Functional investigations of the Ecdysone Receptor and production of ecdysone in the salmon louse *(Lepeophtheirus salmonis)*

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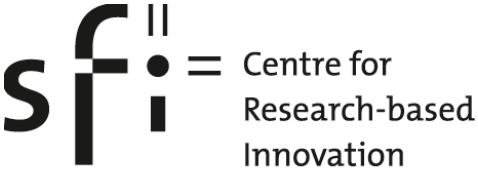

Scientific environment

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Dedicated to my nieces Tuva and Eira

"Much to learn, you still have"

-Yoda-

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Abbreviations

20E	20-hydroxyecdysone
aa	amino acid
AF-1	activation function 1
AF-2	activation function 2
Ab/G	abdomen/genital segment
CT	cephalothorax
DBD	DNA binding domain
Dib	disembodied
E	ecdysone
EcR	ecdysone receptor
EcRE	ecdysone response elements
HRE	hormone response elements
LBD	ligand binding domain
Nvd	neverland
Oct3βR	β3-Octopamine receptor
PonA	ponasterone A
PCD	programmed cell death
PG	prothoracic gland
RT-qPCR	Real-time quantitative PCR
RXR	retinoid X receptor
Shd	shade
USP	ultraspiracle
YO	Y-organ

Abstract

The salmon louse *Lepeophtheirus salmonis* is a marine ectoparasitic copepod naturally infecting salmonid fishes in the Northern hemisphere. At present, salmon louse infections are the most severe disease problem in the salmon farming industry causing significant economical losses. Salmon louse infections on farmed fish have largely been treated with chemotherapeutants which in recent years have lead to the development of resistance towards the majority of available treatment methods. Cases of multi-resistance are reported as increasing, underlining the severity of the situation. Although non-therapeutic methods such as the use of cleaner fish have been implemented into the management of lice infestations, it is clear that new alternative methods are essential to gain control of the parasite in the future.

The ecdysone hormone system and the ecdysone receptor/retinoid X receptor (EcR/RXR) complex are key regulators of molting, development and growth in arthropods. A wide range of studies has demonstrated the importance of this endocrine system in insects and to some extent in malacostraca, but knowledge about ecdysone signalling in copepods is limited. Therefore, we aimed to increase our knowledge about this hormone system in the salmon louse. In this study, the *ecdysone receptor* (*EcR*) and the key ecdysteroidogenic genes *neverland* (*nvd*), *disembodied* (*dib*) and *shade* (*shd*) were identified and functionally assessed using RNA interference studies. *LsEcR* transcripts were expressed in all life stages and in most tissues in both the copepodid (i.e. brain, muscle and immature intestine) and the adult female (i.e. ovaries, sub cuticula, intestine, oocytes and glandular tissue). The wide tissue distribution is expected due to the numerous physiological and biological processes that are regulated by EcR signalling. Interestingly, knock-down of *LsEcR* in nauplia I larvae did not cause immediate molting arrest, but developed into viable copepodids, indicating another partner of RXR. However, further incubation of the *LsEcR* knock-down copepodids on salmon resulted in severe tissue damage and increased mortality. During metamorphosis, an extensive range of tissues is remodelled concurrently with the molting process in order to adapt to the new life

stage. The results obtained from the studies indicate that *LsEcR* is a key regulator of developmental processes associated with tissue remodelling and molting in *L. salmonis.*

Ecdysteroids regulate reproductive processes in arthropods such as vitellogenesis and oocyte maturation. In the salmon louse, vitellogenin and yolk production takes place in the subcuticula before being incorporated into the oocyte. These processes showed to be indirectly affected by knock-down of *LsRXR* which resulted in abnormal egg chambers and no egg-production. This is supported by knock-down studies of *LsEcR* in pre-adult females resulted in hypotrophy of tissues associated with yolk and vitellogenin synthesis, degeneration of the oocytes and termination of egg production. This demonstrates that *LsEcR* plays a key role in the reproduction of the salmon louse. We have not proven that ecdysteroid signalling has a direct effect on the oocytes. However, LC/MS/MS analysis of the ecdysteroid content during oocyte maturation in adult females showed higher levels of ecdysteroids in the abdomen/genital segment (Ab/G) compared to the cephalothorax (CT), suggesting that ecdysteroids may directly affect oocyte maturation and embryogenesis.

The biosynthesis of the ecdysteroids is performed by members of the cytochrome P450 family through several enzymatic reactions. The last step in the pathway is the conversion of ecdysone to the active metabolite by Shd. Knock-down of the biosynthetic genes in other species is associated with embryonic defects and mortality. Interestingly, functional assessment of *LsNvd, LsDib* and *LsShd* in *L. salmonis* nauplia I larvae resulted in molting arrest only in the *LsShd* knock-down animals, while the *LsNvd, LsDib* developed normally into copepodids. Based on the knock-down results and the biology of the salmon louse, we hypothesize that ecdysone are incorporated into the oocytes during maturation and is further converted by LsShd into the active metabolite during development of the lecitotrophic stages. To our knowledge, no-loss of function phenotype has been observed in *nvd* and *dib* knock-down animals during early development in other arthropods, indicating a possible novel regulation pathway in *L. salmonis.*

1. General introduction

1.1 Background

Norway is the largest producer of Atlantic salmon *(Salmo salar)* in the world. The production has more than doubled the last decade from 550 000 metric tons in 2004 to more than 1.2 metric million tons in 2014[1] (www.fao.org; www.ssb.no). With intensive cultivation and a growing aquaculture industry, challenges concerning fish pathogens have become an increasing issue in terms of economical loss and negative environmental interactions. In Norway, the salmon louse (*Lepeophtheirus salmonis)* is one of the most severe pathogens in salmon farming estimated to cost the farming industry as much as 3-4 billion NOK in 2014 http://nofima.no/nyhet/2015/08/kostnadsdrivere-i-oppdrett/. Since the beginning of large-scale salmon farming in the 1970`s, salmon lice infestations have mainly been controlled using anti-sea lice medicaments. However, the efficacy of medical treatments has decreased due to development of lice populations with reduced sensitivity and resistance towards one or more of the anti-parasitic drugs available [4-7]. In the south- and mid-west of Norway where the lice problems are most severe, occurrences of multi-resistance towards all available medicines, including hydrogen peroxide, have been reported [8]. The result has been an increase in the use of chemotherapeutants (e.g. chitinase inhibitors (fluobezurones), nerve toxins (emamectin benzoate, pyrthroids), organophosphates affecting the cholinergic nerve system (azamethiphos) and hydrogen peroxide) causing concerns due to potential negative impact on non-target organisms and the surrounding environment of the fish farm [9]. Additionally, lice larvae originating from fish farms can infect wild salmonids and influence post-smolt survival during costal and oceanic migration. Currently, high sea lice densities as well as escaped farmed salmon have been suggested to be the two most significant threats to the wild salmon populations in Norway. Due to the significant lice problems and reduced efficacy of the existing medicines available, alternative non-medical methods are under development and some of these methods are currently being implemented to

facilitate lice control in salmon farms. The use of cleaner wrasse [10], mechanical delousing and lice-skirts to reduce lice infestations are just a few alternative methods that have been applied by the aqua-culture industry the last few years. Regardless, salmon louse infestations remain persistent making it imperative to develop novel treatment methods to control the parasite population in order to sustain both farmed and wild salmon populations and a growing industry. However, most of these methods are not sufficiently efficient (or the capacity is too low) to bring the lice levels down to the limits enforced by the authorities alone and must be used in combination with other tools. Medical treatment of salmon louse has been the most important tool for parasite control. The last new medicine was introduced in 1999 (SLICE®) and there is a strong demand for new efficient medicine. Increasing basic knowledge about key biological processes in the salmon louse is a long-term effort towards new treatment tools. In arthropods, ecdysone hormones regulate key steps in development, growth and reproduction. Hence, a highly relevant topic for research based innovation towards future sea lice control.

1.2 Salmon louse

Lepeophtheirus salmonis (Krøyer, 1837*)* is a marine ectoparasitic copepod on salmonid fishes from the genera *Oncorhynchus (O. mykiss* (Walbaum, 1792)*)*, *Salvelinus (S. alpinus* (Linnaeus, 1758)*)* and *Salmon (S. salar* (Linnaeus, 1758) *and S. trutta* (Linnaeus, 1758) [11]. Two allopatric subspecies of *Lepeophtheirus salmonis* has been identified; *Lepeophtheirus salmonis salmonis* (Atlantic) and *Lepeophtheirus salmonis onchorynchii* (Pacific) [12]. The salmon louse has a northern circumpolar distribution and is naturally found on wild salmon populations [13]. The parasitic stages feed on mucus, skin and blood [14], causing local erosion in the epidermal tissue of the host, often on and near the head and dorsal fins [15]. At high infestation rates, chronic stress, lesions in the dermis and subcutaneous tissue can occur, compromising the osmoregulation of the host in which can lead to host mortality, especially to post-smolts [13, 15-17]. Salmon louse infestations has negative effects on the host`s reproduction, growth and quality. Damage to the skin

makes the fish more susceptible to secondary infections as the skin acts as their first line of defence against pathogens [18]. The salmon louse itself has also been suggested to be an important vector in transferring diseases between fish [19].

1.2.1 Salmon louse; biology, behaviour and host interactions

The life cycle of the salmon louse (Fig. 1.) includes both a planktonic and a parasitic phase. It consists of eight developmental stages where each instar is separated by a molting event where the exoskeleton is shed [20-22]. The developmental rate is temperature dependent and at 10°C, it takes around 40(♂)-52(♀) days to complete the lifecycle on *S. salar* [23]. The planktonic stages after hatching, nauplius I and II and the free-living copepodid are lecithotrophic (feeding on yolk reserves only [24, 25]. Planktonic stages are passively dispersed by the ocean currents that enables them to spread over great geographic distances and infect farming sites up to 30 km away [26, 27]. To increase host-parasite encounter, the infectious copepodid displays both positive phototactic and rheotaxic qualities. This allows the copepodid to seek towards the upper water column (> 27 ‰ salinity) where they react to pressure waves generated by a nearby swimming fish [28]. It is believed that the copepodids use chemo- and mechano sensors located on their frontal antennas to identify the right host [29, 30]. The copeodid attach to the host using the 2nd antennae which has hook like structures [13] and starts to feed instantly after attachment [31]. During molting into and through the two attached chalimus stages (Fig. 1.), the lice generate and remains attached to the host by a frontal filament [32]. Sexual dimorphism is apparent in the chalimus II stage [33]. After the chalimus stages, the louse molts into the mobile pre-adult I and II and, finally, the adult stage. In the mobile stages, the lice are able to feed over a larger area of the host and thereby increasing the virulence significantly [16]. Males become adults prior to the females and once males are adult, they locate immature pre-adult II female and engage in precopula. The male deposit spermatophores onto the genital complex of the female [34]. Despite deposition of the spermatophore and guarding of the females, polyandry is known to occur which can lead to multiple paternities [35, 36]. A female louse is thought to be able to

produce eggs throughout her lifetime (≤ 15.5 months in the lab) and each pair of egg strings can result in as much as 1200 eggs [36, 37].

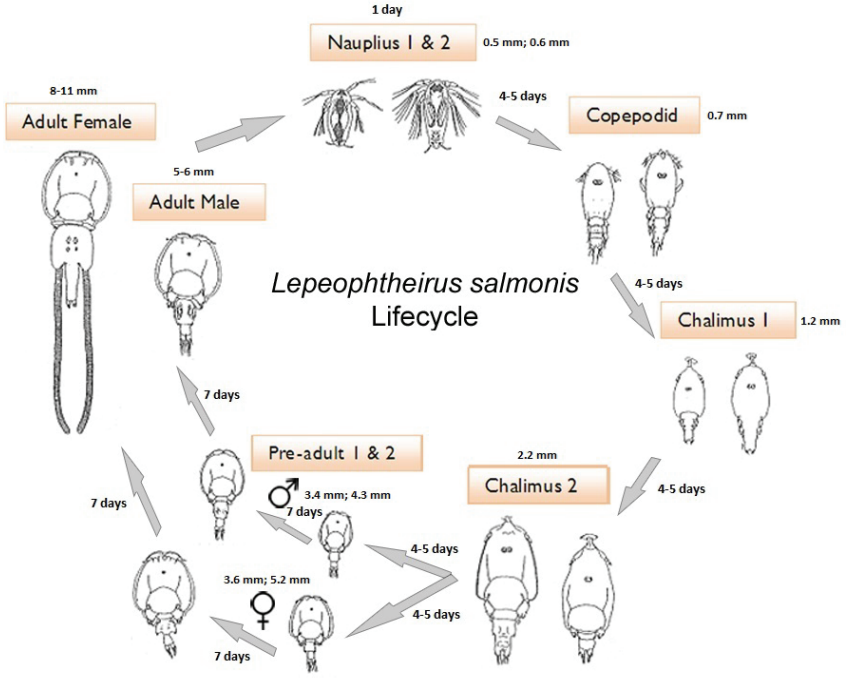

Fig. 1. Representation of the life cycle of *Lepeophtheirus salmonis*. The generation time of adult male and females are 40-52 days. Nauplia I/II and the free-swimming copepodid are nourished by yolk-reserved until the copepod becomes parasitic. The adult female generates a new pair of egg strings every ten days. Both development and reproduction is temperature dependent and the given time-points correspond to 10 °C. The figure is from Schram 1993 and adapted by The Marine Institute of Galtway.

L. salmonis infestations and the degree of damage that is inflicted upon the host largely depend on the amount of lice present and host size leaving post-smolts particularly vulnerable to the parasite [15, 38]. In addition, the effects of salmon louse significantly vary between species due to natural resistance. Juvenile pink (*O. gorbuscha*) and coho (*O. kisutch*) salmon rejects *L. salmonis* copepodids faster than chum (*O. keta*) and Atlantic salmon [39, 40]. Atlantic salmon has been shown to

possess a limited inflammatory response and epithelial hyperplasia compared to the coho and chinook (*O. nerka*) salmon [40]. Recent gene expression analysis of skin from susceptible and resistant species has found that the susceptible species have a lower expression of pro-inflammatory genes compared to more resistant species [39, 41-44]. Additionally, Atlantic salmon has compared to the more resistant salmonids a lower concentration of protective mucosal lysozymes and proteases due to thinner epidermis and less mucosal cells [45]. In addition, some studies have indicated that the mucosa of susceptible hosts stimulates secretion of trypsin-like proteases and prostaglandin E_2 (PGE_2) from the salmon louse, which is thought to modulate host immunity [46-49].

1.2.2 General anatomy of the salmon louse

Cuticle

The exoskeleton of arthropods consists of chitin, sclerotin and calcium carbonate, which pose a unique challenge for growth due to its rigid structural arrangement. In order to grow, arthropods must form a new skeleton before discarding the old cuticle (molt). Molting requires a series of physiological steps, which is initiated by separation of the old cuticula from the underlying epidermal layers. The newly created gap (exuvial space) is filled with molting fluid where the old exoskeleton is digested from underneath, and the protein components are absorbed and reused to build the new exoskeleton. The underlying tissue starts to secrete the new soft exoskeleton that is convoluted in order to expand when old exoskeleton is shed. After shedding, the new soft exoskeleton is inflated due to influx of water and hardening of the new exoskeleton (sclerotinization) begins. The new exoskeleton is folded allowing the animal to increase in size during the instar phase [33].

Subcuticular tissue

The subcuticular tissue (Fig. 2. black frame) is located underneath the exoskeleton (cuticula) and is found throughout the louse. The tissue is thought to perform functions similar to those of the liver and is the site of vitellogenin and yolk-

production in adult females [25, 50]. In addition, genes involved in fatty-acid metabolism and amino acid degradation is associated with the tissue [51] Different types of glandular tissues are present with in the subcuticular tissue [32].

Alimentary canal

No functional gut is present in the nauplius stage but intestinal tissue fully develops during the copepodid stage. The gut stretches all the way from the mouth located anteriorly in the cephalothorax to the rectum of the animal located posterior in the abdomen (Fig. 2.) [52]. Not surprisingly, expression of digestive enzymes i.e. proteases and lysosomal genes are present in the intestine [51, 53]. It has been shown that pancreatic function is located to the intestine in salmon louse [53], which is different from members of the malacostracan, which have hepatopancreas where liver and pancreas function is co-localized.

Reproductive organs

The ovaries (Fig. 2. white frame) and testes are paired organs located on each side of the coalesced eyes in the anterior part of the cephalothorax. The ovaries continuously produce oocytes that are transported via the oviduct to the genital segment where the oocyte matures. Vitellogenesis takes place in the genital segment. Maturation of the oocytes is temperature dependent and takes approximately 10 days at 10 °C. Spermatozytes are produced in the male testes and are transferred to the female via spermatophores that are deposited on the female genital segment [34].

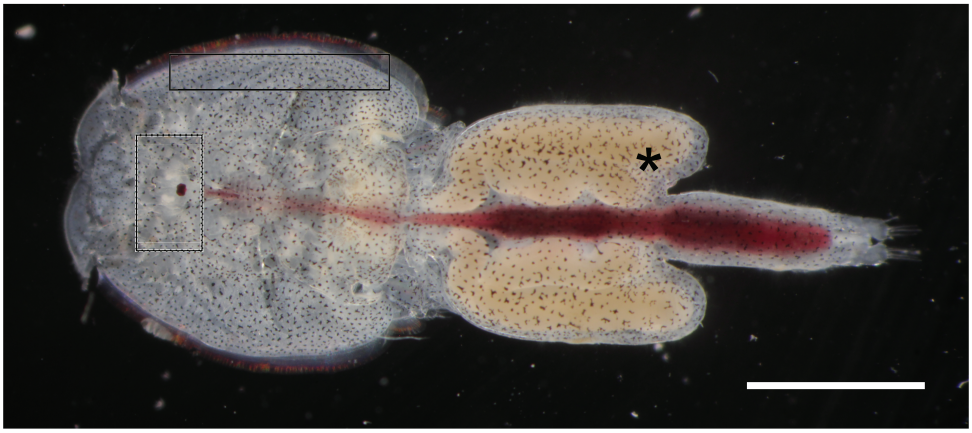

Fig. 2. *L. salmonis* adult female. The sub-cuticular tissue (black frame) is found underneath the cuticula around the edges of the cephalothorax (CT), abdomen/genital segment (Ab/G). The alimentary canal is blood filled and stretches from the CT to the anterior of the Ab/G. The ovaries (white frame) are situated in the front of the coalesced eye. Testes are found in the same position in male lice. The oviducts reach from the ovaries to the Ab/G. Maturing oocytes can be seen in the Ab/G (marked with asterisk). Scalebar = 5 mm

1.3 Nuclear receptors

Nuclear receptors (NRs) make up an ancient superfamily of mostly ligand-dependent transcription factors for cell growth and differentiation, metabolism, homeostasis and embryonic development by directly linking extracellular signals and transcriptional response [54-56]. Mutations in NRs are associated with many common and lethal disorders hence extensive research focus on understanding and modulating the NR functions in order to develop pharmaceuticals that target NRs. Members of the NR superfamily are believed to be present in all metazoans and are classified into six subfamilies based on multiple sequence alignments and phylogenetic analysis of conserved domains [54, 57-61]. NRs like the thyroid receptor, retinoic X receptor and steroid receptors (i.e. glucocorticoid receptor and the oestrogen receptor) are well studied in vertebrates and have given important knowledge to the nature of NRs and their ability to directly regulate gene expression. In insects, the ecdysone receptor (EcR) and its partner ultraspiracle (USP) has been subjected to intensive studies. However, much remains to be learned about the role of NRs physiological pathways

such as development and reproduction in other arthropods. In the human genome, 48 different NRs are identified compared to the *Drosophila* genome containing 21 [62], while 23 NR or NR-like sequences are predicted from the *L. salmonis* genome (Licebase.org: unpublished). The primary feature distinguishing the NRs from other transcription factors is their ability to bind ligands. The ligands include a vide range of small hydrophobic compounds that are derivatives of vitamins, retinoids, fatty acids, lipophilic hormones and cholesterol.

The NR genes presumably evolved from a common ancestor more than 700 million years ago and diversified and duplicated into the subfamilies known today. The evolved ability to bind ligands as well as the ability to homo- and heterodimerize and bind DNA, increased the possibilities and complexities of signal transduction and is considered a potential driving force in the evolution of higher organisms.

In classical signal transduction, an external ligand bind to a membrane-bound receptor that initiates a cascade of events in the cytoplasm, eventually activating nuclear transcription factors. In contrast to these very complex and "time-consuming" pathways, the NRs can shorten the time of signal transduction by their simultaneous ligand and DNA binding ability. This capability allows for signals to be transferred in a one-step response that directly affects the expression of the target gene. Generally, the NR signalling pathway is initiated by diffusion of hydrophobic ligands through the nuclear membrane followed by receptor binding or binding of ligand to the receptor in the cytoplasm, dependent on the receptor. Following binding of a ligand, the receptor complex will translocate to the nucleus or, if already present in the nucleus, bind to specific hormone response elements (HRE) (reviewed in [55]). Regulation of gene expression of NRs is enabled by recruitment of co-activators and co-repressors, which modulate gene transcription by modifying the chromatin architecture [2] (Fig. 3.) [3].

[2] Chromatin structure is modified by ATP-dependent chromatin remodelling complexes and histone modifying complexes. Enzymatic modification includes acetylation, methylation, phosphorylation and ubiquitinylation (e.g. acetylation relax chromatin structure and recruits the transcription machinery in contrast to methylation which condense the chromatin structure and prevents transcription).

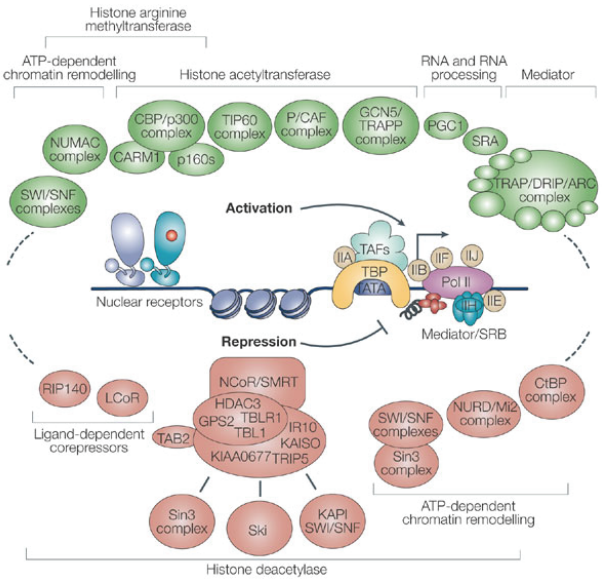

Fig. 3. Co-activator and co-repressor complexes are required for nuclear receptor-mediated transcriptional regulation. The figure illustrates the complexity of eukaryotic transcription.

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1.4 Discovery of the Ecdysone Receptor

In 1974, Ashburner and his colleagues postulated a hierarchical genetic response model for the puffing of polytene chromosomes induced by ecdysone based on the work performed earlier by Peter Karlson [63]. The model was based on studies of salivary glands from *Drosophila* where "puffs" occurred at specific loci on the chromosomes when treated with ecdysone. The "early puff" response was rapid and peaked at four hours in the presence of protein synthesis inhibitors, suggesting the ecdysteroids to act directly on the chromosomes. A set of "late puffs" was observed to follow the "early puff" response, however, the late puffs did not occur in the presence of protein inhibitors. The observations lead Ashburner and his colleagues to suggest that the ecdysone bound to a cognate receptor protein that directly activated "early puff" expression and that the protein product of the initial response induced a larger set of "late puffs" expression [64].

Based on the work of Ashburner and his colleagues, Koelle et al., [65] isolated and characterized the ecdysone receptor (EcR) from *Drosophila* by screening cDNA libraries for members of the steroid receptor family. Although the gene product acquired the properties consistent with an ecdysone receptor binding both active steroid and DNA, the receptor was later found only to be fully functional when dimerized to a second NR, ultraspiracle (USP) a homolog of the retinoid X receptor (RXR) found in vertebrates and crustaceans [66, 67]. The EcR was initially recognized as the molting receptor but considerable research the last few decades has shown that it is also a central regulator of major developmental and biological processes across the arthropod phyla. A number of EcR orthologs have also been identified in nematodes, molluscs, leeches, squid and polychaete worms [68]. The discovery of the ecdysone receptor as a nuclear receptor and target for ecdysone did not only revolutionize the field of arthropod endocrinology but also showed that the NRs evolved prior to the divergence of protostomes and deuterostomes.

1.4.1 Structural domains of the Ecdysone Receptor

The EcR is the ortholog to the vertebrate farnesoid X receptor (FXR) [69] and shares a similar organisation of domains and core modular architecture common to the NRs (Fig. 4.) [70]. Flanked between a highly variable N-terminal (A/B-domain) that harbour a ligand-independent-activation function-1 (AF-1) and the hinge region (D-domain) that plays a role in nuclear translocation [71] and subcellular distribution, is the central DNA-binding domain (DBD; C-domain). The DBD is highly conserved maintaining about 50 % identity between all NRs in the superfamily. The domain contains two zinc-finger motifs that facilitate both sequence-specific interaction with DNA and receptor complex-DNA dimerization [72]. Most importantly, in terms of function, is the ligand binding domain (LBD; E-domain) that includes the ligand-dependent transcription activation function-2 (AF-2). Moreover, some NRs contain a highly variable C-terminal F-domain that may be involved in the in co-factor recruitment [73].

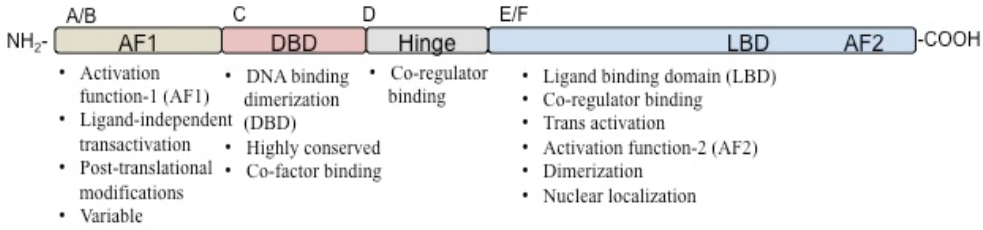

Fig. 4. Schematic overview of the primary structure of NRs- important properties is listed under the corresponding domain. The A/B domain (AF1) is associated with both ligand dependent and ligand independent transcriptional activation mediating crosstalk between signaling pathways. The C-domain (DNA-binding domain (DBD)) is primarily involved in DNA-dimerization and initiates binding of co-factors. The highly variable D-domain links the DBD and E/F region with the conserved ligand binding domain (LBD) which contributes to dimerization and recruitment of co-regulatory factors. The F part of the domain is highly variable and can be absent in some nuclear receptors.

Ligand binding

To date, the structure of five insect EcR-LBD/USP-LBD in complex with ecdysteroids or inhibitors have been determined by X-ray crystallography [74-77]. As expected, all EcR-LBD tertiary structures displays a canonical shape made up of 12 α-helices and an anti-parallel β-sheet that pack together and facilitate the formation of a hydrophobic ligand binding pocket (LBP). Although EcR is capable of ligand-binding in the absence of USP, the affinity for ecdysteroid binding increase significantly in the presence of its heterodimerization partner [78]. After binding of the ligand, dissociation of ligand-receptor complex is prevented by folding of Helix-12 (also called AF-2) across the pocket. The conformational change of the helix exposes an interactive surface enabling recruitment of co-activator proteins and members of the transcription initiation complex beginning transcription. The LBD of EcR is flexible and capable of adapting the LBP to ligands with different chemistries [79]. This feature explains how some arthropods can utilize various ecdysteroids to regulate development at different life stages. The absence or presence of ligand determines how the ecdysone receptor binds to DNA and associates with either co-activators or co-repressors (Fig. 3.) [80-82]. The USP receptor in insects is defined as an orphan receptor because it is locked in an antagonistic conformation preventing

binding of ligand [83]. In contrast, crustacean RXRs has the ability to bind ligands including 9-cis retinoic acid, methyl farnesoate and neurotransmitters [84, 85].

EcR/USP complex-DNA interactions

The DBDs of the EcR/USP binds as a heterodimer to specific ecdysteroid response elements (EcREs) which are half-sites with a one base pair spaced inverted repeat (palindrome; IR1; 5`-AGGTCA-3`) located in the regulatory regions of target genes [86-88]. Upon binding of DNA, the EcR/USP heterodimer adopts an asymmetrical organization that induces a conformational change in the LBD of USP, which stabilises the EcR/USP/DNA complex and aid in the fine-tuning of gene regulation [89].

1.4.2 EcR mediated ecdysone signalling

Ecdysone and ecdysone signalling has mostly been studied in holometabolous insects like the model organism *Drosophila melanogaster* due to the major transitional changes they undergo during metamorphosis. However, some crustacean species like shrimp, crabs, lobster and very recently the salmon louse has been increasingly investigated due to their commercial importance and, therefore, understanding of mechanisms regulating growth and reproduction has been the topics of investigation. Insects and crustaceans differ in many ways e.g. growth, sexual differentiation, reproduction and life cycles but common for both groups are that these biological events are regulated by ecdysteroids [90]. Additional neurohormones and peptide hormones such as hyperglycemic hormones (e.g. molt-inhibiting hormone among others) and farnesoic acids (e.g. methyl farnesoate) are important regulatory factors in addition to external environmental factors (e.g. temperature, nutrition and salinity). Crosstalk between signalling pathways regulated by these factors allows for adaptation of the hormonal response to meet the functional requirements of the target tissue (for more information please see reviews [90-93]). However, as these regulatory functions are beyond the scope of this study they will not be further addressed.

Just like the holometabolous insects, the molt cycle in crustaceans results in extensive physiological changes in addition to changes in the integument. Molting is not just restricted to shedding of the exoskeleton but also the series of events required for synthesis, degradation and exchange of the old exoskeleton to facilitate growth and metamorphosis[3] [94-96]. During metamorphosis, an extensive remodelling takes place in organs like the hepatopancreas, muscles and neurological and adipose tissue in order to morphologically adapt into the new life stage. During this remodelling, some tissue is triggered to undergo programmed cell death and histolysis and some tissue will grow and differentiate while others will not respond at all [97]. All the processes associated with molting and metamorphosis, are triggered by pulses and fluctuating levels of circulating ecdysone (reviewed in [55]). During the molting cycle, the concentration of ecdysone can fluctuate dramatically (e.g. between < 10 ng/ul and > 350 ng/ul) in juvenile lobster *Homarus americanus* [98] in a time-dependent manner. The titer remains low during intermolt and postmolt stages but a peak is reached in the premolt stage with an abrupt drop in ecdysteroid concentration that triggers the shedding of the exoskeleton [55, 99].

The extensive physiological and biological changes that take place during the life cycle is achieved by binding of ecdysone to the EcR/RXR complex, which results as proposed by Ashburner et al., [64] in the regulation of a conserved hierarchical cascade of hundreds of ecdysone-responsive early genes and early-late genes. The ecdysone derivative 20E has in both insects and found to be biologically active hormone during molting [100] in addition to ponasterone A (PonA) present in crustaceans. The gene products include but are not limited to the transcription factors E74, E75, *Drosophila* hormone receptor 3 (DHR3), Broad-Complex (Br-C) and FTZ transcription factor-1 (FTZ-F1) [101-105]. Products of the early genes subsequently regulate ecdysone responsive late-genes that determine the phenotypic effects of the ecdysteroids in a time and tissue specific manner [106]. In addition, in response to the

[3] Not all crustaceans go through complete metamorphosis. In addition, molting pattern may vary between species.

increase in ecdysone level, EcR provides an auto-regulatory loop and activate its own transcription thereby increasing its own expression [65].

EcR is typically found in different forms that allows for differential regulation within time and space. The key role that EcR plays in this comprehensive diversity of physiological and morphological processes is partially due to the various isoforms. This allows for differences in the receptor`s ability to repress and activate expression of down-stream genes and hence influence separate physiological functions. Most of the EcR variants differ mainly in the N-terminal region which is associated with regulation of transcription [107-109], however, splice variations of the hinge region and the LBD has been identified in some crustaceans including, but not exclusive to the fiddler crab *Uca pugilator* [110], the kuruma prawn *Marsupenaeus japonicus* [111], the freshwater prawn *Macrobrachium nipponense* [112] and the water flea *D. magna* [113]. In *Drosophila* and *Manduca sexta*, three EcR isoforms (EcRA, EcRB1 and EcRB2) differing in the length of their N-terminal have been identified with varying biological effects at different time-points [109, 114]. Mutations that block all three variants of EcR cause embryonic lethality while removal of one isoform cause effects in specific physiological processes. In *Drosophila*, EcRA is predominantly expressed in cells that proliferate and differentiate during metamorphosis of adult stages, whereas isoform EcRB1 and B2 are essentially expressed during larval stages in cells that enter apoptosis [115]. During arthropod development, the neuronal tissue undergo crucial remodelling of the mushroom bodies (MBs: the brain memory centre), olfactory circuits and neuromuscular junctions where axons and dendrites are pruned and regrown to fit their new functions. All neuronal remodelling events depend on the EcR, however, in an isoform-specific manner (reviewed in [116]).

Growth and reproduction are two processes that are tightly regulated and connected in arthropods and ecdysteroids play a key role in both of them. The role of ecdysteroids in insect reproductive processes is well established and has shown to be important in vitellogenesis [117, 118], follicle development [119] and ovarian and oocyte development [120]. Depletion of the EcR level is associated with oogenic defects such as the presence of abnormal egg chambers and loss of vitellogenic stages

[120-124]. Although ecdysteroids are primarily considered to be molting hormones in crustaceans, it has become evident from recent studies that they also play a role in reproductive processes (e.g. vitellogenesis and ovarian maturation) [125, 126].

The significant effect of ecdysteroids on gene regulation is also evident through several transcriptomic studies performed in *Drosophila* Kc cells. Consistent with the morphological changes during metamorphosis, genes encoding proteins involved in cell movement and organization associated with the cytoskeleton has shown to be regulated by 20E [127]. In addition, several members of the cytochrome P450 family, stress-response genes, lipid transporters, starvation-genes (i.e. peptidases) and Toll-ligand response genes were regulated by 20E in an EcR- dependent manner [127, 128]. These observations indicate that ecdysone signalling regulates many metabolic processes as well as the immune response. This is in accordance with microarray analysis of *L. salmonis* RXR knock-down lice where genes involved in fatty acid metabolism and transport, steroid biosynthesis and genes involved din different metabolic pathways (e.g. chitin metabolism and digestion) were regulated [129]. These studies demonstrate that ecdysteroids are associated with a large gene-regulatory network, which illustrates the complexity involved in endocrine signalling.

1.5 Biosynthesis of ecdysteroids

Arthropods are incapable of synthesizing cholesterol *de novo* and are dependent on uptake of cholesterol or alkylated plant sterols through the diet for ecdysteroid synthesis. Ecdysteroids are polyhydroxylated steroid hormones that are synthesized by steroidogenic enzymes classified as members of the cytochrome P450 (CYP) family commonly known as the Halloween genes [1, 2, 130-133]. The biosynthesis of ecdysteroid hormones takes place in specific hormone producing tissues or glands such as the PG of insect larva [134], the ovarian follicle cells of adult insects and the Y-organ (YO) in decapods crustaceans [135]. The ecdysteroids are then secreted into the circulatory system and transported to the peripheral tissue where conversion into the active metabolite by *shade* takes place. No tissue like the Y-organ has so far been identified in microcrustaceans such as the copepods.

The biosynthetic pathway of ecdysteroids is complex and can according to Mykles et al., [1] be divided into two stages: (1) conversion of cholesterol to 5β-diketol and (2) hydroxylation of 5β-diketol to the secreted steroid (Fig. 5.). The first part of the biosynthetic pathway where cholesterol is converted into 5β-diketol by the 7,8-dehydrogenase *neverland* is similar in insects and crustaceans but the second part is more complex in crustaceans as they produce a broader range of ecdysteroids [136-140]. The enzymatic conversion of 5β-diketol to the active metabolite is performed by the Halloween genes *phantom* (CYP306A1), *disembodied* (CYP302A1), *shadow* (CYP315A1) and *shade* (CYP314A1) where each gene is believed to perform one specific hydroxylation as mutations in these genes have resulted in low ecdysteroid levels, abnormalities in cuticula formation and embryonic death [130, 141-143]. Additional enzymes are contributing to the biosynthetic steps called "the Black box" which are a series of hypothetical reactions that results in the conversion of 7-dehydrocholesterol to ketodiol. The precise intermediates in these steps are currently unknown, due to their chemical instability, however, the four enzymes CYP307A1/*spook* (*spo*) [144], CYP307A2/*spookier* (*spok)* [144, 145], CYP6T3 [146] and *non-molting glossy/shroud* (*nm-g/sro*) [147] have been characterized and are considered to act during these steps. In decapod crustaceans, it has been suggested that the biosynthetic pathway has branching points at the conversion of diketol and ketodiol (see [1] for extensive description) resulting in four final ecdysteroid products that are converted to either 3-dehydro-20-hydroxyecdysone, 20E or PonA by *shade* dependent on the precursor steroid. For simplicity, only one pathway is presented in Fig. 5.

Although the biosynthesis of ecdysteroids have mainly been investigated in insects and to some degree in decapod crustaceans, orthologs of the Halloween genes have been identified in microcrustaceans such as the branchiopod water flea *Daphnia magna* [148], the copepod *Calanus finmarchicus* [149] and the salmon lice *Caligus rogercresseyi* [150] and *Lepeophtheirus salmonis* (present study).

Octopamine receptor

It has been suggested that ecdysteroids are capable of interacting with G protein-coupled receptors (GPCRs) thereby activating a broad range of signalling pathways. The GPCRs is recognized by a structural motif containing seven transmembrane domains that show considerable diversity in their sequences [151]. One such GPCR is the octopamine receptor (OctR), which is known to bind the biogenic amids octopamine and tyramine, which acts as neurotransmitters, neuromodulators and neurohormones in both vertebrates and invertebrates (reviewed in [152]). The OctR mediates attenuation of adenylyl cyclase, which induce responses of secondary messengers such as cyclic nucleotides (cAMP, cGMP), calcium ions (Ca^{2+}) and inositol-1,4,5-triphosphate (IP_3) that in turn regulates the activities of enzymes and nonenzymatic proteins in a wide variety of signalling pathways. Four different octopamine receptors (Oamb, Oct1βR, Oct2βR, Oct3βR) have been identified in *Drosophila* which all are expressed in the central nervous system but differ in their expression pattern in the peripheral tissues [153]. The presence of several octopamine receptors contributes to many behavioural and physiological reactions [154, 155] and more importantly for this thesis, the biosynthesis of ecdysteroids [156].

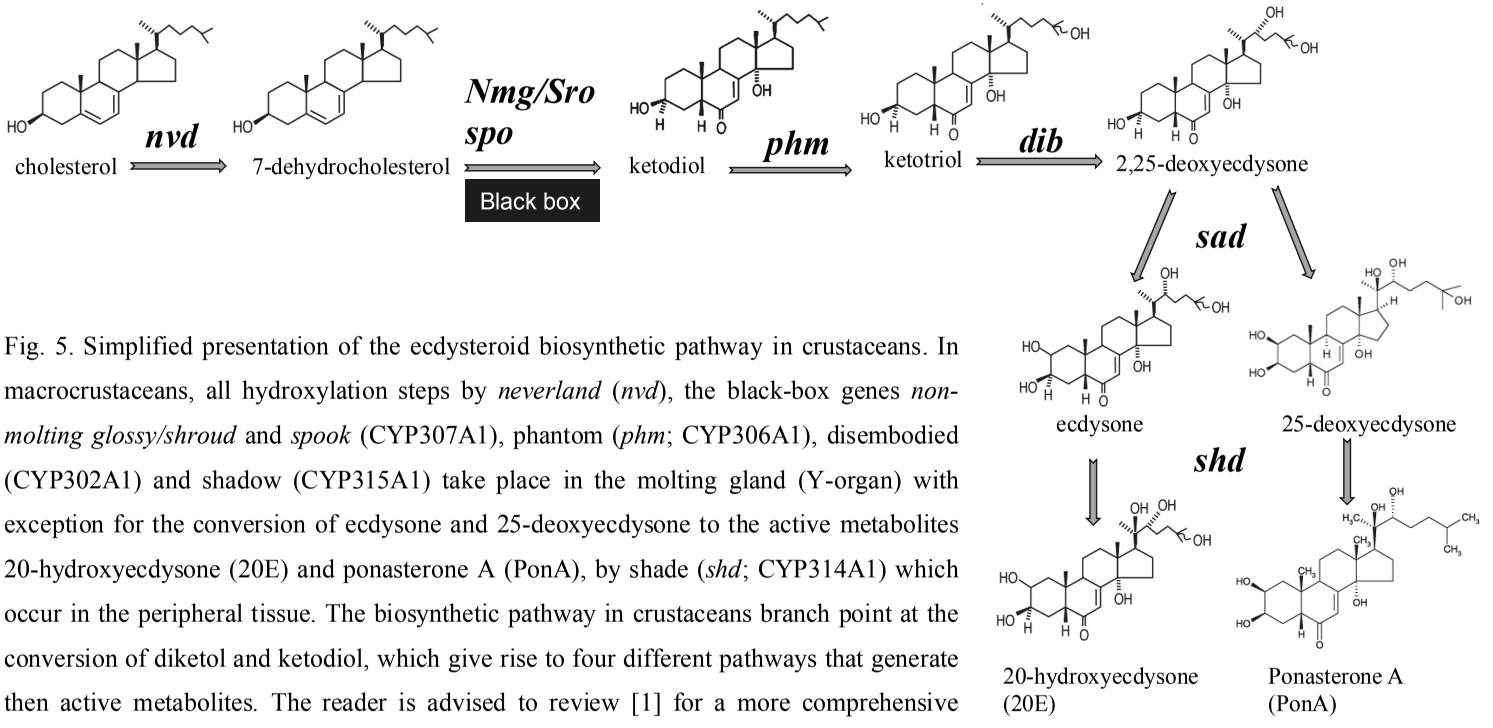

Fig. 5. Simplified presentation of the ecdysteroid biosynthetic pathway in crustaceans. In macrocrustaceans, all hydroxylation steps by *neverland* (*nvd*), the black-box genes *non-molting glossy/shroud* and *spook* (CYP307A1), phantom (*phm*; CYP306A1), disembodied (CYP302A1) and shadow (CYP315A1) take place in the molting gland (Y-organ) with exception for the conversion of ecdysone and 25-deoxyecdysone to the active metabolites 20-hydroxyecdysone (20E) and ponasterone A (PonA), by shade (*shd*; CYP314A1) which occur in the peripheral tissue. The biosynthetic pathway in crustaceans branch point at the conversion of diketol and ketodiol, which give rise to four different pathways that generate then active metabolites. The reader is advised to review [1] for a more comprehensive description. Modified from [2].

1.6 RNA interference as an experimental tool

RNA interference (RNAi) is a natural biological process in which small RNA molecules inhibit gene expression. It was first described by Fire et al., [157] in the nematode worm *Caenorhabditis elegans* and has since then been applied as an experimental tool to study the gene function in cell cultures and *in vivo* in different organisms. This is achieved by introducing double-stranded RNA (dsRNA) into an organism to manipulate gene expression. In this process, the complementary strand of the dsRNA becomes part of the RNA-induced silencing complex (RISC) a multi-protein complex that identifies the corresponding target mRNA and cleaves it at a specific site. Next, the cleaved mRNA is targeted for degradation, which results in the loss of protein expression. The double-stranded RNA can be introduced in several ways such as RNAi vectors, soaking, through food or injection [157, 158].

Since the discovery of RNAi as an analytical tool, a plethora of studies using dsRNA for gene knock-down has been applied in a variety of metazoan species to study the functionality of genes essential in development, growth and reproduction [50, 159-161]. This includes genes involved in ecdysteroidogenesis and ecdysteroid signal transduction such as the *EcR* and *RXR* genes [123, 129, 162-164].

Most RNAi studies in crustaceans have been performed on commercially important decapods but have very recently been employed to microcrustaceans such as the branchiopod *Daphnia pulex* [165, 166] and the copepod *Lepeophtheirus salmonis* [25, 167]. In the *L. salmonis*, protocols have been developed in order to perform functional studies both in larval [167] and pre-adult stages [25]. In larval stages, gene silencing is achieved by soaking nauplius I larva in dsRNA during their molt into the nauplius II stage. The experiments are normally terminated when the control animals reach the copepodid stage and potential phenotypes can be determined. Pre-adult animals, on the other hand, are injected with dsRNA in the CT and put on salmon for approximately 35 - 40 days. The knock-down effect has been detected two days after both treatment methods, however, the degree of knock-down decrease during the time

after treatment and has shown to cease between 14 - 40 days in the adult animals dependent on the gene [167, 168].

RNAi provides an efficient tool to functionally assess genes within a genome and evaluate their role in signalling pathways or physiological processes. Dependent on the target gene, phenotypic traits be a direct effect of gene knock-down [25] however, it is important to keep in mind that phenotypes can be caused by indirect effects of decreased gene expression as reduced expression of some genes can affect many molecular processes. Moreover, it is crucial to distinguish between gene knock-down where gene expression is reduced as opposed to gene knock-out where gene expression is eliminated.

2. Aims of the study

The overall objective of this research was to gain knowledge of the endocrine system in the salmon louse on a molecular level focusing on the ecdysone receptor (EcR). Ecdysone signalling through the EcR/RXR nuclear complex is well known to play vital roles in a multitude of biological processes in all arthropod species. Interference of the ecdysone signalling pathway is associated with molting arrest, embryonic death and disruption of reproductive processes. Therefore, it is of great interest to gain knowledge of the ecdysone signalling pathway in *L. salmonis.* Using RNA interference (RNAi) techniques we can get an in-depth understanding of the functionality of genes involved in the pathway, which in the future can be used in parasite control. The specific objectives for the present study were:

- To characterize the *LsEcR* gene, describe the transcript expression pattern and study its functional role in reproduction using RNAi in adult female lice
- To study the transcript pattern and function of the EcR during molting and development in salmon louse larvae through knock-down studies
- To investigate the temporal expression pattern of the ecdysteroids: ecdysone, 20-hydroxyecdysone and ponasterone A during molting and oocyte maturing using LC/MS/MS
- To identify genes involved in the biosynthesis of ecdysteroids and investigate their function during early developmental stages

List of publications

Paper I: Liv Sandlund, Frank Nilsen, Rune Male, Sindre Grotmol, Heidi Kongshaug and Sussie Dalvin (2015). Molecular characterisation of the salmon louse, *Lepeophtheirus salmonis salmonis* (Krøyer, 1837), ecdysone receptor with emphasis on functional studies of female reproduction. International Journal for Parasitology, 45:175-185

Paper II: Liv Sandlund, Frank Nilsen, Rune Male and Sussie Dalvin (2016). The Ecdysone Receptor (EcR) is a Major Regulator of Tissue Development and Growth in the Marine Salmonid Ectoparasite, *Lepeophtheirus salmonis* (Copepoda, Caligidae). *Molecular and Biochemical Parasitology*

Accepted for publication after minor reviews

Paper III: Liv Sandlund, Rune Male, Tor Einar Horsberg, Frank Nilsen, Heidi Kongshaug and Sussie Dalvin (2016). Identification and functional assessment of the ecdysone biosynthetic genes *neverland, disembodied* and *shade* in the salmon louse *Lepeophtheirus salmonis* (Copepoda, Caligidae).

(Manuscript)

3. Abstract of papers

3.1 Paper I:

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 million US dollars worldwide. To facilitate development of a vaccine or other novel control 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 (EcR) and the retinoid X receptor, ultraspiracle (USP) 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 characterized cDNA with the 5´untranslated region of the predicted *L. salmonis EcR (LsEcR)*. The *LsEcR* cDNA was 1608 bp encoding a 536 aa sequence that demonstrated high sequence similarities level to other arthropod EcRs including *Tribolium castaneum* and *Locusta migratoria*. Moreover, *in situ* analysis of adult female louse revealed *LsEcR* transcript to be localized 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. This is the first report of on the ecdysone receptor in the economically important parasite *L. salmonis*.

3.2 Paper II:

The function of the ecdysone receptor (EcR) during development and molting has been thoroughly investigated in 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 cause major economical expenses 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 fibers and the immature intestine. 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* 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.

3.3 Paper III:

The salmon louse is a marine ectoparasitic copepod on salmonis fishes. Its lifecycle consists of eight developmental stages, each separated by a molt. In crustaceans and insects, molting and reproduction is controlled by circulating steroid hormones such as 20-hydroxyecdysone (20E). Steroid hormones are synthesized from cholesterol through catalytic reactions involving a 7,8-dehydrogenase *neverland* and several cytochrome P450 genes collectively called the Halloween genes. In this study, we have isolated and identified orthologs of *neverland* (*nvd*), *disembodied* (*dib*) and *shade* (*shd*) in the salmon louse *L. salmonis* genome. Tissue-specific expression analysis showed that the genes are expressed in intestine and reproductive tissue. Furthermore, knock-down studies using RNA interference in adult females showed that only *shd* terminates molting in larval stages. However, knock-down of *nvd* affected development of the ovaries and oocyte maturation. In addition, we performed knock-down studies of an ortholog of the *Drosophila* octopamine receptor (*Oct3βR*) a regulator of the Halloween genes, to determine its role during early development. Depletion of the *Oct3βR* ortholog in *L. salmonis* resulted in molting arrest, but did not down-regulate expression of all of the identified Halloween genes.

Our results show that ecdysone biosynthetic genes are present in *L. salmoni*s and that ecdysteroids is necessary for both molting and reproduction in the lice.

4. General Discussion

Steroid hormones have an essential role in regulating biological processes within all animals. In arthropods, one of the main steroid hormones is ecdysone (with its metabolic variants). Ecdysteroid signalling is crucial in arthropod physiology regulating a wide diversity of biological processes. Biologically active ecdysteroid hormones are synthesized from cholesterol via several enzymatic steps before it transducing its signal by binding to the EcR/RXR nuclear receptor complex. A wide range of studies have shown the importance of EcR/RXR signalling in hexapod insects but for many groups of arthropods like the copepods, very limited information exist about this basic biological system. In insects, it has been demonstrated that the ecdysteroid pathways has a key role in developmental transitions [123, 169-171] and reproduction [115, 119, 120, 124, 172, 173]. From arthropods other than hexapods the literature is more limited but some studies have been conducted in decapods, ticks, daphnia and copepods [110, 148, 174, 175] (present study).

In model organisms like *Drosophila,* detailed information exists about ecdysteroid function through a wide range of studies. This includes knock-out studies where it has been demonstrated that mutation and depletion of EcR are associated with embryonic lethality and disruption of reproductive processes. Hence, uncovering the functions of EcR as well as regulation of ecdysone production is an important step towards understanding basic physiological processes in the salmon louse. Emphasis is given to the function of the EcR during molting, development and reproduction through a range of experiments and functional studies (see **paper I**/**II**). To further understand ecdysteroid function, key genes involved in ecdysteroid biosynthesis and regulation (octopamine receptor) have been identified and functionally assessed (**paper III**).

4.1 Identification of ecdysteroidogenic genes and the ecdysone receptor

4.1.1 Characterization of the ecdysone biosynthetic genes: *neverland*, *disembodied* and *shade* in *L. salmonis*

Ecdysteroids are synthesized through several enzymatic steps performed by dehydrogenases and members of the cytochrome P450 family proteins coded by the so-called Halloween genes. In **paper III**, orthologs of the 7,8 dehydrogenase *neverland (nvd)* and transcripts from the Halloween genes *disembodied (dib)* and *shade (shd)* were cloned and sequenced from the *L. salmonis* genome. The amino acid (aa) sequence of all three enzymes showed low sequence identity to their orthologs, which is commonly seen in the CYP family. Only 33 – 40 % sequence identity is found between lepidopteran and dipterian Halloween gene orthologs but their functions are conserved [176]. However, it should be noted that one amino acid substitution of essential residues could change the catalytic specificity. As mentioned in the introduction, the ecdysteroid synthetic pathway is far more complex in crustaceans than insects. Both *dib* and *shd* can hydroxylate several different compounds (reviewed in [1]) indicating that the Halloween genes identified in *L. salmonis* could acquire the same competence. Only one transcript encoding one single ORF has so far been identified for each gene. However, successful sequencing of the 5`UTR was not accomplished in this study, hence, it cannot be excluded that several transcripts exist for the three genes.

4.1.2 Characterisation of the *L. salmonis EcR*

In **paper I**, three mRNA transcripts with highly different 5' UTRs and alternative splicing, but encoding only one ORF of the *L. salmonis EcR* was identified based on sequencing and identity of conserved domains. Comparisons of *L. salmonis* EcR with other EcRs showed primary structural conservation across phyla (Fig. 2., **paper I**). Due to high degree of sequence similarities between the DBDs and to some extent the LBDs, we can assume that the *LsEcR* share similar functions as its homologs. Salmon

louse has one copy of EcR and RXR (**paper I** and [129]) however, a minimum of three different ORF isoforms was identified for *LsRXR.*

In mammals, variations of the 5`UTR of genes are relatively common and can either be expressed using different promoters [177, 178] or by alternative splicing within the UTR [179]. In *L. salmonis* it is evident that different levels of the *EcR* mRNA variants are present at different life stages. This suggests that *EcR* transcription is regulated by different promoters and/or alternative splicing during lice ontogeny. This could allow for rapid changes in gene expression in response to different stimuli e.g. during development and cell differentiation in a spatial and temporal manner (reviewed in [180-182]. However, in order to verify this, targeted studies investigating promoter function should be undertaken.

4.2 Functional assessment of *LsEcR* during molting and development

Developmental processes from embryogenesis to life stage transitions are under the influence of ecdysteroids in arthropods. In order to gain a deeper understanding of ecdysteroid signalling during molting of the salmon louse, attempts were made to measure the level of the three main steroid hormones known to be present in crustaceans: E, 20E and PonA, during the molting cycle of the copepod stage (**paper III**). This was challenging due to the combination of small sample sizes of the larval stages and technical difficulties with the LC/MS/MS. However, from the preliminary experiments, it was established that ecdysteroids are key regulators of molting in *L. salmonis.* PonA was present at higher levels compared to the other measured ecdysteroids in the pre-molting stage, which is in accordance with results from the shore crab *Carcinus maenas* [183]. Although we have not been able to successfully perform time specific measurements of ecdysteroid levels throughout the molting cycle in copepodids, the obtained data gives information of the ecdysteroid content in *L. salmonis* and provides valuable information to our understanding of the ecdysteroid regulatory system in the salmon louse.

Ecdysteroids exert their effect through the EcR/RXR receptor complex and reduced expression of *EcR* during early life stages is associated with molting defects and lethality [123, 169, 184, 185]. Surprisingly, RNAi knock-down of *LsEcR* in nauplia I larva did not cause immediate molting arrest, but resulted in viable copepodids identical to the control group even though significant knock-down of *LsEcR* was achieved (**paper II**). No deviation in swimming behaviour or histological aberrations (in sections) was detected in the *LsEcR* knock-down (*LsEcRkd*) copepodids. However, it should be noted that detection of LsEcR protein in the salmon louse has not been successful in this study and, therefore, we cannot rule out the possibility that residual protein sufficient to mediate ecdysone response is still present. Interestingly, similar results were observed in *LsRXR* knock-down larvae [167]. Although the ecdysone receptor is normally thought to act in a heterodimer with RXR, ligand binding without RXR has been reported [186]. These findings indicate that both members of the EcR/RXR complex can act as a receptor and function under certain conditions without its partner in other species. The same situation may apply in the salmon louse.

However, further incubation of *LsEcR* knock-down animals (on fish) resulted in high mortality and the surviving lice exhibited severe tissue damage in their pre-adult stage (**paper II**). During *Drosophila* metamorphosis, extensive neuronal remodelling by pruning and regrowth of axons and neuronal cell death are essential in order to establish the fully mature brain architecture and connectivity between motor neurons and muscles necessary for muscle growth [187, 188]. Ecdysteroids play a key role in the regulation of neuronal remodelling through the EcR/USP receptor complex [189] hence, it is reasonable to assume that the severe degeneration of both neuronal and muscle tissue found in the *LsEcR*kd lice, is caused by silencing of *LsEcR*. In a recent study, Eichner et al., [129] showed that both an ortholog of advillin, a Ca^{2+}-regulated actin binding protein important in nervous system development and motor neuron protein precursors involved in regulating motor neuron differentiation and survival was down-regulated in *LsRXR* knock-down lice. In addition, genes regulating muscle growth such as *tropomyosin-2, twitchin* and *myosin heavy chain* were down-regulated

accordingly, indicating that also muscle development is regulated, either directly or indirectly, by the ecdysteroid pathway in *L. salmonis*.

Programmed cell death (PCD) has shown to be involved in both neuronal remodelling and organogenesis in insects in response to ecdysone [116, 119, 190]. RNAi studies in *T. castaneum* showed that EcRA plays a critical role in the 20E regulation of midgut remodelling through the EcR/RXR complex [191]. Midgut reorganisation is required in holometabolous insects and amphibians due to dietary changes between larval and adult stages [192]. The salmon louse shift from using yolk as the only energy source in the free-living stages to digesting host mucosa, skin and blood when they infect the host and initiate the parasitic phase of the life cycle. It is highly likely that differentiation of intestinal tissue occurs in order to adapt to the new diet and it is possible that ecdysteroids have a significant role in this process. The midgut epithelium in *Drosophila* and *T. castaneum* is replaced during metamorphosis by PCD and activation of caspases is triggered by induction of BR-C and E93 through ecdysone activation [190]. Eichner et al., [129] found that apoptosis regulating factors (inhibitor of apoptosis 2 protein) are down-regulated after knock-down of *LsRXR*. Considering that EcR and RXR to a large extent govern the same pathways, it is not unlikely that *LsEcR* knock-down could influence midgut development (e.g. through PCD) explaining the large developmental abnormalities found in pre-adults when *LsEcR* was silenced (**paper II**). However, to confirm if abnormal PCD activity creates these developmental aberrations further studies need to be performed (e.g. assess expression levels of caspases and other key genes important in PCD in *LsEcR*kd lice).

We observed a striking difference in phenotype between individual knock-down of *LsEcR* and *LsRXR* compared to the combined knock-down of the receptors. The double knock-down animals (*LsEcR/LsRXR*) failed to enter the copepodid stage whereas single knock-downs successfully molted into apparently healthy infectious copepodids (**paper II**). In order to molt, arthropods are strictly dependent on the ability to remodel chitinous structures and malfunction in chitin metabolism leads to developmental disorders. Ecdysteroids have shown to be both positive and negative

regulators of chitin synthesis [193]. In *Drosophila*, it is indicated that ecdysone through the activation of EcR/RXR has a direct regulatory role of the chitin synthase genes as EcREs are found in their promoter region [194]. The promoter region for the two chitin synthases present in *L. salmonis* has not been examined for EcREs. However, down-regulation of sub-cuticular *LsCHS2* in both *LsEcR* and *LsEcR/LsRXR* knock-down lice strongly suggests that they are regulated by the ecdysteroid pathway through the EcR/RXR complex (**paper II**). RNAi experiments in *Drosophila* embryo showed that the CHS1 gene *krotzkopf-verkehrt* (kkv) is essential for maintaining the structure of procuticula and stabilisation of the epicuticula as well as epidermal morphology. Sclerotization and melanization were additionally impaired in these animals, suggesting that the activity of chitin synthases regulate several enzymes in chitin metabolism [195]. It is indeed possible that the molting arrest of *LsEcR*kd*/LsRXR*kd animals is caused by disruption of chitin metabolism. Furthermore, even though no visible phenotype was observed in the *LsEcR*kd animals in the copepodid stage, several enzymes important in chitin metabolism (*LsCP1, LsCHS2, LsChs1, LsChs2*) were affected which could cause discrepancies in the molting process. This could account for the high mortality observed for the *LsEcR*kd lice during the infection trial (**paper II**).

4.3 Functional assessment of *LsEcR* during reproduction

Ecdysteroid signalling is essential for reproduction in *L. salmonis.* Transcript knock-down of the ecdysone biosynthetic enzyme *LsNvd* (**paper III**) as well as the transcription factor *LsEcR* (**paper I**) inhibits the development of reproductive tissues, but more severely for the latter where egg production is completely abolished and females do not extrude any eggs.

In *D. magna*, ovarian ecdysteroids are transferred into the oocytes as free ecdysteroids or as polar (mostly phosphate esters) or apolar (e.g. long chained fatty acid esters) ecdysteroid conjugates. It is suggested that the purpose of the conjugates is to act as inactive storage forms of maternally derived hormones that can be

hydrolyzed into active ecdysteroids during embryogenesis [125]. Transcripts of both *LsNvd* and very low levels of the two Halloween genes are found in the ovaries (In-situ hybridization and Licebase.org; unpublished) of *L. salmonis* (**paper III**) suggesting that either complete and/or partial biosynthesis of ecdysteroids take place in the reproductive tissue. This is supported by the observed irregularities of the ovaries and follicular epithelium lining the oocytes in the adult female lice when *LsNvd* transcripts are significantly reduced (**paper III**). A complex network of signalling events acts to establish the lining of the follicular cell layer of the oocyte during maturation. In both *Drosophila* and *T. castaneum*, depletion of EcR disrupts development of the follicular cell layer necessary for oocyte maturation and loss of vitellogenic stages thereby preventing embryogenesis. Blocking of EcR signalling in follicular cells prevents proper organisation of the oocyte membrane presumably causing anomalies in the actin cytoskeleton in the microvilli [196]. Microvilli are important in the assembly of the vitelline membrane [197] which stored information of embryo patterning in *Drosophila*. Disruption of the vitelline membrane aborts embryonic development due to loss of eggshell function resulting in sterile females [198]. The only invertebrate where this is described in detail is for *Drosophila* and although the evolutionary distance to *L. salmonis* is large it is tempting to speculate that the lack of normal egg chamber generation and egg string formation in adult female *LsEcR*kd lice (**paper I**) is caused by aberrations in the cytoskeleton. This speculation is further supported by up-regulation of actin depolymerisation factors in *LsRXR*kd lice [129] that indicates impaired cytoskeleton function by increased depolymerisation. Dysfunction of the cytoskeletal components interferes with a cascade of events such as cell differentiation, vesicle/organelle trafficking and synaptic signalling. Based on this, it is possible that the extensive tissue damage observed in *LsEcR*kd animals in the infection trial (**paper II**) is linked to alterations in the actin filaments. In contrast to knock-down of *LsEcR*, knock-down studies of total *LsRXR* transcript in adult females showed that the females were able to generate and protrude egg strings, which either did not hatch or produced offspring that was not viable [129]. Even though *LsEcR* and *LsRXR* transcripts locate to the same tissue and the same degree of knock-down is achieved (~ 60 %), it appears that the absence

of *LsEcR* results in larger consequences for the reproductive processes than the absence of *LsRXR*. Histological assessment of the genital segment of both *LsEcR* and *LsRXR* knock-down lice shows the presence of individual oocytes in the *LsRXR*kd lice, whereas the oocytes are completely disintegrated in the *LsEcR*kd lice. As mentioned earlier, the presence of residual LsRXR protein or homo/ heterodimerization with an alternative partner has to be taken into account.

The last molt of the *L. salmonis* life cycle occurs between the pre-adult II and adult stage hence possible variations in ecdysteroid level in the adult female lice is presumably related to reproductive processes. Since ecdysteroids are key regulators of arthropod reproduction, investigation of the ecdysteroid level in adult female lice was performed. In **Paper III** we demonstrated that the ecdysteroid level is significantly different between the CT and the Ab/G segment of gravid adult females. One explanation for this is that ecdysteroids in CT are important for oocyte production and maturation as well as yolk production while the high levels in the genital segments could serve as a source for maternally provided ecdysteroids. Presence of *LsNvd, LsDib* and *LsShd* mRNA transcripts in unfertilised oocytes suggests that the oocytes are capable of *de novo* synthesis from cholesterol, supporting the high level of ecdysteroids present at the end of *L. salmonis* oocyte maturation in. The rise in the ecdysteroid level confirms previous reports from other crustaceans where ecdysteroids are associated with promotion of the ovary [125, 175, 199]. In adult *Drosophila*, ecdysteroids stimulate both proliferation and maintenance of germline stem cells through EcR signalling. It has also been shown that ecdysone signalling is required in follicle formation in somatic cells (review in [122]), which is the site for vitellogenin synthesis in adult insects. In *L. salmonis*, yolk production takes place in the sub-cuticular tissue. The yolk proteins are taken up by oocytes in the genital segment [25, 50] and are either directly or indirectly dependent on EcR-transduced ecdysteroid signalling to occur (**paper I**). Knock-down of LsRXR had a similar effect on yolk protein production [129]. Since the salmon louse is lecitotrophic, the eggs must be supplemented with sufficient nutrition that ensures proper embryogenesis and development until the parasitic copepodid can take up

external food, which occurs after successful host settlement. Energy conservation by incorporation of nutrients (i.e. aa, lipids, carbohydrates, proteins and hormonal metabolites) into the maturing oocyte is, therefore, beneficial in order to prolong the free-living lifetime of the lice. The presence of ecdysteroids in the oocytes could also explain why only *LsShd* gives a significant phenotype upon larval knock-down whereas *LsNvd* and *LsDib* gave no phenotype (**paper III**: see below).

4.4 Biosynthesis of ecdysteroids in *L. salmonis*

Several enzymes are involved in the biosynthesis of ecdysteroids. In this study, three enzymes neverland, disembodied and shade were investigated that represents three parts of the biosynthetic pathway; the initial, middle and the last steps (**paper III**). The data obtained give us insight into the localisation and function of the enzymes during specific physiological processes. However, it should be mentioned that no functional enzyme assay was conducted in the present study.

4.4.1 Functional assessment of ecdysteroid biosynthetic enzymes in *L. salmonis* early development

In other species, knock-down of *Nvd*, *Dib* and *Shd* are associated with embryonic defects resulting in mortality [130, 141]. Functional assessment of the genes in *L. salmonis* indicates that, of the investigated ecdysteroidigenic genes, only the *shade* gene product is necessary for development during the lecitotrophic stages. The results obtained (**paper III**) indicate that the regulation of ecdysteroid synthesis in *L. salmonis* might differ from that described in previous literature.

As mentioned, the salmon louse free-living larvae rely solely on yolk nutrition that is deposited into the eggs until it becomes parasitic during the copepodid stage. Nauplia I larvae with significant knock-down of *LsNvd* and *LsDib* molted through the nauplius II stage and into the copepodid stage and did not show any morphological aberrations. However, depletion of *LsShd* in nauplia I resulted in immediate molting arrest. We hypothesize that sufficient ecdysone is synthesized in the maturing oocytes and are stored as part of the food package. The ecdysone reserves are then converted

by LsShd into the active metabolite that is used by the LsEcR/LsRXR complex during embryogenesis and development from the free-swimming to the parasitic stage. The hypothesis is supported by the increase in ecdysteroid level observed at the end of oocyte maturation (**paper III**) prior to the extrusion of the eggs and initiation of embryogenesis. In addition, preliminary RNAi knock-down studies of the *L. salmonis shadow* (*LsSad*) (CYP315A1) ortholog showed the same results as those obtained by knock-down *LsNvd* and *LsDib* (data not shown). To the best of our knowledge, the no-loss of function phenotype observed in the *LsNvd, LsDib* and *LsSad*, has not been previously reported in arthropods and shows a novel regulation pathway that is most likely an adaptation to the lecitotrophic life stages of the parasite larvae. Whether this regulatory mechanism is unique to *L. salmonis* or similar in other lecitotrophic parasite larvae remains a topic for future studies.

4.4.2 Functional assessment of *L. salmonis Oct3βR* during early development

Octopamine receptors are known to modulate an extensive variety of physiological processes including learning and memory responses, neuronal development [154], ovulation [200] and ecdysteroidogenesis [156]. We searched the salmon louse genome using the *Drosophila melanogaster Oct3βR* (*DmOct3βR*) sequence and identified a putative ortholog sequence (**paper III**). RNAi knock-down of *LsOct3βR* gene in nauplia I resulted in immediate molting arrest resembling the phenotype observed for the *LsShd*kd animals.

Recent studies of *DmOct3βR* showed that depletion of the gene negatively regulated the ecdysone biosynthetic genes present in the PG [156]. Based on this study, we performed RT-qPCR on the identified biosynthetic genes *LsNvd, LsDib* and *LsShd* and the *LsEcR* and *LsRXR* nuclear receptors. Surprisingly, in the *LsOct3βR* RNAi animals, *LsShd* transcripts were significantly up-regulated while *LsDib* remained unregulated. These results contradict the observations from *Drosophila*, where *dib* was significantly down-regulated [156]. *LsNvd* was however regulated in a similar

manner in both *L. salmonis* and *Drosophila*. The findings in this study indicate that the ecdysteroid biosynthetic pathway in *L. salmonis* is different compared to that of insects, which is in agreement with observations from other crustaceans (review in [1]). Interestingly, the *LsOct3βR* appears to only regulate *LsEcR* and not its partner *LsRXR,* which remained unaffected by the *LsOct3βR* knock-down in the salmon louse. Originally, EcR and RXR are thought to act as heterodimeric partners. However, this study (**Paper I**, [129]) indicate that they are regulated in different ways dependent on the physiological/biological conditions. It is evident from the present study that the *LsOct3βR* is necessary for proper ecdysteroidogenesis and ecdysone signalling in *L. salmonis*.

4.5 The ecdysone pathway and lice control

From the aspect of applied biology, knowledge of the ecdysone receptor and the ecdysone biosynthetic enzymes may contribute to the development of novel treatment methods against the salmon louse. In insects, ecdysteroid-controlled mechanisms like molting and growth has been used as targets for development of pesticides with low environmental impact and vertebrate toxicity, therefore, there is a potential for novel medicines targeting the ecdysone hormone system in *L. salmonis*. The best prospect in the area is to exploit the susceptibility of the ecdysone receptor using antagonists/agonists, administered either by oral or bath treatment, that is specific to the *LsEcR*. However, this requires investigation of the shape and structure of ligand-binding pocket of the *LsEcR*. Teflubenzuron and diflubenzuron are today used as an anti-lice medicine and targets the molting process, by interfering with the chitin synthesis. These chemicals are species unspecific and have shown to have a negative impact on other arthropods such as shrimp, lobster and copepods in the environment [201]. In addition, teflubenzuron and diflubenzuron only work on molting stages hence adult lice and egg strings are not affected. *LsEcR* specific of antagonists/agonists would reduce the probability of influencing other important marine arthropods in addition to targeting all life stages including reproductive processes.

Members of the P450 family are present in both vertebrates and invertebrates and even though their P450 enzyme systems differ, some xenobiotics (e.g. phenobarbital) can induce metabolism in both groups (reviewed in [202]). The Halloween genes are specific to arthropods but are related to vertebrate CYPs. Hence, it could be conflicting to target these enzymes in the salmon louse as the xenobiotics or their metabolites (formed through biotransformation) could have a negative effect on the salmon and other vertebrates.

5. Concluding remarks and future perspectives

The present study has extended our knowledge of EcR-mediated ecdysone signalling in the salmon louse *Lepeophtheirus salmonis* with respect to its key role in the regulation of developmental and reproductive processes.

In **papers I-II**, we identified and functionally assessed the *LsEcR* during early developmental and reproductive stages. From the results, it is concluded that the *LsEcR* play key roles during oocyte maturation and reproduction in adult female louse and loss of LsEcR function terminates egg production. Depletion of *LsEcR* did however, not cause molting arrest between nauplia II and the copepodid stage but resulted in high mortality and severe tissue damage in later stages of development, showing that EcR is essential in growth and survival of the parasitic life stages. Future studies should investigate the interaction between ecdysone biosynthesis and signalling and other potential hormonal regulators of salmon louse development.

Future studies of the LsEcR should aim to determine potential subcellular localisation of the protein outside the nucleus. Detection of protein would also be beneficial in order to determine the half-life of protein after gene knock-down and the presence of residue protein. To get a better understanding of EcR-mediated ecdysone signalling in *L. salmonis,* protein-protein and protein-ligand interactions should also be investigated. This work has been initiated by master students at the Sea Lice Research Centre (SLRC), using a mammalian two-hybrid luciferase system. Although this is an artificial *in vitro* system it will undoubtedly give information of the *L. salmonis* EcR/RXR dimerization and ligand binding properties. Alternative partners for *LsEcR* (and *LsRXR*) should be investigated to give information about possible alternative regulation of ecdysone signalling pathways. Moreover, possible *LsEcR* agonist/antagonist can be tested for future parasite control.

From the functional studies of the biosynthetic genes *LsNvd, LsDib* and *LsShd* in nauplia larvae (**paper III**), only reduction of *LsShd* transcripts caused developmental defects. This lead us to hypothesize that precursors of the active steroid hormones

20E and PonA is synthesized and stored in the maturing oocyte for utilization during embryogenesis and development of the lecitotrophic stages until entry into the parasitic stage. In order to verify our hypothesis, it would be necessary to measure the levels of the metabolites (e.g. cholesterol and diketol) converted by *LsNvd* and *LsDib* during embryogenesis (i.e. from the point of fertilization to hatching of the egg string) as well as the ecdysone level. In addition, infection trials using *LsNvd* and *LsDib* knock-down animals would be beneficial to determine the phenotype and viability of the lice in the parasitic stages when these genes are decreased. At present time, only the three genes involved in ecdysteroidogenesis of *L. salmonis* have been partially investigated. It is apparent that other enzymes of this pathway have to be functionally assessed in order to gain a better understanding of the ecdysone biosynthetic pathway in the salmon louse.

Biogenic amines such as octopamine and tyramine have shown to play a role in the upstream regulation of ecdysteroidogenesis in insects through GPCRs. Recent studies by Ohhara et al., [156] showed that ecdysone biosynthesis is regulated by the GPCR *Oct3βR,* in *Drosophila.* From our *LsOct3βR* knock-down studies, it is apparent that the octopamine receptor also play a role in the regulation of ecdysoteroid biosynthesis in *L. salmonis.* Therefore, further studies of the *LsOct3βR* should, first and foremost, include proper identification and sequencing analysis as well as identification of possible paralogs present in the salmon louse genome. It would be beneficial to perform functional studies of *LsOct3βR* and/or possible paralogs using RNAi in adult female louse in addition to the RNAi in the nauplia stage. The octopamine receptor is well known to be a target for insecticides (e.g. amitraz), hence, the *LsOct3βR* is of great interest as a target for future salmon louse control.

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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

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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

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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 $2^{-\Delta\Delta Ct}$ approach and presented with the 95% confidence interval calculated from the $2^{-\Delta\Delta Ct}$ 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.

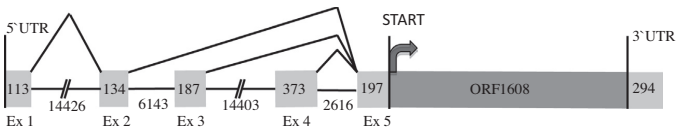

Fig. 1. Schematic representation of the genomic sequence of the *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*). Cloning and sequencing revealed the presence of three different mRNA variations. The exons (Ex; depicted in light grey boxes) were mapped to the genomic DNA and show the gene to extend 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.

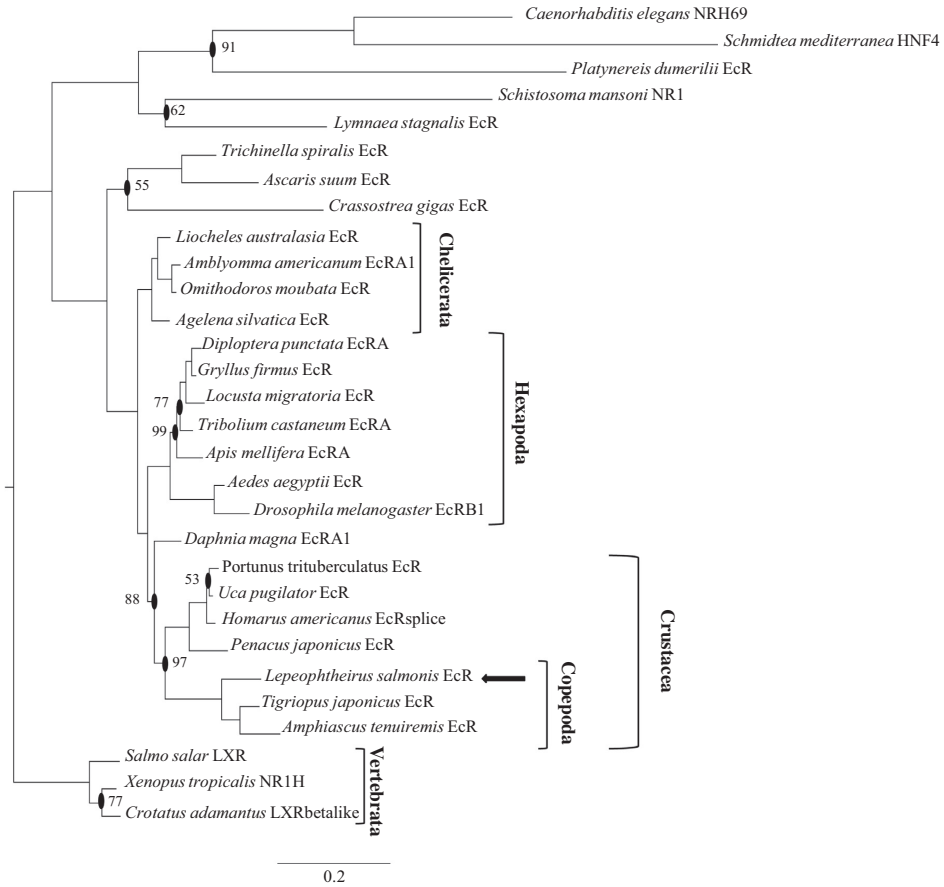

Fig. 2. Phylogenetic analysis of *Lepeophtheirus salmonis* ecdysone receptor (LsEcR). A rooted phylogenetic tree of amino acid sequences of full-length EcR and EcR-like receptors from different species was generated using Bayesian methods. Branch length is proportional to sequence divergence. Branch 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

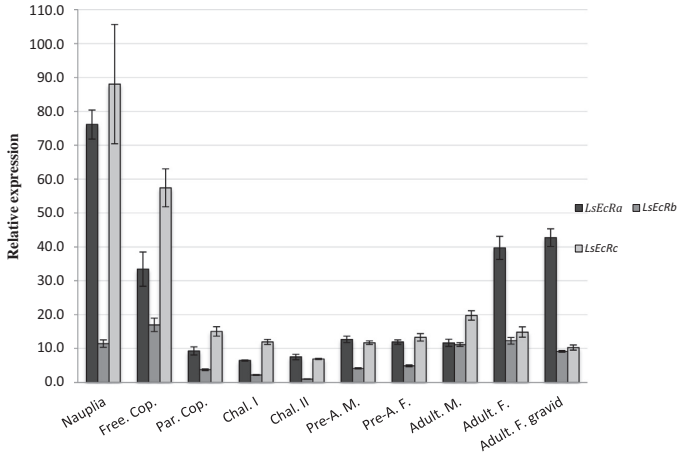

Fig. 3. Quantitative real-time PCR (RTq-PCR) analysis of relative expression of the three mRNA *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) variants (a, b, c) in different developmental stages. Each point represents the mean and confidence intervals (n = 5 parallels of approximately 150 animals 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.

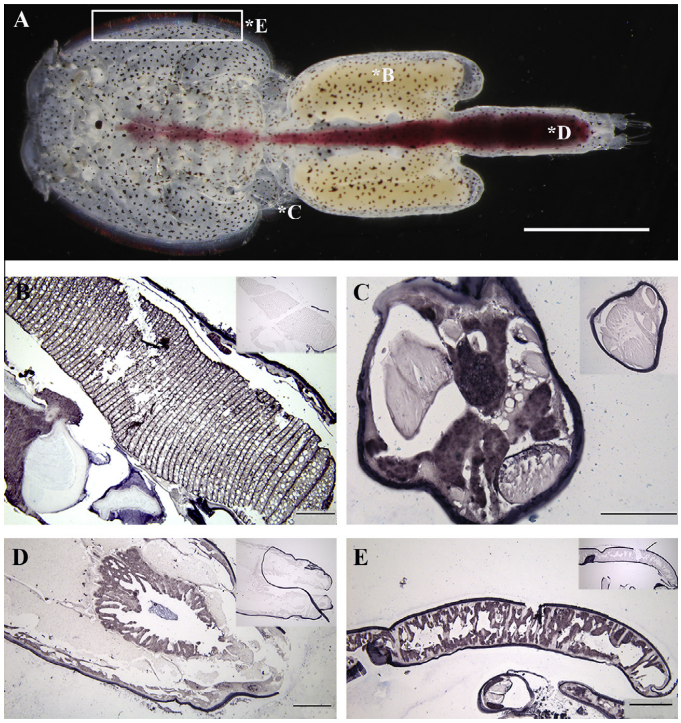

Fig. 4. Localisation of *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) transcripts in an adult female louse. (A) Light microscope image of gravid adult female louse. Letters and asterisks are guides to the corresponding photos of individual tissues. A part of the sub-cuticular tissue is framed 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).

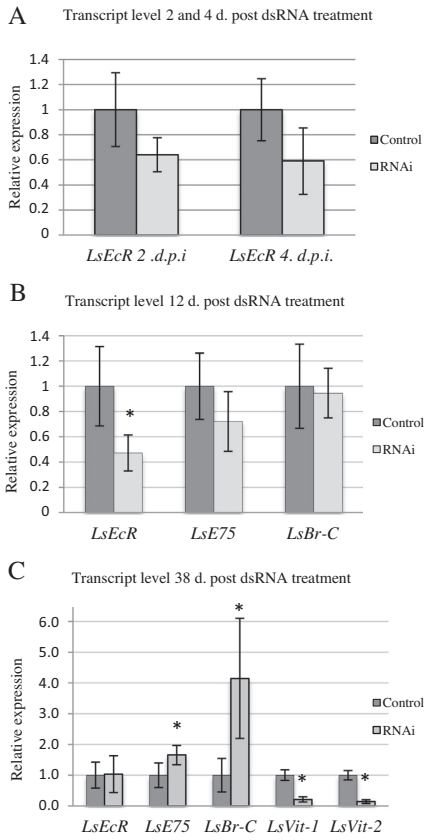

Fig. 5. Transcript level of selected ecdysone receptor (EcR) target genes after injection of double-stranded (ds)RNA. *Lepeophtheirus salmonis EcR* (*LsEcR*) adult female lice were removed from anesthetised fish and analysed after (A) 2, 4, (B) 12 or (C) 38 days (d.) post treatment (i.e. dsEcR injection; 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

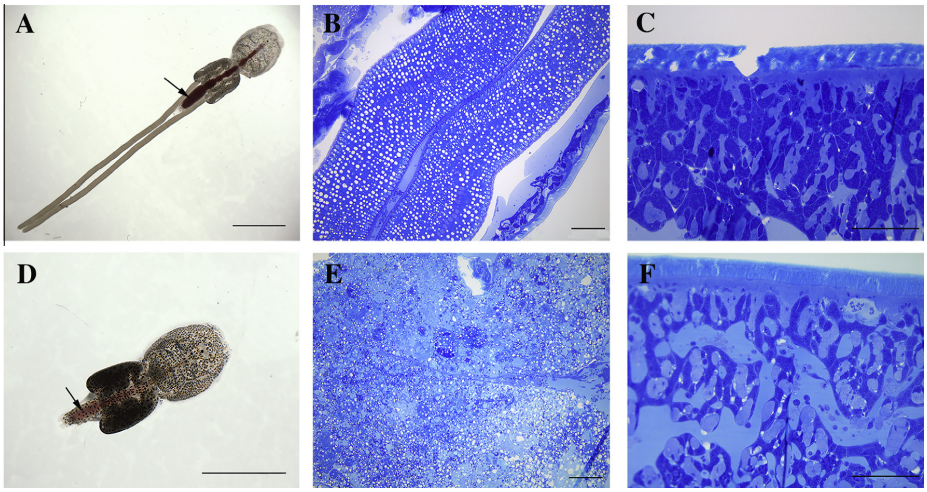

Fig. 6. Functional assessment of the *Lepeophtheirus salmonis* ecdysone receptor (*LsEcR*) by RNA interference (RNAi). The control lice produced normal egg strings (A) that hatched and produced viable offspring. *LsEcR* dsRNA-treated lice (D) showed a distinct phenotype with no production of eggs. It 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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