and larvae of salmon louse exists, but in adult salmon louse kept in full-strength seawater, glycine, alanine, proline and taurine are the quantitatively most important FAA (Hahnenkamp & Fyhn 1985). The relative constant levels observed for taurine during development is also seen in fish larvae (Rønnestad & Fyhn 1993, Rønnestad et al. 1998). The presently reported FAA, including proline, glycine and taurine, belong to the same general set of AA that are involved in osmoregulation and cell volume regulation in marine invertebrates (Hahnenkamp & Fyhn 1985, Huong et al. 2001) and also in freshwater crustaceans such as the prawn *Macrobrachium rosenbergii* (Huong et al. 2001). These AA often dominate the FAA pool and have been shown to increase dramatically with an increase in salinity as part of the osmoconforming strategy (Hahnenkamp & Fyhn 1985, Huong et al. 2001). Further, these AA are typically simple, indispensable AA, and can also serve as energy substrates. Proline can be used as an energy source in insect muscles, and in the beetle *Pachylomerus femoralis*, proline is the only energy substrate used for flight (Auerswald & Gade 2000). Both proline and glycine are used for energy by marine teleost embryos (Rønnestad & Fyhn 1993). These AA therefore possibly accumulate in the FAA pool as part of a strategy to provide the free-swimming salmon-louse stages with readily available sources of energy. It is therefore most likely that the composition of the FAA pool with development in salmon louse is a trade-off between instantaneous and future demands for osmoregulation, substrates for body proteins, other biomolecules and energy, until the louse gains access to nutrients and energy from the host.

Acknowledgements. We are indebted to H. Kongshaug, P. G. Espedal, M. Sula Evjen and I. Fiksdal for technical help in the laboratory and to the editor and reviewers for valuable comments on the manuscript. This work was supported by the FUGE program of the Norwegian Research Council.

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: May 28, 2010; Accepted: January 9, 2011
Proofs received from author(s): April 25, 2011