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Molecular characterisation of the salmon louse, *Lepeophtheirus salmonis salmonis* (Krøyer, 1837), ecdysone receptor with emphasis on functional studies of female reproduction

Liv Sandlund [a,*], Frank Nilsen [a], Rune Male [b], Sindre Grotmol [a], Heidi Kongshaug [a], Sussie Dalvin [c]

[a] Sea Lice Research Centre, Department of Biology, University of Bergen, Thormøhlensgt. 55, 5008 Bergen, Norway
[b] Sea Lice Research Centre, Department of Molecular Biology, University of Bergen, Thormøhlensgt. 55, 5008 Bergen, Norway
[c] Sea Lice Research Centre, Institute of Marine Research, 5817 Bergen, Norway

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ABSTRACT

The salmon louse *Lepeophtheirus salmonis* (Copepoda, Caligidae) is an important parasite in the salmon farming industry in the Northern Hemisphere causing annual losses of hundreds of millions of dollars (US) worldwide. To facilitate development of a vaccine or other novel measures to gain control of the parasite, knowledge about molecular biological functions of *L. salmonis* is vital. In arthropods, a nuclear receptor complex consisting of the ecdysone receptor and the retinoid X receptor, ultraspiracle, are well known to be involved in a variety of both developmental and reproductive processes. To investigate the role of the ecdysone receptor in the salmon louse, we isolated and characterised cDNA with the 5′untranslated region of the predicted *L. salmonis EcR* (*LsEcR*). The *LsEcR* cDNA was 1608 bp encoding a 536 amino acid sequence that demonstrated high sequence similarities to other arthropod ecdysone receptors including *Tribolium castaneum* and *Locusta migratoria*. Moreover, in situ analysis of adult female lice revealed that the *LsEcR* transcript is localised in a wide variety of tissues such as ovaries, sub-cuticula and oocytes. Knock-down studies of *LsEcR* using RNA interference terminated egg production, indicating that the *LsEcR* plays important roles in reproduction and oocyte maturation. We believe this is the first report on the ecdysone receptor in the economically important parasite *L. salmonis*.

1. Introduction

In arthropods, steroid hormones such as 20-hydroxyecdysone (20-E) and ponasterone A (PonA) (hereafter referred to collectively as ecdysone) initiate signalling through a multitude of pathways that regulate different aspects of biological processes such as development and reproduction. The effect of ecdysone is generally mediated by binding to a nuclear receptor (NR) complex consisting of two transcription factors; the ecdysone receptor (EcR, NR1H1) and the retinoid X receptor homolog ultraspiracle (USP, NR2B) (Yao et al., 1992, 1993; Thomas et al., 1993). The ligand–receptor complex regulates the transcription of ecdysone-responsive early and early-late genes such as *E74*, *E75* and Broad Complex (*Br-C*) (Thummel and Chory, 2002; Riddiford et al., 2003) by binding to ecdysone response elements (EcREs) in the promoter region of their DNA sequence. Activation of these transcription factors further trigger the expression of ecdysone-responsive late genes, which define the phenotypic effects of the steroid hormones in a spatial and tissue-specific manner (Thummel, 2002; Qian et al., 2014).

The EcR belongs to the NR protein superfamily that is characterised by five typical NR domains (Evans, 1988; Billas et al., 2009): (i) a highly variable N-terminal (domain A/B) important in activation of transcription, (ii) a highly conserved DNA binding domain (DBD) (domain C) containing two C2C2 zinc finger motifs important in heterodimerisation and recognition of EcREs, (iii) a flexible and variable hinge region (domain D) involved in EcRE recognition and heterodimerisation, (iv) a moderately conserved ligand binding domain (LBD) (domain E) including 12 α-helices and two β-sheets making up a complex tertiary structure that is subjected to conformational changes which enable involvement in ligand binding and dimerisation with other transcription factors, and (v) a highly variable C-terminal of unknown function (F domain) (Hill et al., 2013). Different isoforms of the EcR are found in a selection of arthropods such as the marine copepod *Amphiascus tenuiremis* (Gaertner et al., 2012) and the freshwater decapod *Macrobrachium nipponense* (Shen et al., 2013) that have three and four isoforms, respectively. The temporal and spatial expression

* Corresponding author.
E-mail address: liv.sandlund@bio.uib.no (L. Sandlund).

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differ between isoforms, however the biological functions are unknown. Recent studies on the Chinese freshwater prawn, *M. nipponense*, showed that isoforms *MnEcR-S1* and *MnEcR-S2* were mainly found in testes while isoforms *L1* and *L2* were predominantly detected in the ovaries, suggesting a sex-specific expression pattern for the different isoforms. Knock-down studies of *EcR* performed in *Tribolium castaneum* resulted in impairment of ovarian growth and oocyte maturation as well as possible induction of apoptosis in the follicular cells (Parthasarathy and Palli, 2007). In addition, functional analysis performed in the fruit fly, *Drosophila melanogaster*, revealed defects in ovarian differentiation when EcR levels were reduced (Hodin and Riddiford, 1998).

The *EcR* sequence has been identified in crustacean species such as the decapods *Uca pugilator* (Hopkins et al., 2008) and *Homarus americanus* (Tarrant et al., 2011), the branchiopod *Daphnia magna* (Kato et al., 2007), the copepods *Tigriopus japonicus* (Hwang et al., 2010) and *A. tenuiremis* (Gaertner et al., 2012), and the mysids *Americamysis bahia* (Hirano et al., 2009) and *Neomysis integer* (De Wilde et al., 2013). Even though the receptor has been identified and sequenced in several crustacean species, few functional studies have been performed, leaving the action of the EcR in species other than insects poorly understood.

The endocrine system has been extensively studied in hexapods where ecdysteroids are produced and secreted from the prothoracic glands during metamorphosis (Gilbert et al., 1997) and from ovarian follicle cells after adult female eclosion (reviewed by Belles and Piulachs, 2014). In many crustaceans such as the American lobster *H. americanus*, the hormones are produced and secreted from the Y-organ (Mykles, 2011). In copepods, however, such an organ has yet to be identified which renders the origin of steroid secretion and distribution pathways, for example in the salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837), unknown. One hypothesis suggests the ecdysone steroid is secreted from one organ and transported with vitellogenins to the oocytes where it is stored for use in embryogenesis.

The salmon louse, *L. salmonis*, is a marine ecto-parasite of salmonid fishes (*Salmo* and *Onchorhyncus*) in the Northern Hemisphere (Kabata, 1979). In the salmon farming industry, the salmon louse has become an increasing problem due to the high number of hosts available, which facilitates continuous re-infestation and the spread of lice between farming sites (Heuch et al., 2005). A major concern is the development of resistance to the currently approved pesticides (Fallang et al., 2004; Espedal et al., 2013), which leads to higher consumption of these drugs followed by the spread of resistance within louse populations, thereby creating a loop of negative effects.

The EcR has long been known as a site of action for ecdysteroid agonists such as the bisacylhydrazines (BAH). Ligand binding assays using recombinant EcRs have demonstrated that these chemicals attain large variations in binding affinities between different phylogenetic groups, thus making them target-specific. Their selective specificity and their non-toxic effect on vertebrates have made these agonists important tools in integrated pest management as they have reduced the risk of affecting non-pest species and of causing negative environmental effects (Dhadialla et al., 1998; Hill et al., 2012). Understanding the EcR/USP heterodimer complex and the endocrine signalling pathways in *L. salmonis* could be of great importance for development of vaccines and/or novel medicines against this important parasite.

Here we show that the *L. salmonis EcR* (*LsEcR*) gene codes for EcR from a single exon but contains several alternative 5′ untranslated (UTR) exons that may determine in which organs of the adult female louse the gene is expressed. Moreover, in female lice gene silencing using RNA interference (RNAi) targeted to *LsEcR* gave a distinct phenotype with no production of egg strings. This suggests that signalling mediated by *LsEcR*, either directly or indirectly, plays a key role in oogenesis and that disruption of this signalling pathway may provide a means by which to control louse reproduction and, consequently, infestation.

2. Materials and methods

2.1. Animal culture and sampling

Eggs from the Atlantic salmon louse strain *Lepeophtheirus salmonis salmonis* (Skern-Mauritzen et al., 2014) were hatched and cultivated to copepodid stage in flow-through incubators before infection of Atlantic salmon *Salmo salar* (Hamre et al., 2009). Both lice and fish were kept in seawater with a salinity of 34.5‰ and a temperature of approximately 10 °C. The lice were kept on the fish until they reached the desired developmental stage. Prior to sampling, the salmon were either killed with a blow to the head or anaesthetised in a mixture of methomidate (5 mg/l) and benzocaine (60 mg/l); thereafter lice were removed with forceps. Salmon were held and treated in accordance with the Norwegian legislation for animal welfare.

2.2. Cloning and sequencing of LsEcR

For all stages of salmon lice, total RNA was isolated using TRI Reagent® (Sigma–Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. The total RNA was treated with AmpGrade DNase I (Invitrogen, Carlsbad, CA, USA) and reverse transcribed for preparation of template cDNA using SMARTscribe Reverse Transcriptase (Clontech, Takara Bio, CA, USA). 5′-RACE was performed using the SMARTer™RACE cDNA Amplification kit (Clontech, TaKaRa) with kit primers and an EcR-specific primer (*EcR*_specific_P1; Table 1), according to the manufacturer's recommendations (Sigma–Aldrich). The following PCR program was used: initial denaturation step 94 °C for 2 min and subsequent 35 cycles of amplification (94 °C, 30 s; 68 °C, 30 s; 72 °C, 2 min). The PCR products were run on a 1% agarose gel, purified using a GelElute™ Gel Extraction Kit (Sigma–Aldrich), sub-cloned using a pCR®4-TOPO® vector system (Invitrogen) and transformed into *Escherichia coli* TOP10 cells. Clones were verified by PCR with M13_f and M13_r primers (Table 1), grown overnight and purified using a Miniprep Nucleospin® Plasmid Purification Kit (Macherey–Nagel, Duren, Germany). Plasmids were sequenced using a BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems®, Foster City, CA, USA) and analysed in MacVector (MacVector Inc., North Carolina, USA).

2.3. Sequence comparison and phylogenetic analysis

To investigate the phylogenetic position of the *LsEcR* protein, homologous proteins were found by basic local alignment search tool (BLAST) searches performed in GenBank (National Center for Biotechnology Information (NCBI), Bethesda, USA). A total of 30 EcR protein sequences or EcR-like sequences from different species covering the phyla Annelida, Arthropoda, Chordata, Mollusca, Nematoda and Platyhelminthes were chosen. GenBank accession numbers of selected sequences are listed in Table 2. Multiple sequence alignment was performed using ClustalX2 (Thompson et al., 1997) with the multiple alignment parameter settings of 10 for gap opening and 0.2 for gap extension. The alignment was trimmed in MacVector by removal of parts of the highly variable 5′-A/B domain and converted to Nexus format using Mesquite (Maddison, W.P., Maddison, D.R., 2004. Mesquite: a modular system for evolutionary analysis. v2.5. http://mequiteproject.org). Phylogenetic analysis was performed using MrBayes v3.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) with the

Table 1
Primer sequences and Taqman® assays[a] used in this study.

Primer name[b]	Sequence (5′–3′)	Method
*EcR*_specific_P1	GTTGATCCCTAAGGATCGAAGCTCAGTA	5′-RACE
*EcR*_specific_P2	GAAAGTCGATAACGCAGAATACGCTCTC	
M13_f	GTAAAACGACGGCCAG	TOPO cloning
M13_r	CAGGAAACAGCTATGAC	
*LsEcR*_specific_P3	CCGATTTGCCATTACGTAGGCTTGTAGAGC	3′RACE/in situ/dsRNA
*LsEcR*_specific_P4	CCGCAGCTGCAGCCGACACAACTGTAGAT	in situ/dsRNA
*LsEcR*_specific_P5	CGAGCGTTTCCACTTACTTGCCAT	dsRNA
*LsEcR*_specific_P6	CGCCAACAACGACGACCC TCCACCAACAGCACT	dsRNA
Cod_specific_T7f	ATAGGGCGAATTGGGTACCG	dsRNA
Cod_specific_T7r	AAAGGGAACAAAAGCTGGAGC	dsRNA
LsEF1α_f	CATCGCCTGCAAGTTTAACCAAATT	RTq-PCR
LsEF1α_r	CCGGCATCACCAGACTTGA	RTq-PCR
LsEF1α_TaqMan®	ACGTACTGGTAAATCCAC	RTq-PCR
mRNA *LsEcR* total_f	TCGGGAGAAAGTCCCTCTTCT	RTq-PCR
mRNA *LsEcR* total_r	ACAGCTCCAGTAGGTGTTAAAGGA	RTq-PCR
mRNA *LsEcR* total TaqMan®	TCGCAGTCCATTCTC	RTq-PCR
mRNA *LsEcRα*_f	GTGTAGATGTGTTGTTGAAAGGGAAAAA	RTq-PCR
mRNA *LsEcRα*_r	CCTATCAATGCACCCTTTAATTTTCCAA	RTq-PCR
mRNA *LsEcRα* TaqMan®	AAACACGGCAAATATG	RTq-PCR
mRNA *LsEcRβ*_f	AACGAAACAAAAAAGACAAGTGGAATGT	RTq-PCR
mRNA *LsEcRβ*_r	TCACCCGTTGAGTGACTTCTTT	RTq-PCR
mRNA *LsEcRβ* TaqMan®	CATCTCCGCAGAACTT	RTq-PCR
mRNA *LsEcRγ*_f	CATCATCAGAGTCTCTGCAATCAAT	RTq-PCR
mRNA *LsEcRγ*_r	TTTTGGACCAATCGTTCTAGAAAACTTTTT	RTq-PCR
mRNA *LsEcRγ* TaqMan®	CCTCACCCACTTTTGC	RTq-PCR
*LsE75*_f	CCTTGACCAATTTTCAGAACGGTTT	RTq-PCR
*LsE75*_r	AATCCAGGGATCCGCTTGG	RTq-PCR
*LsE75*_TaqMan®	CACGTTCGCCAAGTTT	RTq-PCR
*LsBR-C*_f	CTCCATTGTACATAAAACAGAGTAGTGACT	RTq-PCR
*LsBR-C*_f	CAGTACCTCATCAACATCCTTTGCT	RTq-PCR
*LsBR-C*_TaqMan®	AATGCCTCGCAAATAG	RTq-PCR
*LsVit-1*_P1	ACATCGACTACAAAGGAACTCAGAAC	RTq-PCR
*LsVit-1*_P2	GGAAGCATGTAACGAATGAACTCA	RTq-PCR
*LsVit-1*_TaqMan®	AGATTTTCTTTAGCTTCTGGATACAAACCTGCTCCA	RTq-PCR
*LsVit-2*_P1	AATGAGCAATTTAGTTGAGAAAACTTGT	RTq-PCR
*LsVit-2*_P2	CAATCTCGCTTTGAGCATCACA	RTq-PCR
*LsVit-2*_TaqMan®	TGGATAAATCACGTCAAGTTACTTACCCTACCGC	RTq-PCR

RACE, rapid amplification of cDNA ends; TOPO, DNA topoisomerase I; dsRNA, double-stranded RNA; RTq-PCR, real-time quantitative PCR.
[a] Taqman® assays were provided by Applied Biosystems, Branchurg, NJ, USA.
[b] All general primers were purchased from Sigma–Aldrich, St Louis, MO, USA.

General time-reversal inverted gamma (GTR + I + G) amino acid (aa) substitution matrix. The Monte Carlo Markov Chain (MCMC) was run with two simultaneous runs and four simultaneous chains for 1,000,000 generations to approximate the posterior probability. The MCMC temperature was set to 0.5. FigTree v1.4 (A. Rambaut, 2007, http://tree.bio.ed.ac.uk/software/figtree/) was used to evaluate the consensus tree with percent posterior probability values estimated on each branch node. To root the tree, sequences from vertebrates, *S. salar*, *Xenopus tropicalis* and *Crotatus adamantus*, were used as outgroups.

2.4. Analysis of expression levels of the 5′UTR mRNA splice variants of LsEcR at different life stages using real-time quantitative PCR (RTq-PCR)

Five parallels of different life stages of the salmon louse were sampled prior to ontogenetic analysis; nauplia I/II ($n \approx 150$), free-living copepodids ($n \approx 150$), parasitic copepodids ($n = 10$), chalimus I ($n = 10$), chalimus II ($n = 10$), pre-adult male I/II ($n = 1$), pre-adult female I/II ($n = 1$), adult male ($n = 1$), immature adult female lice ($n = 1$) and gravid female lice ($n = 1$), and stored on RNAlater™ (Ambion Inc., Austin, TX, USA). Total RNA was isolated using TRI Reagent® (Sigma–Aldrich) according to the manufacturer's protocol. Concentration and purity of RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Thermo Fisher Scientific, Wilmington, DE, USA). RNA quantity and quality was checked by standard O.D. 260/280 and O.D. 260/230. The normalised stocks (500 ng/μl) were treated with DNase I (Amplification Grade, Invitrogen). Two parallel cDNA synthesis reactions were set up using an AffinityScript cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) to a final concentration of 10 ng/μl. PCR was performed using 2.5 μg of cDNA, 5 μM *LsEcR*-specific TaqMan® probe (Table 1) and 2× TaqMan® Universal PCR mix (Applied Biosystems®) in a total volume of 10 μl. The RTq-PCR of the mRNA *LsEcR* variants was carried out independently but simultaneously with the housekeeping gene, elongation factor 1 alpha (*EF1α*; Frost and Nilsen, 2003) as the reference. RTq-PCR was performed with parallel series of each sample. Standard curves (cycle at threshold (C_t) versus log quantity), slope evaluation and transcription levels of the mRNA *LsEcR* variants were compared with *EF1α* using the *Applied Biosystems 7500* Real-Time PCR System (Applied Biosystems®). Results were analysed by the $2^{\wedge -\Delta\Delta Ct}$ approach and presented with the 95% confidence interval calculated from the $2^{\wedge -\Delta\Delta Ct}$ values.

2.5. Localisation of LsEcR transcript

Localisation of *LsEcR* mRNA in adult female lice was accomplished using in situ hybridisation carried out according to Kvamme et al. (2004) with some modifications. PCR product with T7 promoters generated from *LsEcR*-specific cDNA was used as a template for a single stranded digoxigenin (DIG)-labelled RNA probe (667 bp) synthesis (Table 1, primers: *LsEcR*_specific_f, *LsEcR*_specific_r). Probe concentration and quality was determined

Table 2
List of amino acid (aa) sequences from all species used to determine the phylogenetic relationship of *Lepeophtheirus salmonis* ecdysone receptor (*Ls*EcR).

Classification	Species	EMBL Accession No.	Product size (aa)
Annelida	*Platynereis dumerilii*	ACC94156	496
Chelicerata	*Liocheles australasia* (Australian rainforest scorpion)	AB297929	539
	Agelena silvatica	GQ281317	533
	Ornithodoros moubata	AB191193	567
	Amblyomma americanum (Lone star tick) isoform1	AF020187	560
Crustacea	*L. salmonis*	KP100057	536
	Tigriopus japonicus	ADD82902.1	546
	Penacus japonicus	AB295492	499
	Uca pugilator (Sand fiddler crab)	AF034086	518
	Amphiascus tenuiremis	JF926564	458
	Homarus americanus (American lobster)	HQ335007	541
	Daphnia magna (Water flee) isoform1	AB274821	693
	Portunus trituberculatus (Gazami crab)	JQ250795	503
Hexapoda	*Locusta migratoria* (Migratory locust)	AF049136	541
	Gryllus firmus (Sand cricket)	GU289704	416
	Apis mellifera (Honey bee)	AB267886	567
	Drosophila melanogaster (Fruit fly) isoformB1	NP_724460	878
	Tribolium castaneum (Flour beetle) isoformA	CM000284	549
	Aedes aegyptii (Yellow fever mosquito)	AY345989	776
	Diploptera punctata isoformA	JQ229679	538
Mollusca	*Crassostrea gigas* (Pacific oyster)	EKC19773.1	471
	Lymnaea stagnalis (Great pond snail)	ADF43963.1	478
Nematoda	*Caenorhabditis elegans*	NP_492615.2	373
	Trichinella spiralis	XP_003376657.1	573
	Ascaris suum	ADY42534.1	496
Plathyhelminths	*Schmidtea mediterranea*	AFF18489	655
	Schistosoma mansoni	ARR29357.1	715
Vertebrata	*Salmo salar* (Atlantic salmon)	FJ470290	462
	Xenopus tropicalis	NP_001072853.1	441
	Crotatus adamantus (Pit viper)	AFJ50856.1	435

by spectrometry (Nanodrop ND-1000) and a spot test, respectively. Briefly, paraffin sections were baked at 60 °C for a minimum of 20 min and treated with Histoclear (National Diagnostics, Atlanta, GA, USA) prior to rehydration of tissue and proteinase K treatment for 10 min, followed by tissue fixation in 4% formaldehyde in PBS, acetic anhydride treatment and dehydration. Hybridisation mix (100 µl) containing 20 ng of DIG-labelled RNA was added to the tissue and left overnight in a vacuum chamber at 60 °C. DIG-labelled probes were visualised using secondary antibody labelled with an anti-DIG alkaline phosphatase-conjugated FAB fragment and a chromogen substrate containing nitroblue tetrazolium (NBT) (Roche Diagnostics GMbH, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics). Sense RNA was used as a negative control.

2.6. LsEcR knock-down using RNAi

Two primer pairs with and without a 5′ T7 promoter extension were used to generate PCR products of the *LsEcR* open reading frame (ORF). Fragment 1 (667 bp; Table 1, primers: *LsEcR*_specific_P3 and *LsEcR*_specific_P4) and fragment 2 (815 bp; Table 1, primers *LsEcR*_specific_P5 and *LsEcR*_specific_P6) localised to the hinge and A/B region, respectively. An Atlantic cod (*Gadus morhua*) gene fragment, CPY185 (850 bp), was used as a control (Table 1, primers: Cod_specific_T7f and Cod_specific_T7r). The PCR products were used as templates with T7 RNA polymerase to synthesise dsRNA fragments as described by the MEGAscript® RNAi Kit (Ambion Inc.). The concentrations of sense and anti-sense strands were measured by spectrometry (NanoDrop Technologies Inc.) before equimolar amounts of each strand were pooled to generate dsRNA. A solution containing 50 µl of dsRNA was added to 5 µl of saturated Trypan blue to the final concentration of 600 ng/µl of dsRNA. Pre-adult female and male lice were collected with forceps from anesthetised salmon. Pre-adult II female lice were then injected with 1 µl of the dsRNA solution in the cephalothorax using custom-made injection needles. These were pulled by utilising a 1 mm Borosilicate glass tube with an inner diameter of 0.5 mm (Sutter Instrument, Novato, CA, USA) on the P-2000 laser-based micropipette puller system (Sutter Instrument). Needles were ground and opened using a Micropipette Grinder EG-44 (Tritech Research, Los Angeles, CA, USA), and coupled to a microinjector before use. By blowing air into the needle, the dsRNA fragments were dispersed in the louse, visualised by dispersion of blue colour within the cephalothorax. After injection, the lice were kept in seawater for 6 h before they were placed on anesthetised fish together with male lice, in a 1:1 ratio (female $n = 13$). Three parallel experiments were set up for each gene. Lice were kept on one salmon, each in single fish tanks (50 L) with seawater for either 2, 4 or 12 days, or until the female adults had produced a second set of egg strings (approximately 38 days), when the remaining lice were removed from the fish. Lice were harvested at different time points in order to detect any reduction in mRNA levels and to study the function in sexually mature lice. Egg strings, when present, were collected and placed in individual incubators for hatching. Live lice were transferred and stored on either RNAlater™ (Ambion Inc.) for RTq-PCR, fixed in phosphate buffered 4% formaldehyde at 4 °C overnight for in situ hybridisation or fixed for light microscopy (see Section 2.7). To confirm hatching, egg strings were observed daily. Phenotypes were evaluated throughout nauplia and the copepodid stages. The number of recovered lice from each experiment is listed in Table 3.

2.7. Histology

Specimens for light microscopy were fixed by immersion in a mixture of 10 ml of 10% formaldehyde (fresh from paraformaldehyde), 10 ml of 25% glutaraldehyde, 20 ml of 0.2 M cacodylate buffer and 60 ml of PBS, and the pH was adjusted to 7.35. Thereaf-

Table 3
Summary of recovered lice and phenotypic traits observed using RNA interference experiments.

	Recovered female lice	Blood in intestine	Lice producing egg strings	RTq-PCR[d]
Control: Fragment 1: 2 days[a]	10	Not registered	–	9
dsRNA: Fragment 1: 2 days[a]	10	Not registered	–	6
Control: Fragment 1: 4 days[a]	7	Not registered	–	6
dsRNA: Fragment 1: 4 days[a]	8	Not registered	–	7
Control: Fragment 1: 12 days[a]	13	11	–	10
dsRNA: Fragment 1: 12 days[a]	19	6	–	17
Control: Fragment 1: 38 days	23	23	23	10
dsRNA: Fragment 1: 38 days	14	10 (7[b])	1[c]	13
Control: Fragment 2: 38 days	16	16	16	10
dsRNA: Fragment 2: 38 days	16	5 (3[b])	0	11

dsRNA, double-stranded RNA.
[a] Lice had not reached mature adult stage, hence no egg string production.
[b] Barely visible blood in intestine.
[c] Egg strings did not hatch.
[d] Number of lice submitted to real-time quantitative PCR (RTq-PCR).

ter specimens were rinsed in PBS and dehydrated in a series of ethanol solutions (50%, 70% and 96%), before being embedded in Technovit 7100 (Heraeus Kulzer GmbH & Co, Germany). Sections (1–2 μm) were stained with Toluidine blue.

Digital micrographs were acquired with a ColorView III camera (Soft Imaging System GmbH, Münster, Germany) mounted on an Olympus BX61 Microscope (Olympus, Tokyo, Japan), and processed using Adobe Photoshop CS6 (Adobe Systems, San Jose, California, USA).

2.8. Detection of transcript levels in dsRN- treated lice by RTq-PCR

RTq-PCRs using TaqMan® probes (Table 1) were used to detect total expression of *LsEcR*, Vitellogenin-1 (*LsVit-1*), Vitellogenin-2 (*LsVit-2*), ecdysone induced protein 75 (*LsE75*) and Broad-Complex (*LsBr-C*) from dsRNA-treated lice harvested from the RNAi experiments (samples listed in Table 3). Total RNA was isolated and samples prepared as described in Section 2.4. Two micrograms of cDNA from RNAi lice were added to the RTq-PCR mix (Applied Biosystems®) to a total volume of 10 μl. Each louse was analysed separately as described in Section 2.4. The number of lice analysed from each RNAi experiment is listed in Table 3.

2.9. Statistical analysis

From the RNAi experiments, significant differences between the control groups and the treated groups were determined using the Kolmogrov–Smirnov test (non-parametric, un-paired: compared cumulative distributions) by employing Prism6 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical evaluation of the mRNA *LsEcR* splice variant at specific life stages was performed by two-way ANOVA analysis utilising SPSS software V. 21 (IBM® SPSS® Statistics, Armonk, NY, USA).

3. Results

3.1. Sequence analysis and molecular phylogeny of the LsEcR

In order to obtain full-length *LsEcR* cDNA, 5′ and 3′ Rapid amplification of cDNA ends (RACE) PCR was run using EcR-specific primers (Table 1, *EcR* specific_P1 and *EcR* specific_P3) based on expressed sequence tag (EST) sequences. A 2932 bp cDNA was retrieved with a 5′ UTR of 1044 bp, a 280 bp 3′ UTR and a 1608 bp ORF consisting of one exon, encoding 536 aa. The predicted molecular weight was 60.4 kDa (Expasy, ProtParam Tool, http://web.expasy.org/protparam/). Cloning and sequencing of the RACE products revealed the existence of three mRNA variants, *LsEcRa*, *LsEcRb* and *LsEcRc*, differing in their 5′UTR (Fig. 1). A BLAST search revealed the deduced protein sequence encodes the EcR of *L. salmonis* and exhibits 61% identity to the full-length aa sequence and 82% and 77% for the DBD and LBD, respectively, with the copepod *T. japonicus* (ADD82902.1). The deduced aa sequence of LsEcR contained domains characteristic of nuclear receptors, namely an A/B domain associated with transcriptional activation, DBD (C-domain, aa 170–261), a hinge region (D-domain) and a LBD (E/F-domain, aa 303–535) containing a short aa sequence (ATGMRA) recognised as the activation factor-2 domain (AF-2; aa 235–240). Alignments of the domains to conserved domains in NCBI (Marchler-Bauer et al., 2011) proved the retrieved cDNA sequence form *L. salmonis* encodes the nuclear receptor LsEcR.

Phylogenetic analysis of the aa sequence of LsEcR was performed by conducting a Bayesian analysis of a full-length aa alignment of EcR and EcR-like receptors from a variety of species (listed in Table 2). From the rooted bootstrap tree (Fig. 2), LsEcR grouped together with the copepods *T. japonicus* and *A. tenuiremis* and was separated from the decapods. The water flea *D. magna* (Branchiopoda) EcR form a separate clade and is the closest sister group to the copepods, followed by Hexapoda and Chelicerata.

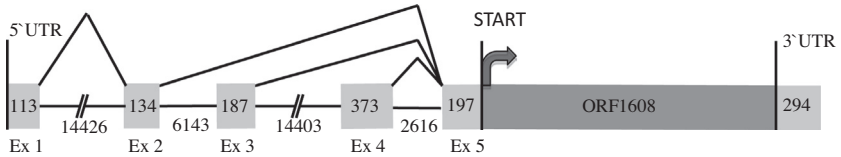

Fig. 1. Schematic representation of the genomic sequence of the *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*). Cloning and sequencing revealed the presence of three different mRNA variations. The exons (Ex; depicted in light grey boxes) were mapped to the genomic DNA and show the gene to extend over 38.5 kbp. The open reading frame (ORF) is depicted in dark grey and the translation start site is marked with an arrow. Introns are depicted as lines between exons with lengths in numbers of nucleotides. The three mRNA variants are represented with connecting lines; mRNA *LsEcRa* consists of exons 1, 2 and 5; mRNA *LsEcRb* of exons 3 and 5 and mRNA *LsEcRc* of exons 4 and 5. All mRNA variations share a common exon (Ex 5) linked directly to the coding sequence consisting of only one exon and no introns.

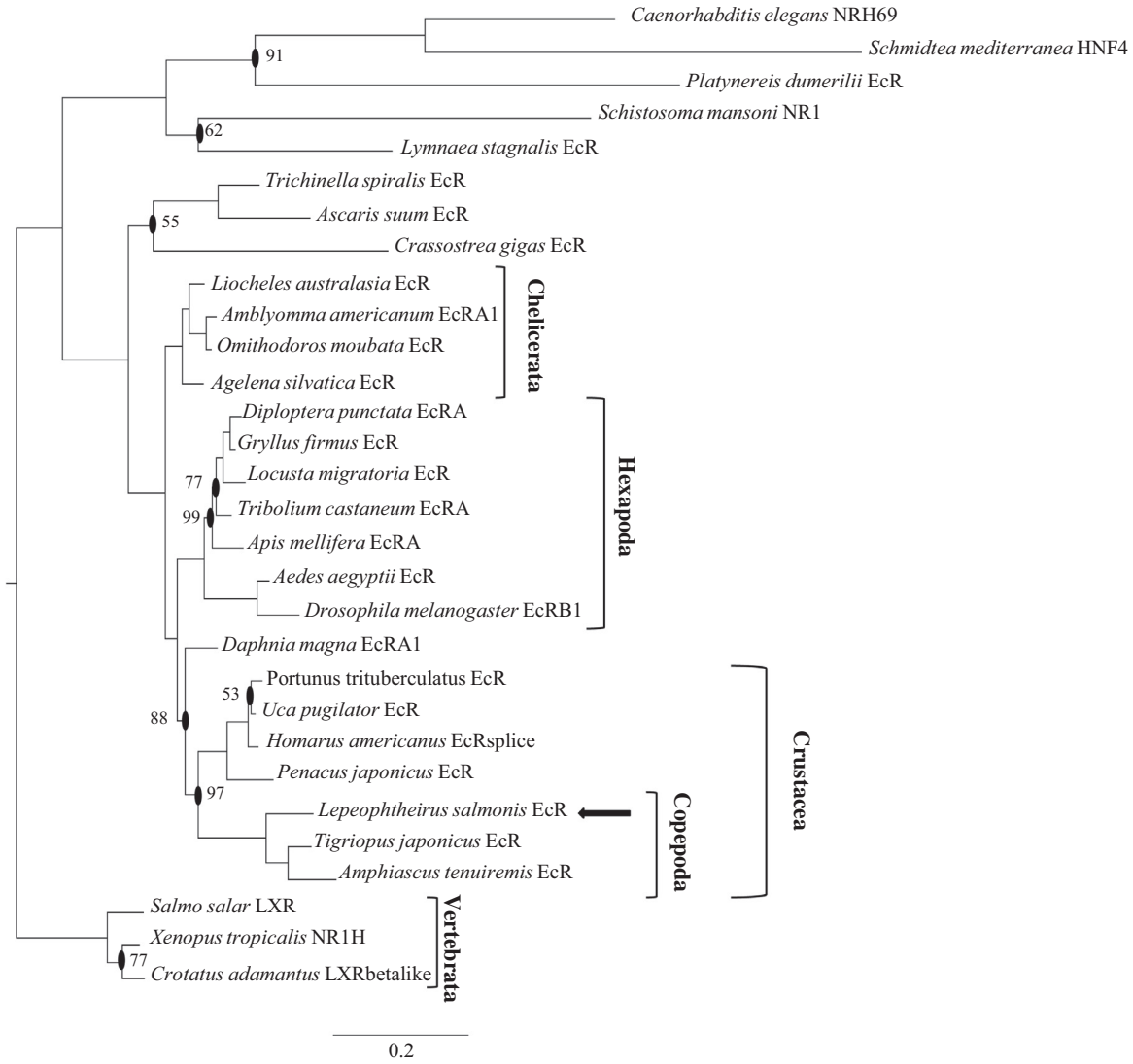

Fig. 2. Phylogenetic analysis of *Lepeophtheirus salmonis* ecdysone receptor (LsEcR). A rooted phylogenetic tree of amino acid sequences of full-length EcR and EcR-like receptors from different species was generated using Bayesian methods. Branch length is proportional to sequence divergence. Branch numbers and bars represent bootstrap values in percent and 0.2 substitutions per site, respectively. LsEcR is marked with an arrow. *Salmo salar* LXR, *Xenopus tropicalis* NR1H and *Crotatus adamantus* LXRbetalike receptors were used as an outgroup. The GenBank accession numbers of all EcR sequences used are listed in Table. 2.

3.2. Expression pattern analysis of 5′UTR mRNA splice variants of LsEcR

The expression pattern of the different mRNA splice variants of *LsEcR* was evaluated in different developmental stages in *L. salmonis*. Thus, ontogenetic analysis was performed using RTq-PCR on RNA extracted from nauplia I/II, free-living copepodids, parasitic copepodids, chalimus I, chalimus II, pre-adult male I/II, pre-adult female I/II, adult male and immature adult female lice, and gravid female lice. Specific Taqman® assays (Table 1) were designed to discriminate between the three 5′UTR mRNA splice variants (Fig. 3). In general, the highest relative expression was detected in the nauplia I/II and free-living copepodids for all three splice variants with *LsEcRa* and *LsEcRc* significantly more highly expressed compared with *LsEcRb*. The expression pattern decreased from copepodid to the chalimi stages before an increased expression occurred in the pre-adult and adult stages, with the relative expression of *LsEcRa* being significantly higher compared with *LsEcRb* and *LsEcRc*. The expression of *LsEcRa* was significantly higher in immature and gravid adult female lice compared with pre-adult female/male and adult male lice.

3.3. LsEcR transcript is expressed in a variety of tissues

In situ hybridisation analysis performed on paraffin sections of an adult female louse demonstrated that *LsEcR* transcript was present in most tissues except for the muscle tissue (Fig. 4B–E). Expression was observed in the ovaries, immature/mature eggs present in the genital segment (Fig. 4B), different glandular tissues of unknown function present in the legs (Fig. 4C) and the anterior part of the cephalothorax, intestine (Fig. 4D) and in the subcuticular tissue (Fig. 4E). Unspecific colouring of the outer cuticular tissue was observed both for the sense and anti-sense probes.

3.4. Down-regulation of LsEcR by RNAi inhibits the production of offspring

Functional studies using RNAi were performed to assess the effect of LsEcR in reproduction of the salmon louse. First, an experiment was set up using fragment 1 (Table 1) to determine the degree of down-regulation in *L. salmonis*. In total, 39 pre-adult II female lice were injected with a ds*LsEcR* fragment and 39 were injected with dsRNA from a cod and left on the fish for 2, 4 and

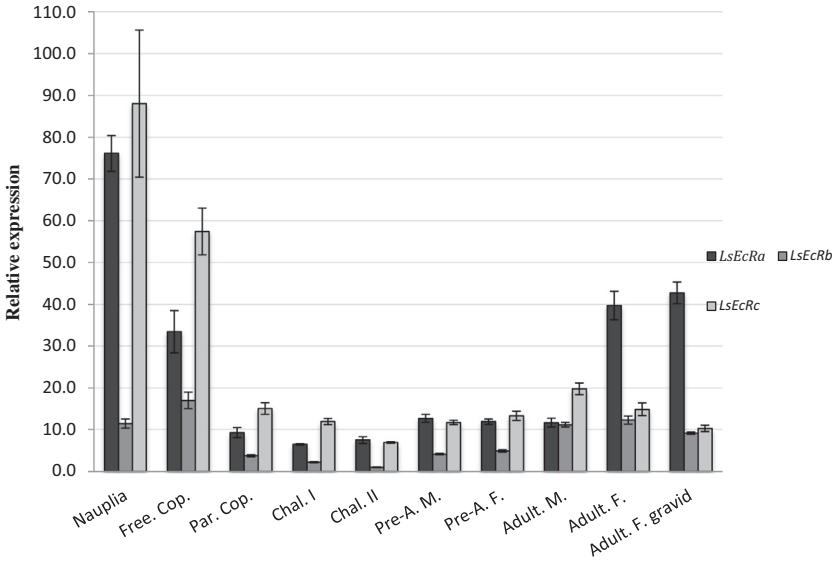

Fig. 3. Quantitative real-time PCR (RTq-PCR) analysis of relative expression of the three mRNA *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) variants (a, b, c) in different developmental stages. Each point represents the mean and confidence intervals (n = 5 parallels of approximately 150 animals for the nauplia and free-living copepodid (Free. Cop.) stages, 10 animals for the parasitic copepodid (Par. Cop.), chalimus I (Chal. I) and chalimus II (Chal. II.) and one animal for each of the pre-adult male (Pre-A. M.), pre-adult female (Pre-A. F.), adult male (Adult M.), adult female (Adult F.) and gravid adult female (Adult F. gravid)) stages. The relative expression of *LsEcRb* at the chalimus II stage was set to 1.

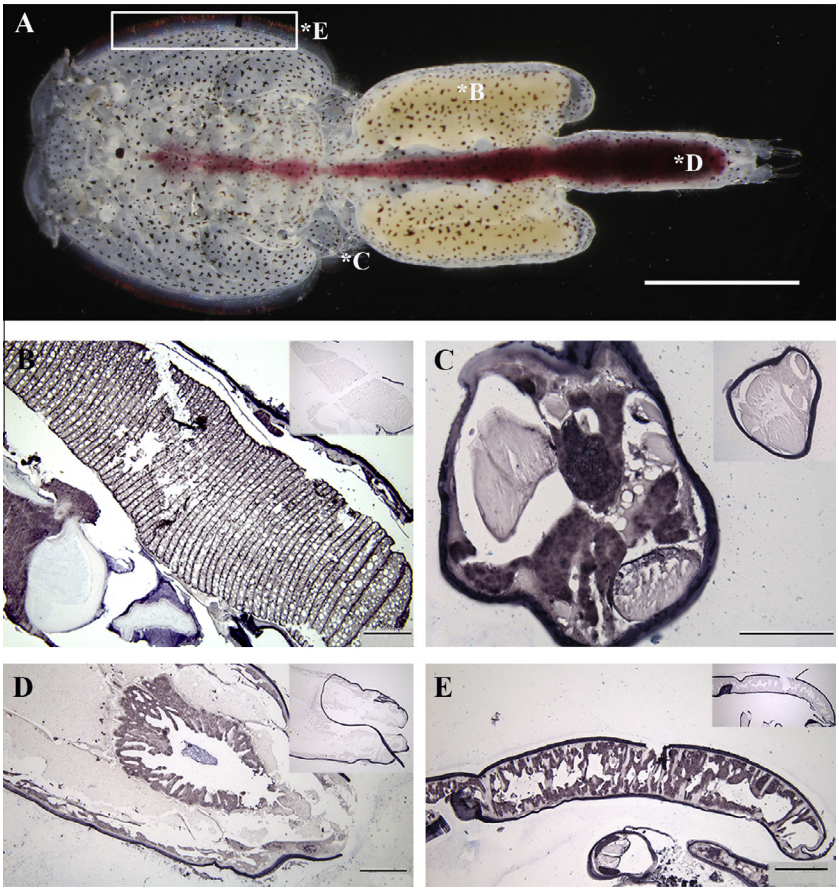

Fig. 4. Localisation of *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) transcripts in an adult female louse. (A) Light microscope image of gravid adult female louse. Letters and asterisks are guides to the corresponding photos of individual tissues. A part of the sub-cuticular tissue is framed to better visualise localisation. (B–E) In situ hybridisation using *LsEcR*-specific anti-sense RNA was used for detection of transcript. Negative controls (sense RNA) are shown (insets). Positive staining was seen in mature eggs (B), unidentified glandular and surrounding tissue in the legs (C), intestine (D) and sub-cuticular tissue (E). Unspecific colouring of the outer cuticular layer was seen using both sense and anti-sense probes. Scale bar = 5 mm (A); 200 μM (B, D, E) and 100 μM (C).

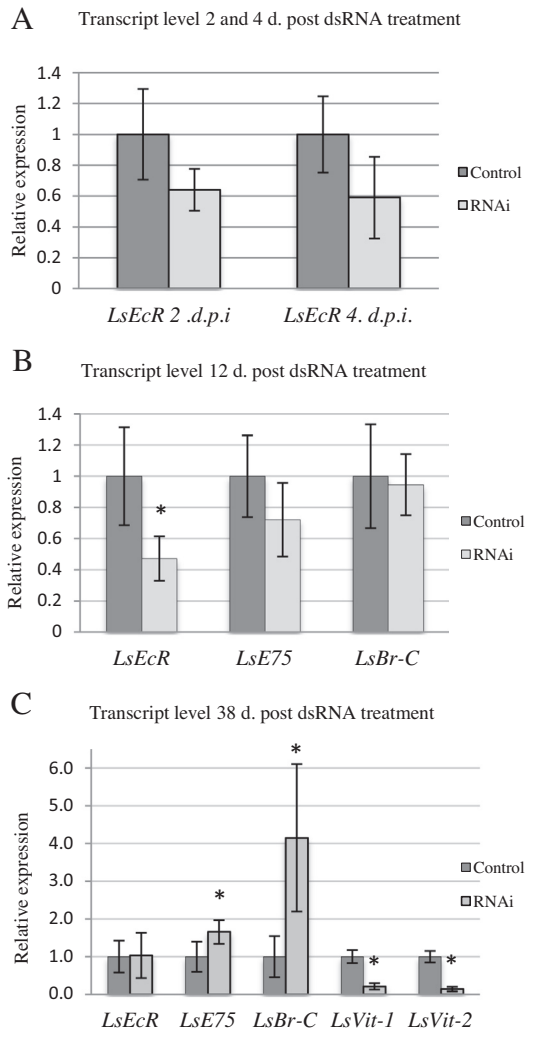

Fig. 5. Transcript level of selected ecdysone receptor (EcR) target genes after injection of double-stranded (ds)RNA. *Lepeophtheirus salmonis EcR* (*LsEcR*) adult female lice were removed from anesthetised fish and analysed after (A) 2, 4, (B) 12 or (C) 38 days (d.) post treatment (i.e. dsEcR injection; d.p.i.). Quantitative real-time PCR (RTq-PCR) analysis of the relative expression of *LsEcR* and selected downstream genes *LsE75* and *LsBroad-complex* (*LsBr-C*) (B, C) *LsVitellogenin-1* (*LsVit-1*) and *LsVitellogenin-2* (*LsVit-2*) (C) was evaluated. (C) The graph is representative of two experiments. The expression levels of the respective genes in the control groups were set to 1. Mean ± confidence interval of treated lice is shown. Each louse was analysed separately and confidence intervals represent individual differences. Numbers of lice analysed are listed in Table. 3. *Statistically significant ($P < 0.05$). Statistical analysis was performed using a Kolmogorov–Smirnov test.

12 days. No phenotype or reduced survival rate was observed for the immature female animals compared with the control group; however, RTq-PCR showed that the *LsEcR*-gene was significantly knocked down (by 53% at day 12; Fig. 5B). At days 2 and 4, however, no significant knock-down was observed (Fig. 5A). A second experiment was set up using two different fragments in order to exclude any non-target effects. Fragments 1 and 2 were injected and run in two separate experiments for 38 days under the same criteria as the first experiment. RTq-PCR analysis of the second experiments terminated at 38 days and did not detect any significant regulation of the *LsEcR* (Fig. 5C). However, at 38 days the *dsLsEcR*-treated lice showed a characteristic phenotype where no production of offspring was observed. Female lice injected with dsRNA from cod had no phenotype and produced viable offspring (Fig. 6A, D). We also observed that ds*LsEcR*-treated lice were found with less blood in the intestine (Table 3 and Fig. 6D), which deviates from what is observed to be normal in adult female lice in our laboratory system, where most females have a blood-filled gut (Table 3 and Fig. 6A). Histological sections from ds*LsEcR*-treated lice revealed that the oocytes did not display a normal stacking pattern like the control lice and an individual ova could not be detected (Fig. 6B, E). The lining of the developing oocytes was disintegrated, leaving the area filled with a mesh of fat and yolk granules. The cellular structure of the sub-cuticular tissue was observed to be hypotrophic compared with the control lice (Fig. 6C, F) giving an impression of a reduction in tissue.

The binding of ecdysone to the EcR/USP complex is known to regulate several down-stream genes. The expression level of the known down-stream genes *LsE75* and *LsBr-C* was evaluated in lice after dsRNA from LsEcR was injected. No significant regulation was detected for *LsE75* or *LsBr-C* from the *dsLsEcR*-treated lice after 12 days (Fig. 5B). In contrast, both *LsE75* and *LsBr*-C were up-regulated in *dsLsEcR*-treated lice after 38 days (Fig. 5C) (Kolmogorov–Smirnov, $P < 0.05$). RTq-PCR analysis was also conducted on *LsVit-1* and *LsVit-2* from lice treated for 38 days and both genes were significantly knocked down (Fig. 5C) (Kolmogorov–Smirnov, $P < 0.05$). The expression of the two vitellogenins was only evaluated in lice treated for 38 days as *LsVit-1* and *LsVit-2* are only expressed in mature female lice (Eichner et al., 2008).

4. Discussion

In the present study, we isolated a cDNA for the EcR in *L. salmonis*. The genetic composition of *LsEcR* proved to be similar to the *EcR* gene found in *T. japonicus* (Hwang et al., 2010) with only one exon spanning the ORF and with introns only detected in the UTRs. Putative full-length protein sequence alignment (Table 1) and phylogenetic analysis (Fig. 2) cluster the *LsEcR* together with the copepods *T. japonicus* and *A. tenuriemis* in the Malacostraca group, with the water flee *D. magna* (Branchiopoda) as the closest sister group. Identical aa found in the LBD of EcRs between species are consistent with the widespread use of ecdysone as the hormone initiating developmental processes. Identity searches and determination of phylogenetic position of the retrieved *L. salmonis* cDNA sequence classify it as an ecdysone receptor.

The 5′UTR region of the retrieved cDNA revealed the existence of three *LsEcR* mRNA splice variants, all starting from different exons. This suggests that those are regulated by different promoter regions. Selective promoter regions are well known from steroid hormone receptors such as the human oestrogen receptor (ER) (Kwak et al., 1993; Bockmuhl et al., 2011) and have been shown to possess different tissue specificity and to be activated by different signals (Ayoubi and VanDeVen, 1996). The mechanisms involved in 5′UTR mediated regulation is poorly understood and has to our knowledge not been studied in crustaceans. It is possible that the *LsEcR* mRNA splice variants are expressed in different tissues or regulated by specific signals in the salmon louse. However, further studies are required in order to understand how the different *LsEcR* mRNA splice variants are regulated.

The existence of multiple EcR isoforms that differ in their spatial and temporal expression are common in many crustacean species (Durica et al., 1999; Tarrant et al., 2011; Verhaegen et al., 2011). Expression profiling using RTq-PCR, performed on embryos and adults of the water flea (*D. magna*), revealed that the EcRB isoform was expressed at a higher level during embryogenesis compared with EcRA, while the opposite expression pattern was observed in adult fleas during molting (Kato et al., 2007). In the salmon louse, of the three variants of *LsEcR* mRNA transcripts present, the *LsEcRb* variant had relatively low expression throughout all life stages compared with *LsEcRa* and *LsEcRc* that were observed to have the highest relative expression in the nauplia I/II and the

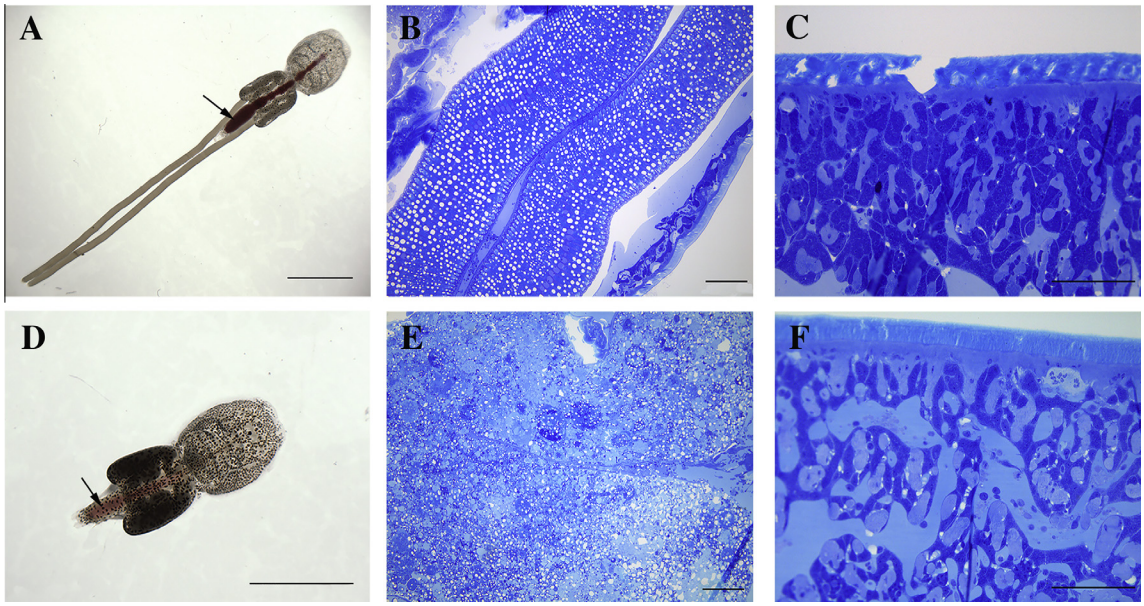

Fig. 6. Functional assessment of the *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) by RNA interference (RNAi). The control lice produced normal egg strings (A) that hatched and produced viable offspring. *LsEcR* dsRNA-treated lice (D) showed a distinct phenotype with no production of eggs. It was also observed that the *LsEcR* dsRNA-treated lice attained a thicker genital segment as well as less blood in the intestine (marked with arrow, D), compared with the control (marked with arrow, A). (B and C, E and F) Toluidine stained sections showed the normal stacking pattern of the eggs seen in the control (B) which was lost in the dsRNA *LsEcR*-treated lice (E). The sub-cuticular tissue was hypotrophic in the *LsEcR* dsRNA-treated lice (F) compared with the control lice (C). Scale bar = 5 mm (A, D), 200 μM (B, E) and 1000 μM (C, F).

free-living copepodid stage (Fig. 3). A similar expression pattern was observed for the *EcR* in the free-living copepod *T. japonicus* (Hwang et al., 2010). A second peak in expression was observed for *LsEcRa* in immature (T1) and mature females (T6) (classification of maturing female louse after Eichner et al. (2008)) which could indicate that the *LsEcRa* transcript is used more predominantly in female maturation and reproduction. Overall, the differential expression of the three *LsEcR* mRNAs could suggest that those play different roles in different biological processes.

To investigate the spatial distribution of *LsEcR* transcript in the adult female louse, we performed in situ hybridisation. With our protocol, the presence of *LsEcR* transcript was, with the exception of muscle tissue, evident in most tissues such as glandular and sub-cuticular tissues and oocytes (Fig. 4A–E). The wide distribution of *EcR* transcripts has similarly been demonstrated in the kuruma prawn *Marsupenaeus japonicus* (*Mj*) and the soft tick *Ornithodoros moubata* (*Om*) using RTq-PCR and RT-PCR, respectively (Asazuma et al., 2007). In contrast to our results, *MjEcR* and *OmEcR* were also detected, in low quantities, in muscle tissue (Asazuma et al., 2007), however this may be explained by the difference in sensitivity between the methods.

From our knock-down studies of *LsEcR* in reproducing female lice, it has become apparent that the nuclear receptor either directly or indirectly affects a variety of biological processes. In the salmon louse, the sub-cuticular region has been demonstrated to be an active tissue with functions similar to the liver (Edvardsen et al., in press). Yolk proteins such as the vitellogenins are produced in the sub-cuticular tissue before they are incorporated into the oocyte during oocyte maturation (Dalvin et al., 2009, 2011). The reduction of vitellogenin 1 and 2 transcripts observed in *dsLsEcR* knock-down lice (Fig. 5C) could most likely be explained by the major changes occurring in the sub-cuticular tissue (Fig. 6F). At the same time, when depriving the female lice of *LsEcR*, reproduction was halted and eggs failed to mature in the genital segment. Similar observations were reported from *EcR* knock-down studies of *T. castaneum*, where a 50–75% reduction in the vitellogenin transcript level resulted in a decrease in egg development (Xu et al., 2010). Moreover, development of a follicular cell layer necessary for oocyte maturation was disrupted, resulting in an arrest of the oocyte in the pre-vitellogenic stage (Parthasarathy et al., 2010). The same observations had previously been recorded in *D. melanogaster* where *EcR* deficiency resulted in abnormal egg chamber development and loss of vitellogenic stages (Carney and Bender, 2000). Loss of egg production in *L. salmonis* is presumably not a function of reduced yolk production, but either a direct or indirect of effect of *LsEcR* depletion in the oocytes.

In insects, eggs mature in the ovaries to gametes that contain all the proteins and maternal mRNA needed to initiate and maintain metabolism and development of the eggs before fertilisation. From work performed in *D. melanogaster* and *T. castaneum*, it was shown that components of the ecdysone hierarchy such as EcR were expressed and required in germline cells for progression through oogenesis (Buszczak et al., 1999; Carney and Bender, 2000; Freeman et al., 1999). The observation of *LsEcR* transcript in the oocyte implies the presence of maternal transcript in the eggs. The absence of normal egg development in *dsLsEcR*-treated lice provides a good indication that the Ec-EcR pathway plays an important role in reproduction and development of offspring in the salmon louse. The specific mechanism for loss of egg development is currently unknown and further studies are necessary to understand the complexity of the Ec-EcR hormonal pathway.

RNAi is a well established genetic tool for functional studies in different organisms. However, with the exception of plants and the nematode *Caenorhabditis elegans*, little is known about the systemic RNAi response mechanisms in non-traditional model organisms (Miller et al., 2012). In the *dsLsEcR*-treated lice, significant down-regulation of *LsEcR* was not observed until 12 days after injection. Our results deviate from knock-down studies performed on the putative prostaglandin E synthase 2 (*LsPGES2*) in *L. salmonis*, where reduction in the transcript level was most prominent after 48–72 h (Campbell et al., 2009). However, it should be noted that the optimal requirements for knock-down differ among genes depending their locations and turnover rates. After 38 days, the RNAi effect had ceased but a distinct phenotype, only observed

after the prolonged period of *LsEcR* knock-down in adult females, was evident. At the same time, the *LsEcR* response genes *LsE75* and *LsBr-C* were significantly up-regulated in the *dsLsEcR*-treated lice compared with the control lice. The increased expression of the response genes could be a secondary response as a cause of *LsEcR* depletion and disruption of several biological processes. However, as these genes naturally have very irregular expression patterns, further studies are necessary in order to determine the relation between ecdysone pathway and the response genes in *L. salmonis*.

In summary, we report the identification of an EcR from the salmon louse *L. salmonis* and demonstrate the presence of the *LsEcR* transcript in all life stages of the parasite. In situ hybridisation, together with functional knock-down studies, indicates that the *LsEcR* plays a key role in regulation of female reproduction and oocyte maturation. The Ec-EcR hierarchy is a very complex system with a multitude of factors interacting through different pathways. The essential role EcR plays in this hierarchy makes it a good target for pesticide development, as knock-down of EcR results in severe physiological changes in the animal, including the termination of egg production. However, further studies are necessary in order to elucidate the functional role of LsEcR and to fully understand the complexity of the Ec-EcR hierarchy in the salmon louse.

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