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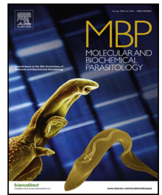

The ecdysone receptor (EcR) is a major regulator of tissue development and growth in the marine salmonid ectoparasite, *Lepeophtheirus salmonis* (Copepoda, Caligidae)

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ABSTRACT

The function of the ecdysone receptor (EcR) during development and molting has been thoroughly investigated in some arthropods such as insects but rarely in crustacean copepods such as the salmon louse *Lepeophtheirus salmonis* (*L. salmonis*) (Copepoda, Caligidae). The salmon louse is an ectoparasite on Atlantic salmon that has major economical impact in aquaculture due to the cost of medical treatment methods to remove lice from the fish. Handling of salmon louse infestations is further complicated by development of resistance towards available medicines. Understanding of basic molecular biological processes in the salmon louse is essential to enable development of new tools to control the parasite. In this study, we found *L. salmonis EcR* (*LsEcR*) transcript to be present in the neuronal somata of the brain, nuclei of muscle fibres and the immature intestine of the salmon louse. Furthermore, we explored the function of *LsEcR* during development using RNA interference mediated knock-down and through infection trials. Our results show that knock-down of *LsEcR* in the salmon louse is associated with hypotrophy of several tissues, delayed development and mortality. In addition, combined knock-down of *LsEcR/LsRXR* resulted in molting arrest during early larval stages.

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1. Introduction

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod on salmonids with a life cycle that includes both free-living and parasitic stages. The louse feeds on the skin, mucosa and blood of its host and can cause skin erosion and open wounds that may lead to death if the parasite infection becomes severe. Due to its high fecundity, salmon louse has become an increasing threat to the salmon farming industry where high fish densities and all year round production provide favourable conditions for large numbers of the parasite [1,2]. The free-living stages of the parasite can spread between pens, different farming sites and also to wild salmonid fish causing environmental concerns. Infestation of wild fish with salmon louse reared on fish in aquaculture has been argued to cause reduction in wild populations nearby aquaculture sites [3–7].

The salmon louse life cycle consists of eight developmental stages named nauplia I and II, copepodid, chalimi I and II, pre-adult I and II and the adult stage [8,9] that are separated by a molt. During molt a new exoskeleton is synthesized and synchronized with shedding of the old exoskeleton. The molting process is a cyclic event that is carefully regulated and requires the precise coordination of specific hormones to be successful. The hormone considered as the main determinant of the molting process in crustaceans is the steroid hormone 20-hydroxyecdysone (20E), reviewed by (Chang et al., 2011) [10]. The effect of 20E is exerted through a nuclear receptor complex consisting of the ecdysone receptor (EcR) and the retinoid X receptor (RXR) referred to as ultraspiracle (USP) in insects [11,12]. This heterodimer also plays a key role in the regulation of many other physiological processes such as development, reproduction and limb generation [13–16]. Upon activation by ecdysteroids, the EcR/RXR complex directly regulates a small set of "early" genes that further regulates a larger set of "late" genes in a genetic hierarchy [17,18]. Multiple splice

Abbreviations: *L. salmonis*, *Lepeoptheirus salmonis*; *LsEcR*, *Lepeoptheirus salmonis* ecdysone receptor; *LsRXR*, *Lepeoptheirus salmonis* retinoid X receptor; RT-qPCR, real-time quantitative PCR; RNAi, RNA interference; dsRNA, double stranded RNA.

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variants with different expression patterns of *EcR* and *RXR* have been identified in both insect and crustaceans such as *Drosophila melanogaster* [19], *Homarus americanus* [20], *Daphnia magna* [21] and *Marsupenaeus japonicus* [14]. Differences in spatial and temporal expression patterns between the isoforms have shown to correlate with specific responses and have distinct functions during development [22–24]. In the salmon louse, three *LsEcR* transcripts differing only in their 5′UTR region has been characterized [25] whereas several *LsRXR* splice variants of the open reading frame were identified by Eichner et al., 2015 [26]. During metamorphic reorganisation of the insect central nervous system, 20E regulates a wide variety of cellular responses such as neuronal proliferation, maturation, cell death and remodelling of larval neurons into their adult forms. Inactivation of the two *Drosophila EcR-B1* and *EcR-B2* isoforms gave defects in the early stages of neuronal remodelling during metamorphosis [22], while heat shock induced *EcR* null mutants were arrested in late embryogenesis or unable to go through molt and metamorphosis depending on their developmental stage [23,27].

The role of the EcR in developmental processes and molting is very well established in insects and to some extent in decapods, however, molecular data regarding the specific function of EcR in copepods is still limited [21]. RNA interference (RNAi) has been successfully applied to investigate gene function in salmon louse and serves as an important tool to understand the biology of the parasite [28–31]. Knock-down of *LsEcR* in adult female salmon lice resulted in degeneration of vitellogenin producing tissue and egg strings and therefor abolishment of reproduction, proving the important role of LsEcR in female reproduction [25]. Due to the important role of EcR in many vital biological processes as a ligand activated transcription factor, it is important to explore the function of *LsEcR* in molting and development and as a possible target for pesticides. In this study, we show that knock-down of *LsEcR* expression in *L. salmonis* larvae results in comprehensive tissue damage and mortality in later developmental stages. In addition, we show that simultaneous knock-down of *LsEcR* and its partner *LsRXR* in nauplia interrupts the molting process. In addition, knock-down of *LsEcR* indirectly or directly affects the expression of genes important in chitin synthesis. This suggests that *LsEcR* plays a key role in molting, growth and tissue development of the salmon louse.

2. Materials and methods

2.1. Animals

Eggs from the Atlantic salmon louse *Lepeophtheirus salmonis* [32] were hatched in wells with flow-through seawater and kept in culture on Atlantic salmon (*Salmo salar*) as previously described [33]. The salmon was kept in single tanks with seawater (34.5 ppt) at approximately 10 °C [34]. All experiments were conducted in accordance with the Norwegian legislation for animal welfare.

2.2. In situ hybridisation

Copepodids were fixed in 4% paraformaldehyde in phosphate buffered saline for 24 h and transferred to 70% ethanol for a minimum of 24 h before being embedded in paraffin wax. Localisation of *LsEcR* transcript in copepodids was performed as described by Trösse et al., 2014 [31] with modifications described in Sandlund et al., 2015 [25]. *LsEcR* specific cDNA was used as template to generate PCR product with T7 promoter extensions followed by synthesis of single stranded digoxigenin-labelled RNA probes (667 bp) (Primers listed in Table 1.). Probe concentration and quality was determined by spectrometry, employing a Nanodrop ND 1000 (Thermo Fisher Scientific) and a spot test, respectively. In brief, sections were dewaxed in Histoclear (National Diagnostics) before rehydration of tissue and proteinase K treatment (10 min) followed by fixation in 4% formaldehyde in PBS, acetic anhydride treatment and dehydration. 100 μl hybridization mix containing 20 ng digoxigenin-labelled RNA were added to the tissue and incubated in a vacuum chamber at 60 °C overnight. Chromogenesis was carried out using nitroblue tetrazolium (NBT) (Roche Diagnostics GMbH) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche). Sense RNA was used as a negative control.

2.3. Protein detection

Rabbit polyclonal antibodies (pAb) were raised against a synthetic LsEcR peptide that corresponding to a unique region of the LsEcR, residues 264–277 (AADTTVDPKSNNNG), by GenScript (Piscataway, NJ, USA). The pAb successfully detected the LsEcR protein at the expected Mw of 60.3 kDa by Western blot analysis. Several trials of immunofluorescence studies using the same antibody were not successful.

2.4. Functional studies using RNAi

Double stranded RNA (dsRNA) fragments for *LsEcR* and *LsRXR* (*LsEcR* fragment: position 782–1458, Ac. no. KP100057.1; *LsRXR* fragment: position in 323–945, Ac. No. KJ361516) were generated by T7 RNA polymerase (MEGAscript® RNAi Kit, Ambion inc) from PCR templates with T7 promoter regions. An 850 bp gene fragment from Atlantic cod (*Gadus morhua*) was used as negative control (Primers are listed in Table 1.). RNAi on salmon louse larvae was performed as described [30]. Five biological parallels of nauplia I ($n = 5 \times 30$) were soaked in either 1.5 μg of control dsRNA fragment or *LsEcR* dsRNA alone or together with equal amounts of *LsRXR* dsRNA, in 150 μl of seawater and left for incubation overnight at 9 ± 1 °C. The next day, samples were checked for the presence of exuvia, shed during the molt from nauplia I to nauplia II stage, and nauplia II was transferred to flow through incubators and left for seven days post treatment (d.p.t.) when the control had molted into copepodids. Copepodids were inspected under a binocular microscope (Olympus SZX 0.5 and 1.6× Olympus objective) and photographed (Canon EOS 600D). The animals were photographed, collected and stored on RNAlater™ (Ambion) or fixed in Karnovsky's reagent for embedding in plastic.

2.5. Infection trial

Copepodids (100/fish) were released in tanks holding one salmon/tank. The water level was held low for the first 10 min of the infection after which water flow was returned to normal. Water from the tanks was filtered through a double filter system (20 μm) to collect copepodids that did not successfully attach to the fish. Three fish were infected with control lice and three fish were infected with *LsEcR* dsRNA treated lice (knock-down of *LsEcR* was verified before infection). The experiment was terminated 27 days post infection when the control lice had developed to the pre-adult/adult stage. The fish was anaesthetised in a mixture of methomidate (5 mg/l) and benzocaine (60 mg/l) and lice were carefully removed with forceps, counted, inspected under a microscope and documented as described in Section 2.4. Both cephalothorax length (CL) and total length (TL) of the control and *LsEcR*kd lice were measured as described by Eichner et al., 2015 [35] in order to determine any differences in size.

2.6. Extraction of RNA and cDNA synthesis

For RNAi experiments, 30 copepodids were homogenised using 1.4 mm zirconium oxide beads (Bertin) in TRI Reagent® (Sigma-

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

Primer name[b]	Sequence (5'-3')	Method
*LsEcR*_specific_P1	CCGATTTGCCATTACGTAGGCTTGTAGAGC	in situ/dsRNA
*LsEcR*_specific_P2	CCGCAGCTGCAGCCGACACAACTGTAGAT	in situ/dsRNA
*LsEcR*_specific_P3	CGAGCGTTTCCACTTACTTGCCAT	dsRNA
*LsEcR*_specific_P4	CGCCAACAACGACGACCC TCCACCAACAGCACT	dsRNA
*LsRXR*_specific_P1	CGGAATTGGGATGTCTACGAGCCATCATA	dsRNA
*LsRXR*_specific_P2	CTTCCTCTGACTCACTATAGAAGCATA	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
LsEcR total_f	TCGGGAGAAAGTCCCTCTTCT	RTq-PCR
LsEcR total_r	ACAGCTCCAGTAGGTGTTAAAGGA	RTq-PCR
LsEcR total TaqMan®	TCGCAGTCCATTCTC	RTq-PCR
LsRXR total_f	CCTAGTTGAACTCATCGCCAAAATG	RTq-PCR
LsRXR total_r	TGAAGAGTATGATGGCTCGTAGACA	RTq-PCR
LsRXR total TaqMan®	CCGCTTTGTCCATTTGCAT	RTq-PCR
*LsHR38*_f	CCGTTCTCACAACTTCCTTTACCAT	RTq-PCR
*LsHR38*_r	CGTCGAAATCGATGTCAGTTTTGC	RTq-PCR
LsHR38 TaqMan®	CCCACGGCAAGACAT	RTq-PCR
*LsE74*_f	GGTCACGTTAAAGATGGGTCAATTT	RTq-PCR
*LsE74*_r	CCAACAGGAGTACTAACACAACTGAT	RTq-PCR
*LsE74*_TaqMan®	CAGCGCCTCGTTCAC	RTq-PCR
*LsE75*_f	CCTTGACCAATTTTCAGAACGGTTT	RTq-PCR
*LsE75*_r	AATCCAGGGATCCGCTTGG	RTq-PCR
*LsE75*_TaqMan®	CACGTTCGCCAAGTTT	RTq-PCR
*LsCPD*_f	TGTCAAATAAGCGTGAGGTTAAGGAA	RTq-PCR
*LsCPD*_f	GCGCGTGAATACCACAATCC	RTq-PCR
*LsCPD*_ TaqMan®	ACCCAAACGATCTTCC	RTq-PCR
*LsChit1*_f	TCCATTCATTTGTACACATGTGGCTTA	RTq-PCR
*LsChit1*_r	CATTGTAAGGGTCAAGGAGTCGAAT	RTq-PCR
LsChit1 TaqMan®	CAGACCAGCAAATCCA	RTq-PCR
*LsChit 2*_f	GTTAATTTCTTAAACAAGTATAACTTCGACGGTT	RTq-PCR
*LsChiti 2*_r	GGAACACCACCTCGTTTAGCA	RTq-PCR
LsChit2 TaqMan®	TTCCCAGTCAATATCC	RTq-PCR
*LsChs1*_f	TTTCGAGGTAAAGCACTTATGGATGAT	RTq-PCR
*LsChs1*_R	AGCCATCTATCTTCTCCTTGATCGT	RTq-PCR
*LsChs1*_TaqMan®	ATGCCTTGGTTCTTCC	RTq-PCR
*LsChs2*_f	AGCAGTGACTATGCTTTTAGATTTGATGA	RTq-PCR
*LsChs2*_r	CTGAGCCTAAAGGATGAATTCTTCCA	RTq-PCR
*LsChs2*_TaqMan®	CACTGCGCCGGTTTT	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.

Aldrich), according to the manufacturers protocol. RNA was extracted from the water phase using RNeasy micro kit (Qiagen) as described by the manufacturer. Samples were stored at −80 °C. Concentrations of RNA was determined by NanoDrop ND-1000 spectrophotometer at 260 nm (NanoDrop Technologies Inc.) Two parallel reactions of cDNA synthesis were achieved using AffinityScript qPCR cDNA synthesis Kit (Agilent Technologies) according to the manufacturer's protocol. A five fold dilution of cDNA was stored at −20 °C.

2.7. Detection of transcript level in dsRNA treated lice by real time quantitative-PCR (RT-qPCR)

RT-qPCR was carried out using *Applied Biosystems 7500* Real-Time PCR System (Applied Biosystems®) under standard conditions (initiation: 50 °C 2 min, holding: 95 °C 10 min, 40 cycles of 95 °C 15 s followed by 60 °C 1 min) as previously described in Sandlund et al., 2015 [25]. Transcription levels of all genes investigated were normalised to the established salmon louse standard gene *eEF1*α [36]. Eight dilutions of RNA were used to create a standard curve and all RT-qPCRs were carried out in duplicate. Samples were analysed independently but simultaneously as *eEF1*α using the same cDNA and mastermix (TaqMan® Universal PCR Master Mix; Applied Biosystems). Five biological parallels of copepodids (n = 5 × 30) were run for each dsRNA treatment group. Results were analysed by the $2^{\wedge-\Delta\Delta Ct}$ approach and presented with 95% confidence interval calculated from the $2^{\wedge-\Delta\Delta Ct}$ values. *T*-tests were used to determine differences in expression between the control group and dsRNA treated animals. A p-value of 0.05 was set as threshold.

2.8. Histology

Copepodids for light microscopy were fixed in Karnovsky's fixative, rinsed in PBS and dehydrated in a series of ethanol solutions (50%, 70% and 96%), before embedding in Technovit 7100 (Heraeus Kulzer Teqhnique) as described by [35]. Sections (1–2 μm) were stained with Toluidine blue (1% in 2% borax). Sections were mounted using Histomount (Invitrogen).

Microscopy and imaging were acquired with an Axio Scope. A1 light microscope connected to Axiocam 105 color camera (Zeiss International) and processed using Adobe Photoshop CS6 (Adobe Systems).

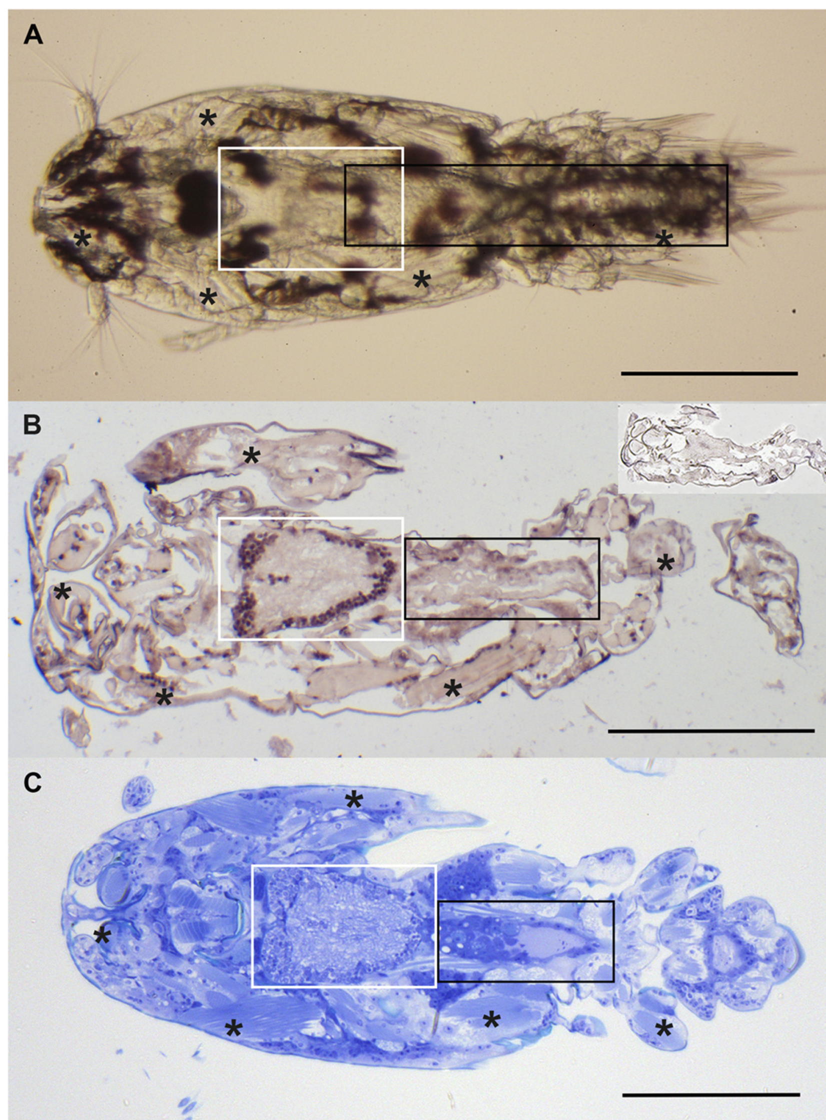

Fig. 1. Localisation of *LsEcR* transcript in *Lepeophtheirus salmonis* copepodid larvae. A) Light microscope image of a copepodid larvae (seven days) to show the outline of the animal. B) In situ hybridisation using *LsEcR*-specific probes was used for detection of transcript. Negative control (sense RNA) is shown (insert). Transcript was detected in the neuronal somata of the brain (white frame) and myonuclei throughout the copepodid (asterisk). A weak positive staining was also observed in the immature intestine (black frame). Weak unspecific colouring of the outer cuticular layer was seen using both the sense and the anti-sense probes. C) A histological section of a copepodid was shown for better visualisation of selective tissues. White and black frames are used to better visualize the localisation of neuronal and intestinal tissue, respectively and asterisks are used to mark muscle tissue. Scale bars = 200 μm (A–C).

3. Results

3.1. *LsEcR transcript expression in* L. salmonis *copepodids*

Sections of free-living copepodids (7 days old) were used for in situ hybridisation in order to identify the localisation of *LsEcR* transcripts in copepodids. Expression of *LsEcR* transcript was detected in the neuronal somata of the brain (Fig. 1B; white frame) as well as in myonuclei throughout the animal (Fig. 1B; muscle fibres are marked with asterisks). In addition, a weak staining of the immature intestine could also be observed (Fig. 1B; black frame).

3.2. *Knock-down of LsEcR by RNAi did not inhibit molting from nauplia II to copepodid larvae*

To assess the function of *LsEcR* during early development and molting, RNAi experiments were performed by soaking nauplia I larvae in dsRNA fragment specific to *LsEcR*. The experiment was terminated seven d.p.t. when control animals had molted to the copepodid stage. Two separate experiments were run showing similar results. The ds*LsEcR* treated group developed normally from nauplia II to the copepodid stage and did not show any loss-of-function phenotype (Fig. 2B) compared to the control animals (Fig. 2A) seven d.p.t. RTq-PCR analysis was performed in order to determine the degree of *LsEcR* knock-down (*LsEcR*) and confirmed a significant decrease of *LsEcR* transcript by an average of 57% (Fig. 3A). Histological sections of the *dsLsEcR* treated lice were examined to identify any differences in internal morphology compared to the control, however no abnormalities were observed (results not shown).

The binding of ecdysone hormones to the EcR/USP complex is known to directly and indirectly regulate several other genes including the transcription factors *LsE75* and *LsE74* and the hormone receptor *HR38*. Consequently RT-qPCR was performed in

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Fig. 2. Selection of representative animals obtained from the RNAi experiment using molting nauplius I larvae. A) Control group. B) Larvae treated with double-stranded RNA (dsRNA) targeted to *LsEcR* alone developed into copepodids and did not show any abnormal phenotype compared to the control group (A). C) Larvae treated with fragments targeting both *LsEcR* and *LsRXR*, was observed to have developed copepodid like features (marked with white arrow) inside the exuvia (marked with black arrow) and was viable at day seven post soaking. However, the molting from nauplia II to copepodid was arrested and the animals started to die after 12 days. Morphology of a normally developed nauplia II larvae is shown in D (for reference only). Scale bars = 200 μm (A–D).

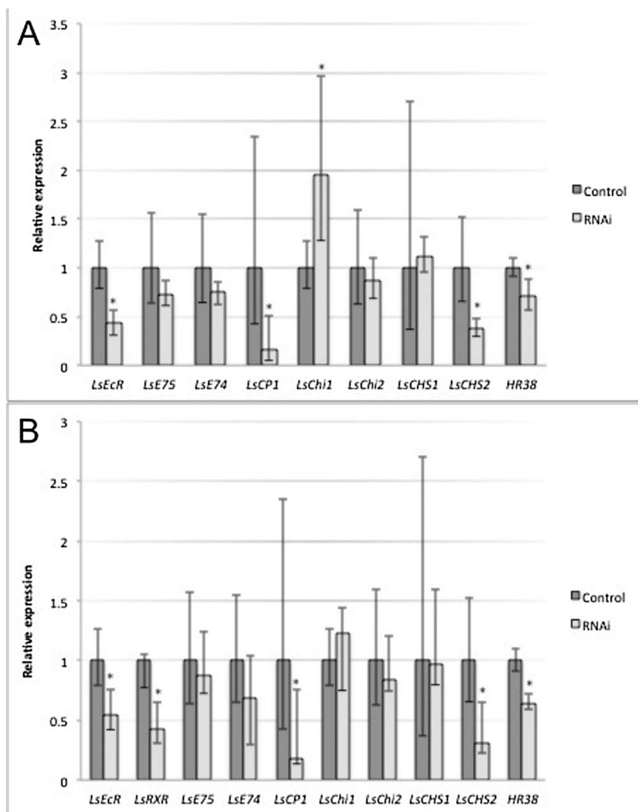

Fig. 3. Relative expression of the ecdysone receptor (EcR) and the retinoid X receptor (RXR) together with possible target genes regulated by the *LsEcR/LsRXR* complex, after treatment with double-stranded RNA (dsRNA). *Lepeophtheirus salmonis* EcR (*LsEcR*) and *LsEcR/LsRXR* treated copepodids were collected 7 days post treatment. Quantitative real-time PCR (RTq-PCR) of *LsEcR, LsRXR* and the possible target genes *LsE75, LsE74, LsChi1, LsChi2, LsCHS1, LsCHS2, LsCP1* and *LsHR38* were evaluated. GenBank Accession Numbers: *LsEcR*: KP100057, LsRXR: KJ361516, *LsChi1*: AJD87505.1, *LsChi2*: AIE45495.1, *LsCHS1*: KX349436, *LsCHS2*: KX349435, *LsE75*: KX260137 *LsE74*: KX349434, *LsCP1*: BT078319.1, *LsHR38*: KX349433. The graphs are representatives of two individual experiments. The expression levels of the respective genes in the control groups were set to 1. Values are expressed as mean ± confidence intervals of five biological replicates (n = 30 copepodids/sample). *Statistically significant ($P < 0.05$). Statistical analysis was performed using a *T*-test.

dsRNA treated lice on these genes and several genes directly involved in the molting process: *LsChitinase1 (LsChi1), LsChitinase2 (LsChi2), LsChitin synthase1 (LsCHS1), LsChitin synthase2 (LsCHS2), LsZinc carboxypeptidase (LsCP1)* transcripts were measured in dsRNA treated lice. A significant down-regulation was observed for *LsCP1, LsCHS2* and *LsHR38* while a significant up-regulation was detected for *LsChi1* in the *LsEcR*kd animals (Fig. 3A) (*T*-test, $P < 0.05$).

3.3. *Simultaneous knock-down of LsEcR and LsRXR isoforms resulted in molting arrest of nauplia II larvae*

An additional experiment was set up to investigate the effect of simultaneous knock-down of *LsEcR* and *LsRXR* in salmon louse larvae, as described above. In contrast to the *dsLsEcR* treated lice, molting of the *LsEcR/LsRXR* knock-down lice was interrupted and the treated lice did not molt from nauplia II (Fig. 2D) to the copepodid stage (Fig. 2A). The *dsLsEcR/dsLsRXR* treated lice had developed some features similar to that of the control copepodid such as segmentation of the abdomen (Fig. 2C; marked by white arrow) but they were not able to emerge from the old cuticula (Fig. 2C; edge of exuvia marked by black arrow). RT-qPCR confirmed a significant knock-down of both *LsEcR* and *LsRXR* by 46% and 57%, respectively (Fig. 3B) (*T*-test, $P < 0.05$). In addition, the same genes mentioned in Section 3.2 were analysed by RT-qPCR analysis in the ds*LsEcR*/ds*LsRXR* treated animals. Similar to the *LsEcR* knock-down animals, *LsCP1, LsCHS2* and *LsHR38* were significantly down-regulated in the ds*LsEcR*/ds*LsRXR* treated animals (Fig. 3B) (*T*-test, $P < 0.05$). However, in contrast to the *dsLsEcR* treated lice no significant up- or down-regulation was observed for the *LsChi1* or *LsChi2* in the ds*LsEcR*/ds*LsRXR* treated animals (Fig. 3B) (*T*-test, $P < 0.05$).

3.4. *Infection trial using LsEcR knock-down animals*

An infection trial with *LsEcR* knock-down copepodids was performed. Atlantic salmon was infected with either control or *LsEcR*kd copepodids and cultivated on the host fish until the control group had developed into pre-adult I and II females and pre-adult II and adult males (27 d.p.i.). At termination, no abnor-

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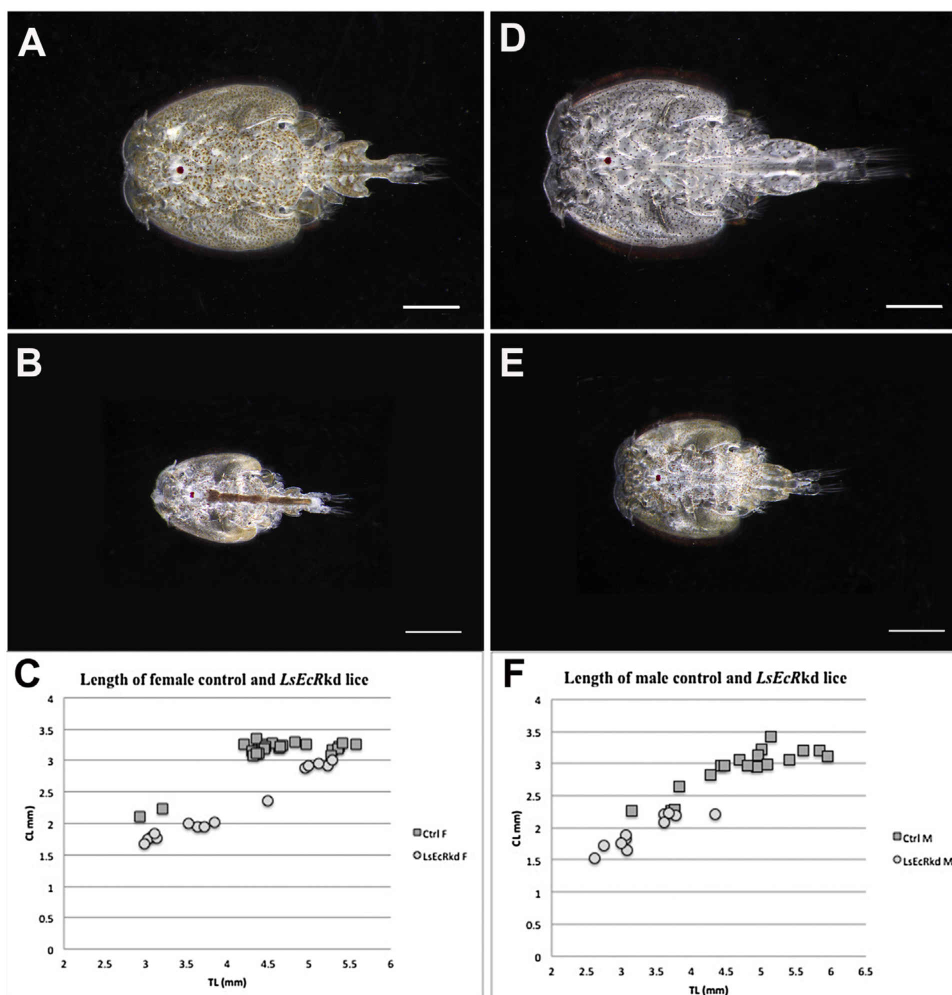

Fig. 4. Representative animals obtained after infection trial using *LsEcR* knock-down (*LsEcR*Kd) copepodids. A, D) shows pre-adult II female (A) and male (D) control animals 27 days after infection. B, E) represents *LsEcR*kd female (B) and male (E) animals. C) The female *LsEcR*kd lice could be divided into two groups that were either significantly smaller or the same size as the control group. F) All male *LsEcR*kd lice were significantly smaller compared to the control. Statistical analysis was performed using a *T*-test; $P < 0.05$. Scalebar = 1 mm A–B, D–E.

malities could be observed by light microscopy in the ds*LsEcR* lice. However, high mortality was evident and a significantly lower number of *LsEcR*kd lice (n = 29) were recovered from the host compared to the control group (n = 96). The *LsEcR*kd males were significantly shorter in CL and TL (Fig. 4E, F; *T*-test, $P < 0.05$) compared to the control group (Fig. 4D, F) with a few individuals that were even shorter than normally developed pre-adult I males [9]. In contrast, the female *LsEcR*kd lice could be divided into two groups. One group that had significantly smaller CL and TL (Fig. 4B, C; *T*-test, $P < 0.05$) compared to the control and one group with CL and TL size similar to the control group (Fig. 4C; *T*-test, $P > 0.05$). Histological sections from female and male ds*LsEcR* treated lice (Fig. 5F–J) both revealed abnormal morphology compared to the control (Fig. 5A–E). Most striking was the severe reduction of muscle tissue of the ds*LsEcR* treated animals (Fig. 5F). The intestine of the ds*LsEcR* treated animals (Fig. 5H) resembled the intestinal tissue observed in copepodids rather then the anatomy described for adult animals (Fig. 5C) [37]. Hypotrophy of the cellular structure of the sub-cuticula (Fig. 5G), the neurological tissue (Fig. 5J framed), female ovaries (Fig. 5I) and male testes (Fig. 5J marked with asterisks) was apparent in the knock-down animals compared to the control (Fig. 5B–E). Histological examinations of *LsEcR*kd females that were the same size as the control group showed similar morphology as the other *LsEcR*kd animals.

4. Discussion

While extensive knowledge of the EcR function in a variety of developmental and physiological processes in insects has been obtained, the functional importance of this receptor in copepods remains largely unstudied. Here we report on the transcriptional distribution and function of the newly characterized *L. salmonis EcR* (*LsEcR*) [25] during molting and development of the salmon louse. Not surprisingly, analysis of the spatial distribution of *LsEcR* in copepodids using in situ hybridization demonstrated a wide expression pattern the intestine, neuronal somata of the brain and in the nuclei of the muscle cells (Fig. 1B). A similar wide distribution pattern of *LsEcR* transcript was observed in the adult female salmon lice [25] and the copepod *Marsupenaeus japonicus* [14]. The extensive expression of *LsEcR* is in accordance with the broad range of biological processes involving this nuclear receptor such as molting, remodelling of neurons [38,39] and muscle tissue during metamorphosis [40].

Knock-down studies of *LsEcR* using RNAi caused near 60% down-regulation (Fig. 3A) of *LsEcR* transcript in the salmon lice larvae,

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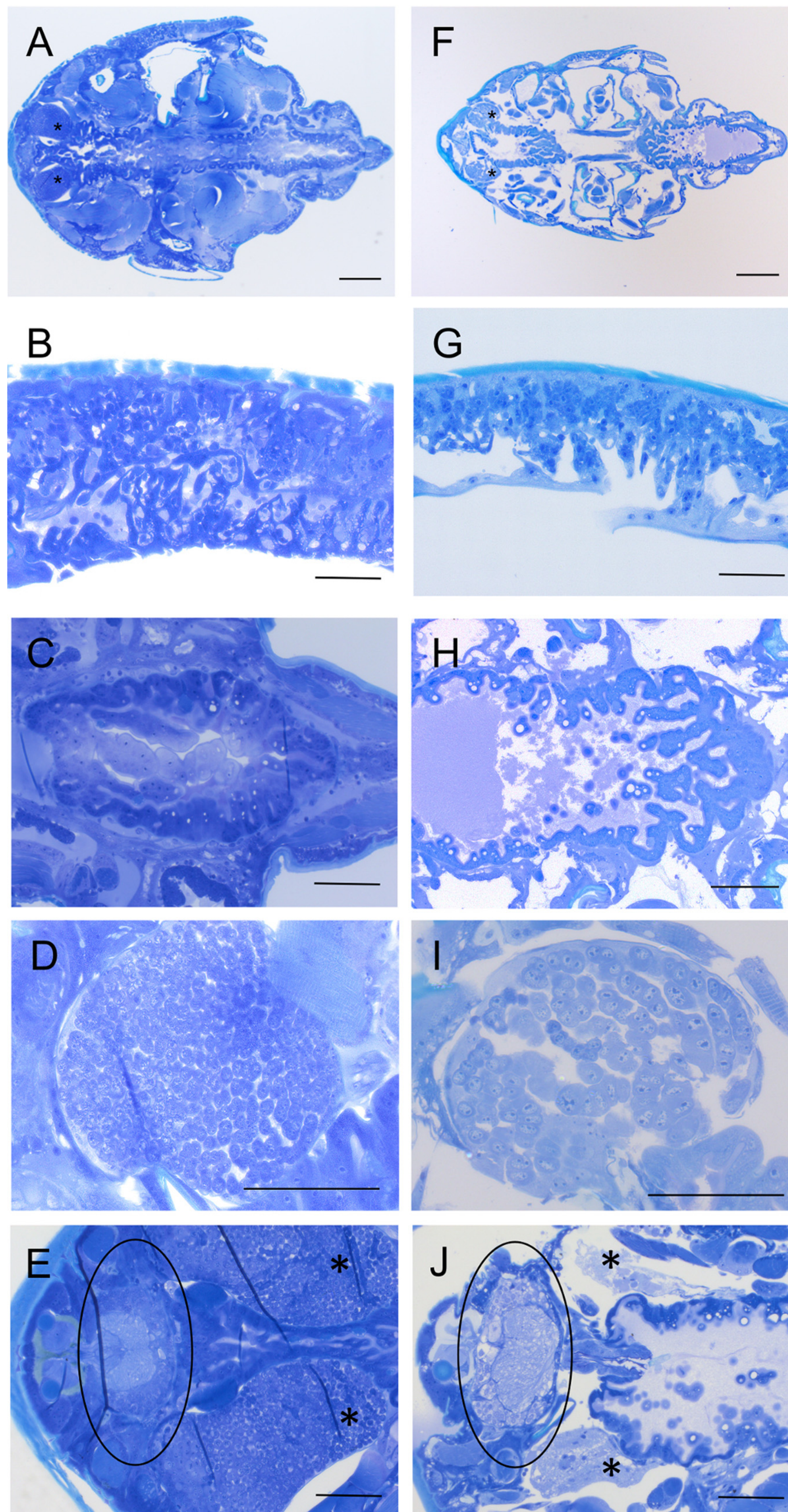

Fig. 5. Histological examination of *Lepeophtheirus salmonis EcR* knock-down (*LsEcR*kd) animals. Toluidine stained control sections and sections from *LsEcR*kd animals are shown in A–E and F–J, respectively. A and F) shows sections of the whole animal with letters as guides to the corresponding photos of individual tissues. E and J) are sections of males where testes are marked with asterisks and brain tissue is framed with a black circle. Testes and ovaries are located at the same position in their respective gender (A, F marked with asterisk). Muscle tissue was observed throughout the control animal (A) in contrast to the *LsEcR*kd animals where a severe reduction of muscle tissue was evident (F). The sub-cuticular tissue (G) ovaries (I), testes (J*) and neuronal tissue (J framed) were hypotrophic compared to the control (B, D, E*, E framed) while the intestinal tissue of the *LsEcR*kd animals appeared to resemble that found in coepodid larva (H) rather then the control (C). Scale bar = 300 μm (A, F) and 100 μM (B–E, G–H).

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but did not result in molting arrest or any observable morphological abnormalities in the copepodids (7 d.p.t.). Similar results have been reported in *LsRXR* knock-down copepodids after dsRNA bath treatment [30]. The normal phenotype was surprising as depletion of both *EcR* and *RXR* transcripts in larvae from several insect and crustacean species both cause severe developmental defects and molting arrest that eventually lead to death of the animal [27,41–43]. Studies have shown that both EcR and USP are able to interact with DNA either as homodimers or monomers [44,45]. In addition, USP alone has shown to bind ligand and exert its function without EcR [46]. These results from our experiments indicate that *LsEcR* and *LsRXR* have other partners or manage to retain its function alone. The apparent normal development of the *LsEcR*kd copepodids could be due to insufficient knock-down of *LsEcR* or the presence residual LsEcR protein. However, due to unsuccessful detection of LsEcR protein, we cannot determine if the extent of protein depletion corresponds to the decrease in *LsEcR* transcript. Molting arrest was however achieved by simultaneous knock-down of both *LsEcR* and *LsRXR*. To our knowledge, not many knock-down studies of the nuclear receptor complex have been performed in crustaceans. Studies of *EcR*/*RXR* depletion in the fiddler crab *Uca pugilator* showed similar results with arrest of growth during early blastemal development and during limb generation. In addition, the ds*EcR*/ds*RXR* injected crabs failed to molt and subsequently died [43].

Despite the lack of observable abnormalities in the ds*LsEcR* treated copepodids (7 d.p.t), the infection trial using the same copepodids resulted in 70% mortality and developmental defects that obstructed the surviving lice from normal development. Whether high mortality is caused by disruption of the molting process or other physiological mechanisms is uncertain. In addition to the high mortality, the CL and TL of the recovered animals were significantly shorter for the males and one group of the females (*T*-test: $P < 0.05$) compared to the control. This could either reflect a decrease in size and/or a delay in development. Similar results were observed in the cotton mirid bug, *Apolygus lucorum,* where the duration of development increased in the third, fourth and fifth instar after siRNA treatment targeted to EcR. Interference of *AlEcR* additionally caused a lethal phenotype where mortality increased from the fourth nymph instar to adult animals [47]. A significant increase in developmental times was also observed for larva and pupal stages of *Spodoptera exigua* after injection of dsRNA in the fifth instar [48]. During metamorphosis of holometabolous insects, neurons undergo a crucial remodelling process dependent on EcR (reviewed in Boulanger et al., 2015) [49]. The ecdysone titer and the pattern of EcR isoform expression have also shown to be a fundamental factor during regrowth of muscle during metamorphosis in *Manduca* [40] and midgut morphogenesis during embryogenesis in *Drosophila* [50]. Histological examinations showed a lack of muscle tissue and hypotrophy of neuronal-, sub-cuticular and gonadal tissue in the *LsEcR*kd lice (Fig. 5F–J). Our results indicate that *LsEcR* play a similar role in the salmon louse and is required either directly or indirectly in the development of different tissues. Knock-down of *LsEcR* in pre-adult II females resulted in failure in normal egg development and offspring generation [25]. The hypotrophic gonads found in both female and male ds*LsEcR* treated lice in this study suggests that the animals would not be able to successfully reproduce and that knock-down of *LsEcR* transcript in larval stages affects maturation of the gonadal tissue of the treated animals and thereby the ability to reproduce. Similar destruction of the gonadal tissues was observed in adult female *LsRXR* knock-down lice [26]. Due to the significant reduced development of internal tissues, reduced growth or even mortality is expected. This is in accordance with present results.

In crustaceans, chitin synthases, chitinases and carboxypeptidases are important enzymes in chitin metabolism that is crucial for reorganisation and exchange of the exoskeleton [51,52]. A significant decrease of *LsCP1* and *LsCHS2* transcript (Fig. 3) were evident in the *LsEcR* and *LsEcR*/*LsRXR* knock-down animals suggesting them to play a role in molting. The results are supported by previous expression studies in *L. salmonis* larval stages [30] and RNA-seq data (unpublished; Licebase.org) that confirms expression of *LsCP1* and *LsCHS2* in all molting stages. No down-regulation was observed for *LsCHS1* that is mainly expressed in the adult female intestine (unpublished; Licebase.org). A similar difference in *CHS* transcript expression was demonstrated in the shrimp *Pandalopsis japonica* [53]. Ontogenetic analysis of *LsChi1* and *LsChi2* showed that expression varies within the intermolt and postmolt stages and peaks between premolt nauplius I and postmolt nauplius II [35]. *LsChi1* were significantly up-regulated (Fig. 3.), respectively in the *LsEcR*kd animals. Previous RNAi studies of *LsChitinases* demonstrated them to affect remodelling of the exoskeleton [35], supporting our results that regulation of the *LsChi*1 gene in *LsEcR*kd copepodids may contribute to the high mortality rate and phenotype observed in animals from the infection trial. No regulation was observed for the *LsChi* genes in the *LsEcR*kd/*LsRXR*kd group. However, due to the physiological differences between the *LsEcR*kd and the *LsEcR*kd/*LsRXR*kd groups (Fig. 2B–C) a direct comparison of transcript expression of down-stream genes is not possible. Of the early response genes E74, E75 and HR38 (Fig. 3.) only the latter was significantly down-regulated in both treatment groups. This could be because of secondary responses caused by depletion of *LsEcR* and *LsEcR*/*LsRXR* and disruption of several biological processes. Comparison of expression of the response genes between different *LsEcR*/*LsRXR* knock-down experiments show that the expression of these genes are dependent on the degree of *LsEcR*/*LsRXR* knock-down indicating that the response genes are tightly regulated by the nuclear receptor complex.

In summary, we found that *LsEcR* transcript is expressed in several tissues in the *L. salmonis* copepods. This is in accordance with its expected role in many molecular biological processes. Functional studies using RNAi showed that knock-down of *LsEcR* in *L. salmonis* larva results in increased mortality and severe histological changes. In contrast, simultaneous knock-down of *LsEcR*/*LsRXR* resulted in molting arrest. This shows that EcR clearly has an important function in molting and development of the salmon louse *L. salmonis*. The last few years, development of increased tolerance and resistance to available anti-sea lice drugs has made it crucial to develop novel methods to control salmon lice infestations. In insects, the EcR has long been used as a target for ecdysone agonists such as the bisacylhydrazines. These chemical compounds attain great target specificity due to their variations in binding affinity between different ecdysozoans [54,55] hence, there is a potential for novel medicines targeting the ecdysone hormone system in *L. salmonis*. However, further investigations of the structure of the ligand binding pocket of *LsEcR* are necessary in order to reduce the potential endocrine disruption of other aquatic invertebrates. The findings identify *LsEcR* as a possible target for chemicals disrupting developmental processes and may be valuable in the development of new tools to fight the parasite.

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