The molecular regulation of molting in the salmon louse (*Lepeophtheirus salmonis*)

Joakim Brunet

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

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Manuscript and publication

Paper I: Brunet. J, Tolås. I, Eichner. C, Dulgheriu. D, Horsberg. T.E, Male. R. (2021) The ecdysteroid Ponasterone A serves as the major regulator of molting in the salmon louse, an ectoparasitic crustacean (*Lepeophtheirus salmonis*) (**manuscript**).

Paper II: Brunet J, Eichner C, Male R (2021) The *FTZ-F1* gene encodes two functionally distinct nuclear receptor isoforms in the ectoparasitic copepod salmon louse (*Lepeophtheirus salmonis*). PLOS ONE 16(5): e0251575. https://doi.org/10.1371/journal.pone.0251575

Abbreviations

20E	20-hydroxyecdysone
AF-1	Activation function 1
AF-2	Activation function 2
bHLH-PAS	Basic Helix-loop-Helix/Per-ARNT-SIM
CHH	Crustacean hyperglycemic hormone
CoRNR	Corepressor/nuclear receptor
CTE	C-terminal extension
DBD	DNA-binding domain
Dib	Disembodied
E74	Ecdysone-inducible protein 74
E75	Ecdysone-inducible protein 75
EcR	Ecdysone receptor
ETHR	Ecdysis-triggering hormone receptor
FoxO	Forkhead Box Class O transcription factor
FTZ-F1	Fushi-Tarazu Factor-1
GPCR	G-protein coupled receptor
HR3	Hormone receptor 3
HR4	Hormone receptor 4
HRE	Hormone response element

LBD	Ligand-binding domain
LXR	Liver X-receptor
MIH	Molt-inhibiting hormone
NTD	N-terminal domain
Nvd	Neverland
PG	Prothoracic gland
Phm	Phantom
PonA	Ponasterone A
PTTH	Prothoracicotropic hormone
RAR	Retinoic acid receptor
RXR	Retinoid X-receptor
Sad	Shadow
Shd	Shade
Usp	Ultraspiracle
UTR	Untranslated region
YO	Y-organ

Abstract

The salmon louse, *Lepeophtheirus salmonis*, is a copepod and an ectoparasite that infects salmonids in the Northern hemisphere. At present, salmon louse infestations are the biggest threat to the health and welfare of wild and farmed fish, causing huge financial losses to the salmon farming industry annually. Salmon louse infestations are predominantly controlled using chemotherapeutants, however, extensive use has resulted in widespread resistance. Non-chemical methods such as freshwater treatment, cleaner fish, and lice skirts have been introduced, but there is a need for novel treatment methods as the non-chemical methods are not sufficient. More knowledge of salmon louse biology can be the key for developing new chemotherapeutants and finding new treatment targets.

Molting is a critical aspect of arthropod physiology, a process where the animal replaces the cuticle for a larger one to allow for further growth. The timing of molting is controlled by a class of arthropod steroid hormones known as ecdysteroids. In insects, pulses of the ecdysteroid 20-hydroxycedysone (20E) controls molting by binding and activating the nuclear receptor dimer complex consisting of the ecdysone receptor (EcR) and Ultraspiracle (Usp). Once activated the EcR/Usp receptor complex initiates a transcriptional cascade consisting of a selection of ecdysteroid regulated transcription factors that regulate the gene networks controlling molting. Feed-forward and feedback loops in the ecdysteroid induced transcriptional cascade ensures that the transcription factors are expressed in a specific sequential order and temporal window. Manipulation of this cascade results in molting arrest and developmental defects in the arthropod species studied. The molecular mechanisms regulating the ecdysteroid induced transcriptional hierarchy have been extensively studied in insects, but little is known about the molecular regulation of these transcription factors in copepods and crustaceans in general. For this purpose, the goal of the study was to expand the knowledge of the ecdysone endocrine system in the salmon louse.

While 20E is the main regulator of molting in insects, both 20E and the ecdysteroid Ponasterone A (PonA) have been shown to have physiological activity in crustaceans.

In the current study, it was demonstrated through the implementation of a two-hybrid reporter gene assay and *in vivo* exposure of nauplii to ecdysteroids, that PonA is the ligand for the salmon louse ecdysone receptor complex, and that 20E apparently has little physiological activity in the salmon louse. The gene expression of the transcription factors Hormone Receptor 3 (HR3), Hormone Receptor 4 (HR4), Ecdysone-inducible Protein 74 (E74), Ecdysone-inducible Protein 75 (E75), and Fushi Tarazu Factor-1 (FTZ-F1), together with ecdysteroid hormone levels, were measured in high resolution through the pre-adult I and nauplius II molt cycle. These ecdysteroid regulated transcription factors displayed specific temporal expression profiles relating to levels of ecdysteroid hormones. Manipulation of the natural expression profiles of the ecdysteroid regulated factors with PonA revealed that *HR3* and *HR4* are early response genes to increased levels of PonA, while increases in *E74*, *FTZ-F1* and *E75* expression appear to be secondary and tertiary responses to high levels of PonA. Quantification of PonA across a molt cycle revealed, similar to observations from other arthropods, that a drop in ecdysteroid treatment precedes ecdysis. Treatment with PonA within the nauplius II stage resulted in molting arrest, indicating that a drop of hormone levels is required to initiate ecdysis.

The nuclear receptor FTZ-F1 has been shown to be crucial in the regulation of molting in both insects and nematodes, but its function has never been characterized in crustaceans. Molecular cloning of the *FTZ-F1* gene in the salmon louse revealed that it encodes two nuclear receptor isoforms with unique N-terminal domains, generated by alternative promotor usage and splicing. Re-analysis of available FTZ-F1 sequences in ecdysozoans suggests that the *FTZ-F1* gene structure to produce two N-terminal isoforms is conserved in all subphyla apart from nematodes. Knockdown of the two *FTZ-F1* isoforms, *αFTZ-F1* and *βFTZ-F1*, revealed that *βFTZ-F1* is an important regulator of both molting and oocyte development in the salmon louse. Knockdown of *βFTZ-F1* in nauplii and pre-adult I resulted in molting arrest, while knockdown in pre-adult II females resulted in the destruction of oocytes at the vitellogenic stage in adult females. Transcriptome sequencing and differential gene expression analysis suggest that *βFTZ-F1* regulates processes involving cuticle detachment and ecdysis.

Combined, the results expand the current knowledge of how ecdysteroid signalling regulates molting in the salmon louse. Increased knowledge of the endocrine control of molting in the salmon louse can provide the basis for the discovery of new chemotherapeutants to control salmon lice infestations.

1. Introduction

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod crustacean that infects and feeds of salmonid fish. Due to widespread resistance to available chemical treatments, there is a need for more sustainable methods for combating sea lice. More knowledge about the salmon louse at the molecular level could be the key to discover innovative tools for sea lice prevention. The focus of this thesis was to study the molecular mechanisms of molting in the salmon louse. The process of molting is a fundamental aspect of the life cycle of arthropods, a group that make up over 84% of the known animal species on Earth, including the salmon louse (1). It is a complex process involving the precise regulation of hundreds and thousands of genes (2, 3). The molting process consists of many interdependent pathways and components, and as such, contains numerous targets for potential pest control. Disruption of any part of the molting process is likely to result in the death of the animal.

To study molting in the salmon louse, the focus of the thesis was to characterize the function of nuclear receptors known to regulate molting in other arthropods. The introduction will begin by describing sea lice biology and relevant anatomy associated with nuclear receptor function. The introduction will then discuss in more detail nuclear receptors, the hormones that control molting, and how molting is regulated in arthropod species.

1.1 The salmon louse problem in aquaculture

The world Atlantic salmon production has increased dramatically since the 1990s from a global production of approximately 230 thousand tons in 1990 to 2.2 million tons in 2018 (4). The salmon louse feeds on the mucus, skin and blood of both wild and farmed salmonids (5, 6). The first salmon lice infestations of farmed salmon were reported during the 1960s with the start of the use of floating net pens that allowed for the exchange of water and content between the salmon farms and the environment (7). The use of a group of pesticides known as organophosphates had initial success but reports on developing resistance started emerging in the 1990s (8, 9). As a result,

organophosphate usage was discontinued in Norway in the late 1990s, and the early 2000s in Canada and Chile (10). Since then, different drugs have been implemented to combat sea lice, among them; pyrethroids, emamectin benzoate, benzoyl ureas, and hydrogen peroxide (10-12). Extensive use of these compounds has resulted in the development of resistances to all compounds except for the benzoyl ureas, diflubenzuron and teflubenzuron (13-17). However, resistances to benzoyl ureas are found in other arthropods and as such could develop in the salmon louse (18). The organophosphate azamethiphos was re-introduced in Norway in 2008, but by 2013 a surveillance program revealed widespread distribution of resistance once again (19). There is also an environmental impact from the usage of these compounds as they are also toxic to non-target crustaceans (20). The dispersion and impact vary depending on the compound, with benzoyl ureas and organophosphates being the predominant compounds found in sediments in the vicinity of the salmon farms (20-22). Non-medicinal treatments like the use of cleaner fish, cage skirts, fresh-water treatment, and thermal lasers have been employed to counter sea lice growth (23), but the cost effectiveness of some of these treatments are an issue of debate (24-26), and in the case of the thermal laser shown to have no significant effect after widespread employment in the industry (27). The economic damage of sea lice parasitism to the Norwegian aquaculture was estimated to be 436 million USD in 2011 (28).

1.2 Salmon louse biology and host interaction

The salmon louse is a copepod crustacean belonging to the arthropod subphylum. Like other arthropods, the salmon louse relies on the process of molting to facilitate further growth. A process where the cuticle is degraded, recycled, and replaced by a newly synthesized cuticle through each successive developmental stage. The salmon louse life cycle consists of 8 developmental stages, each separated by a molt (5, 6, 29) (Fig. 1). The length of the life cycle depends on the environmental temperature (30), with female lice becoming adults after 72 days at 6 °C and after just 13 days at 21 °C. Salmon louse females develop at a slower rate compared to males (30, 31). The adult male louse copulates with the female by attaching a spermatophore on near the copulatory

pores on the female genital segment, which the female uses to fertilize eggstrings as they are released from the genital segment into external egg sacs attached with a hook (32, 33). The female produces two eggstrings which can contain hundreds of eggs each. As the eggs mature in the eggstrings, the color darkens from white to black, until nauplius I larvae proceed to hatch starting from the distal end of the eggstring. During the planktonic stages, the lice is reliant on maternally deposited egg yolk for nourishment (6). After progressing through the two nauplius stages, the lice molt into the infective copepodid stage. Copepodids find their hosts through several senses, reviewed in (34). They have been shown to respond to light and light reflections from host fish, as well as responding to water currents produced by moving fish. In addition, copepodids use chemical cues through the use of olfactory receptors in their antennas to locate and attach to the desired host. Initial reversible attachment to the host is done with a hooked second antenna, and it is speculated that the lice will then "taste" the host and can return back to the water if it finds the host unsuitable (35). Permanent attachment is done just before the molt to chalimus I with the production of a frontal filament that is anchored beneath the epithelium of the host with a basal plate created from a glue-like secretion (36). The stem of the filament is covered by an external lamina that is continuous with the cuticle of the chalimus. Following the two chalimus stages, the lice progress through two pre-adult stages where they are no longer restricted to one site through the filament, but able to move around and graze on the host and are only dependent on the use of a filament for molting. It is hypothesized that the lice are able to avoid an immune response by modulating the hosts immune system through secretions from tegumental and labial glands at the attachment site (37, 38). Characterization of the secretory/excretory products (probably also from intestine) revealed a large number of proteins associated with proteolytic activity such as serine proteases and metalloproteases, which are believed to facilitate blood feeding in addition to host tissue and mucus degradation (37, 39). The prostanoid prostaglandin E2 (PGE2) has also been identified in sea lice secretory/excretory products and was shown to modulate immune response genes in a macrophage-like cell line from Atlantic salmon (40, 41). However, the role of PGE2 in host immune modulation is still debated (42). The physiological effects from sea lice infection are more profound

once the lice reach the pre-adult and adult stages (43, 44). Prolonged and severe infections by numbers of around 30 or more pre-adult lice, depending on the fish size, may lead to chronic stress, anemia, and mortality as a result of osmotic dysregulation from lesions and blood loss (43, 44).

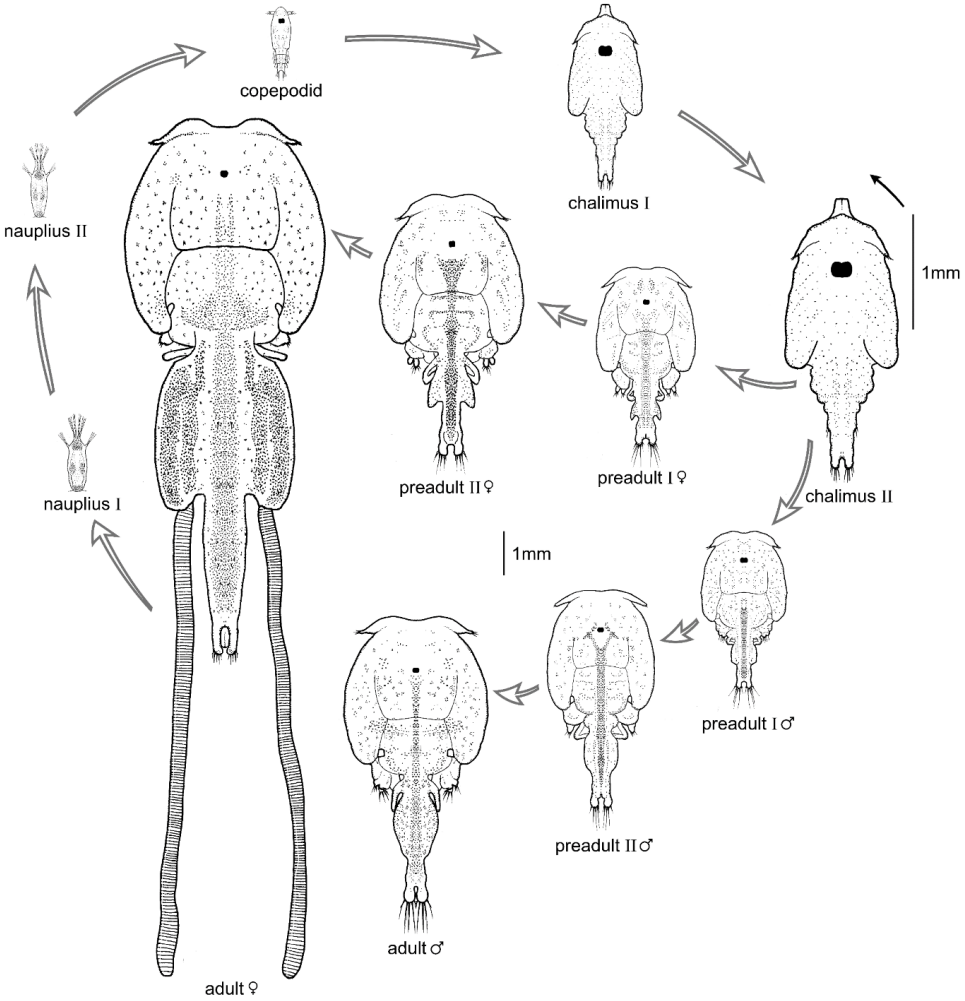


Figure 1: The life cycle of the salmon louse (Lepeophtheirus salmonis). The salmon louse life cycle consists of eight developmental stages: two planktonic naupliar stages, an infectious copepodid stage, two immobile chalimus stages, two mobile pre-adult stages, and an adult reproductive stage. Figure taken from (45).

1.3 Salmon louse anatomy

1.3.1 General appearance

The salmon louse undergoes substantial growth through its eight life stages, from a nauplius I larvae with a length of roughly 0.5 mm to a fully grown reproductive female of 11 mm. The adult salmon louse body consist of four main parts; cephalothorax, thoracic segment, genital segment and abdomen (5, 6). The cephalothorax forms a broad shield which includes all the body segments up to the fifth thoracic segment. The cephalothorax in the free moving pre-adult stages functions as a suction cup to hold the salmon louse attached to its host. Although most of the growth occurs during molts, some growth is observed within an instar (31). The intra-instar growth is predominantly in the length and width of the abdomen, while the length of the cephalothorax remains largely the same. The change in this ratio (CT/TL) between the length of the cephalothorax (CT) and the total length (TL) of the lice can be used as a proxy for intra-instar age (31, 46). The lice become sexually distinguishable at the pre-adult I stage, with males being smaller compared to females, a size difference that increases for each pre-adult stage and is at its largest at the adult stage (5, 6). The genital segment also become sexually dimorphic with the male genital segment being barrel shaped compared to the more triangular shape of the female genital segment. Mature males and females are easily distinguishable as the females are on average twice the size, with the genital segment of females expanding dramatically as they fill with developing oocytes (5).

1.3.2 Cuticle and sub-cuticular tissue

The cuticle is a multi-layered structure covering the body of arthropods consisting of a mixture of chitin, proteins, and lipids. The composition of the cuticle can change based on the life stage and required physical properties, with the degree of strength and toughness dependent on the degree of covalent crosslinking of chitin and cuticular proteins (47). The cuticle forms the exoskeleton of the animal, protecting the organism from predators and pathogens, allows for retention of water, as well as providing structural support in the form of an attachment framework for muscles and internal organs (47). Studies of the ultrastructure of the chalimus II salmon louse larvae

revealed that the salmon louse cuticle, similar to observations in other copepods (48), consists of a multi-layered non-chitinous epicuticle overlaying a laminated procuticle, which in turn overlies a single cell layered epidermis (49). The epicuticle is covered by a mucoid layer named the fuzzy coat (49). The epicuticle (80-120 nm) itself consists of 4 layers. Between the epicuticle and the laminated procuticle a transitional pro-cuticular layer is observed. The pro-cuticle comprises the main part of the cuticle (2.4 µm), and in the more sclerotized sections of the cuticle it appears in two distinguishable zones labelled the inner and outer procuticle (49). The epidermis is comprised of a single layer of cells separated from the procuticle by an apical membrane consisting of wrinkled folds. In areas of the salmon louse body where the cuticle is flexible, notably the hindgut, foregut, joins, and setae, the cuticle consists only of an epicuticle or epicuticle in combination with a transitional procuticle layer.

The subcuticular tissue is situated below the basal lamina of the epidermis and consists of a mixture of cells, often multinucleated, of variable shape, organized in an irregular pattern along with different glandular structures and muscles (38, 49-51). The subcuticular tissue is the site of synthesis of vitellogenin, and the suggested site of fatty acid metabolism in the salmon louse (51, 52).

1.3.3 Reproductive organs

The ovaries of the female salmon louse are located dorsally in the cephalothorax on either side of the anterior part of the intestine, just behind the eyes. The ovaries are pear-shaped and have an internal structure consisting of densely coiled tubules. Oogenia differentiate to primary oocytes from the posterior to the anterior end of the oocytes while attached to the membrane wall of the coiled tubules. The tubules are surrounded by the syncytial germarium consisting of large cells with small nuclei. The primary oocytes enter the oviduct in a stacked fashion at the anteroventral surface of the ovary, initiating the process of vitellogenesis (32). The oviducts pass parallel to the intestine on both sides towards the genital segment. Upon entering the genital segment, the oocytes expand as vitellogenesis continues and in a stacked fashion forms coils of vitellogenic oocytes that fill the lumen on both sides of the genital segment. The genital segment also contains sausage like glands extending from the anterior to the posterior

end of either side of the genital segment, called cement glands. The cement produced in these glands have a glue-like property and is used to cover the eggs and hold the eggstrings together and attached to the mother (33). The cement gland and oviducts open into the cuticle lined genital antrum together with the receptaculum seminis, which is where the female fertilizes the oocytes with sperm from the attached spermatophore supplied by the male salmon louse (32).

The salmon louse testes are in an analogous area to the female ovaries in the male salmon louse. Like the ovaries, the testes have a pear-like shape and can be divided into three zones based on the degree of differentiation from spermatogonia to spermatids. The differentiation occurs in a posterior to anterior direction with the posterior part consisting of spermatogonia and anterior part consisting of spermatids along with accessory and secretory cells that provide mechanical support and nutrition to the germ cells (32). In the middle and anterior part of the testes, dense amorphous globules named A-globules form from degenerating spermatocytes, and together with the young spermatozoa forming in the anterior testes travel down the vas deference that runs parallel to the intestine and into the genital segment (32). The A-globules lines the inner wall of the spermatophore sac, fusing to form an inner secretion which is used to seal shut the copulatory duct once the spermatophore is attached and spermatozoa have been discharged into the receptaculum seminis of the female salmon louse (32).

1.4 Nuclear receptors

1.4.1 Structure and architecture

Nuclear receptors are a protein family of transcription factors that play an important role in many physiological processes in animals such as metabolism, homeostasis, reproduction, development, and metamorphosis (53, 54). The primary function of a nuclear receptor is to regulate the expression of genes in response to hormonal signals. There are many different nuclear receptors, each responding to different hormones or ligands (55). Receptors without known ligands are labelled orphan receptors, and some receptors function independently of ligands (56, 57). All nuclear receptors share a common modular domain structure consisting of 4-5 domains; A-F (Fig. 2) (58).

The A/B domain or N-terminal domain (NTD) is an unstructured and disordered domain. The sequence of the NTD is not conserved and the length of the domain varies substantially among the different nuclear receptors. The NTD contains the Activating Function 1 (AF-1), a region that assists in the transactivation of transcription and interacts with co-regulators to regulate gene expression. The NTD is also targeted by post-translation modifications such as acetylation, phosphorylation and SUMOylation, modifications that can either drive or repress transcription (59).

The C domain or DNA-binding domain (DBD) is the most conserved region of the nuclear receptors in both sequence and structure (60). The DBD consists of two subdomains each containing four conserved cysteine residues that coordinate a zinc ion to produce a DNA-binding zinc-finger motif. Each zinc-finger is followed by an amphipathic α-helix and a loop. The helix from the first subdomain binds specific sequences of DNA regulatory sites called hormone response elements (HREs) located in promoter or enhancer elements of target genes. The helix inserts itself in the major groove of the DNA double helix, while the helix of the second subdomain makes a non-specific interaction with the DNA backbone (61). The peptide loop in the second subdomain contains a dimerization loop, which has residues participating in the dimerization and the stability of nuclear receptor dimers (62). DBDs of nuclear receptors that bind DNA as monomers use the C-terminal extension (CTE) following the second zinc-finger to make additional sequence specific contacts within the minor groove of DNA (63, 64).

The D domain or hinge region is a linker between the DBD and the ligand binding-domain that varies in length, and like the NTD, the sequence of this domain is not conserved among nuclear receptors. The hinge region is also a site for regulatory post-translational modifications (59), and contains a nuclear localization signal (NLS) (65-67).

The E or LBD is structurally conserved globular domain containing 12 α-helices and a β-turn that form 3 antiparallel helical sheets named an α-helical sandwich (68). The overall fold forms a large ligand-binding pocket (LBP) at the base of the domain, which

between different nuclear receptors varies in volume (69). Comparing the LBD structure of nuclear receptors reveals that the largest structural difference occurs at the base of the LBD where the LBP is located (69), highlighting how nuclear receptors have evolved to fit a variety of specific ligands such as heme (70), steroids (71), fatty acids (72), and xenobiotics (73), and in some cases evolved to fit no ligands (57). Binding of a ligand to the LBP induces a confirmational change in the domain, overall stabilizing the three-dimensional structure and repositioning helix 12, known as the Activating Function 2 (AF-2) or activating function helix, to a position to interact with different transcriptional co-regulatory proteins to regulate gene expression (68). Co-activators interact with a hydrophobic groove at the AF-2 through an α-helix containing a short LXXLL motif (X = any amino acid) (74). The co-activator complex activates transcription by remodelling the chromatin structure and driving the formation of the transcriptional initiation complex at the promotor of target genes to be expressed (75). Transcriptional co-repressors also interact with the LBD at the AF-2 surface area, but using a conserved motif known as the Corepressor/nuclear receptor (CoRNR) box, which inhibits the AF-2 helix assuming its active conformation in the absence of a ligand (76, 77). The LBD also contains a dimerization surface important for the dimerization between the LBDs of nuclear receptor partners (58).

The F-domain is located at the C-terminus of a nuclear receptor, and like the NTD it is highly variable in size and is non-existent in some nuclear receptors. Its functional roles remain largely unknown (78).

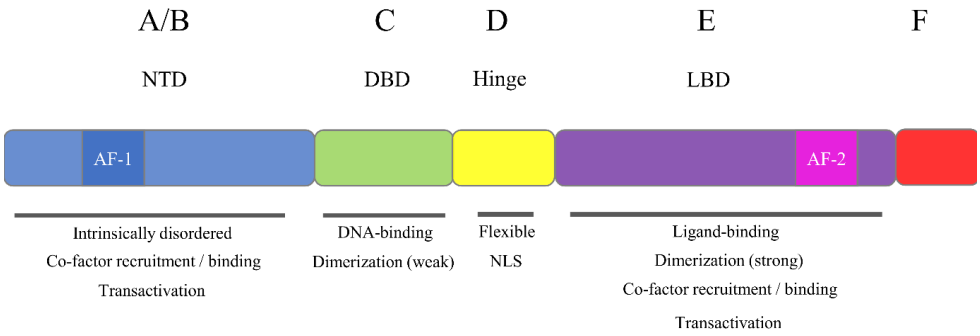


Figure 2: Organization of nuclear receptor domains and their functions. Nuclear receptors generally consist of 5 structural domains. A/B: N-terminal domain (NTD), C: DNA-binding domain (DBD), D: An unstructured and flexible hinge region, E: Ligand-binding domain (LBD), F: Variable C-terminal domain. Abbreviations: NLS = nuclear localization signal, AF = activating function.

1.4.2 Nuclear receptor function

Nuclear receptors can bind DNA as monomers (63), but are mostly found as homodimers (61) or heterodimers in complex with Retinoid X-receptor (RXR), which is the most promiscuous nuclear receptor and is a partner in the majority of the nuclear receptor heterodimer complexes (79). Nuclear receptors are divided into 4 types based on their mechanism of function and DNA-binding properties (Fig. 3).

Type 1 nuclear receptors are activated by steroid hormones and are typically retained in the cytoplasm bound to chaperone proteins. Upon binding of the ligand, they are released from their bound chaperones and translocate to the nucleus where they bind DNA as a homodimer to HREs that consist of palindromic inverted repeats (80, 81). Type 2 nuclear receptors are not sequestered in the cytoplasm but are retained in the nucleus regardless of ligand activation (82). When bound by a ligand, the receptors exchange co-repressor proteins for co-activators to promote the assembly of the transcriptional machinery. This type of nuclear receptor binds HREs that are direct repeats or inverted repeats, commonly as heterodimers with RXR as a partner (79). Type 3 nuclear receptors operate similarly to type 2 but binds DNA as homodimers instead of heterodimers on direct or everted repeats (83). Type 4 nuclear receptors bind

DNA as monomers on HREs containing extended 5’ half sites, recognizing specific sequences in the minor groove of DNA with the CTE of the DBD (63).

Nuclear receptor HREs consists of two hexameric half-sites separated by a variable spacer, or in a few cases a single extended half-site with three additional nucleotides on the 5’ end for binding monomeric nuclear receptors (58). The specificity of nuclear receptor binding to HREs are influenced by the sequence of the half-site, their orientation, and the sequence and length of the spacer between the half-sites (84). Nuclear receptors, particularly type 2 RXR and RAR heterodimers, are promiscuous and show overlap in their affinity for many HREs with different spacer lengths and sequence (85). However, affinity for the HRE does not necessarily equal function as the DNA binding site can allosterically affect the receptor, influencing its ability to interact with co-regulators and regulate gene expression (86, 87). In addition, pioneering factors have been shown to prime DNA response elements to their corresponding nuclear receptors, enabling the receptors to bind HREs and regulate gene expression (88). This, in combination with tissue- and cell specific expression of nuclear receptors (89) and availability of hormone ligands, demonstrates the complexity of how nuclear receptor specificity is achieved to regulate specific genes.

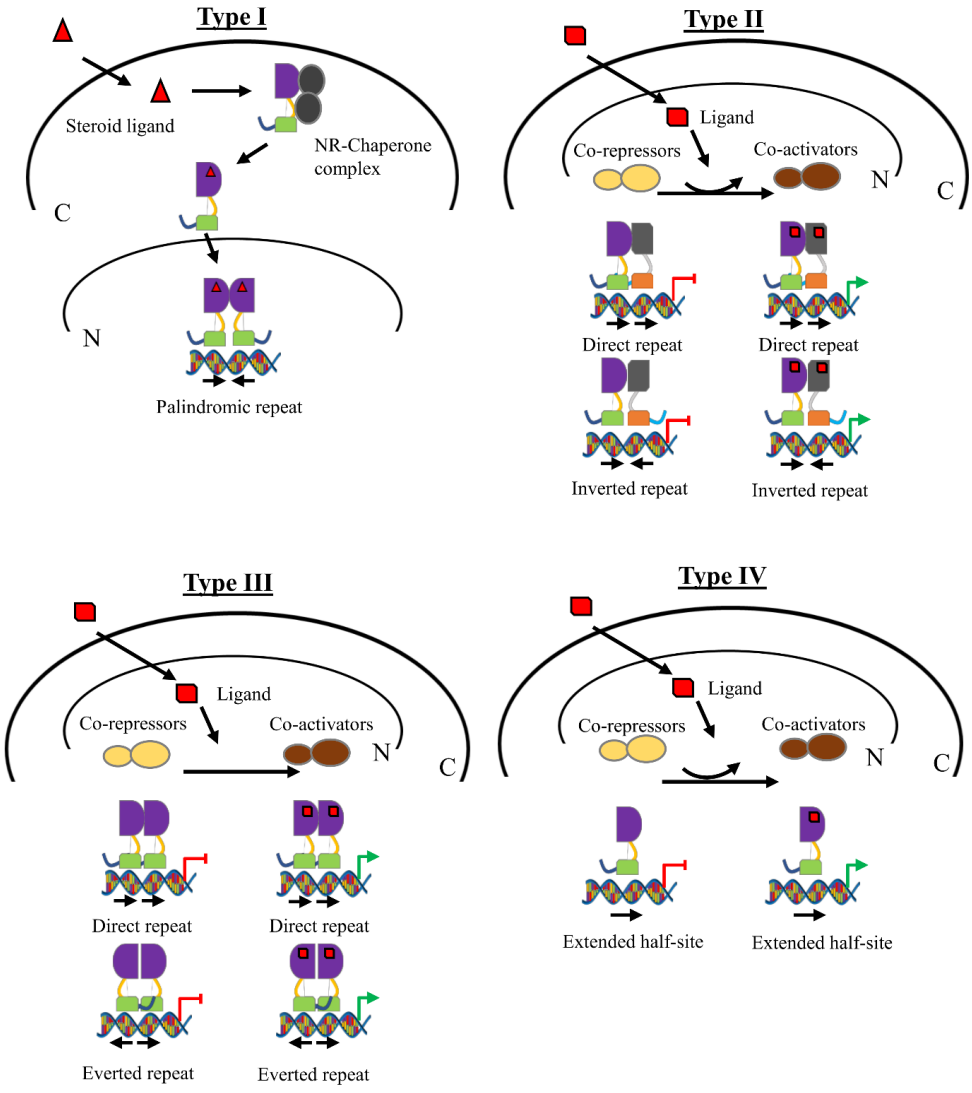


Figure 3: Summary of nuclear receptor signalling mechanisms. Nuclear receptors can be divided into four types (I-IV) based on their mechanism of function and DNA-binding properties. Type I illustrates homodimeric steroid hormone receptor function. Type II illustrates typical RXR heterodimer nuclear receptor function. Type III illustrates homodimeric receptors that function similar to Type II. Type IV illustrates nuclear receptors that bind DNA as monomers using an extended DNA half-site. C=cytoplasm, N=nucleus. Adapted with permission from (90).

1.4.3 Evolution and diversification

Nuclear receptors are thought to have originated in early multicellular animals as we find nuclear receptors not only in bilaterians, but also in sponges (91) and jellyfish (92). As animals evolved, nuclear receptors radiated and are found in all animal phyla. The ubiquitous presence of nuclear receptors, the key role they play in cell growth and differentiation, and evolved complexity of ligand binding, places nuclear receptors as important drivers in the evolution of animals (93). Based on the structural conservation of the two conserved nuclear receptor domains, the nuclear receptor superfamily can be divided into six subfamilies (NR1-NR6) (92). It is debated whether nuclear receptors gained the ability to bind ligands or certain receptors lost the ability (94), but there is no correlation between the position of the nuclear receptor on the phylogenetic tree and its ligand specificity (92). Correlation between binding mode and ancestry, however, is present. For example, monomeric binding is present in all subfamilies, but most members of NR3 bind DNA as homodimers on palindromic elements, while members of NR2 bind direct repeats, additionally most RXR heterodimers are members of NR1 and NR4 (95). All subfamilies appear to be present across the animal lineage, except for sponges where only NR2 members have been identified thus far (91, 96). This indicates that the diversification of the nuclear receptor subfamilies happened early in animal development, predating the division between protostomes and deuterostomes. The exact evolutionary relationship between the different subfamilies is unknown and still debated, but the presence of RXR in sponges and lack of RXR heterodimer ability among NR5 nuclear receptors suggests that NR2 and NR5 split early in animal evolution, with NR2 giving rise to NR4 and NR1, and NR5 diverging into NR6 and NR3 (55). Following the duplications giving rise to the different subfamilies, a second wave of duplications occurred during the Cambrian explosion, a period where all major animal phyla originated and diversified. In the ancestors of vertebrates, duplications resulted in genomes containing one or more paralogues of nuclear receptor genes, resulting in the presence of several paralogues of among others the steroid hormone receptors (glucocorticoid, progesterone, oestrogen, androgen) and the retinoic acid receptors (alpha, beta, gamma) seen in present day vertebrates (93). The number of nuclear receptors varies among different species, with the nematode (*Caenorhabditis*

elegans) having 270 predicted nuclear receptor genes (97), humans 48 (98) and the fruit fly 18 (99). In the ecdysozoan lineage, NR1 nuclear receptors have evolved critical roles associated with molting and metamorphosis, two common characteristics among ecdysozoans (100). The ecdysone receptor (EcR), ortholog to the human Liver X-receptor (LXR), has evolved to become a master regulator of molting as a heterodimer in complex with the RXR ortholog Ultraspiracle (Usp) (99, 101). While LXR binds oxysterols as its ligand, the EcR/Usp dimer complex and its transcriptional responses are activated and regulated by ecdysteroids, sterol derived steroid hormones that emerged in the ecdysozoan lineage (102).

1.4.4 Nuclear receptors as research targets in the salmon louse

The fact that nuclear receptors are crucial for many aspects of animal development and that their activity is regulated by small lipophilic compounds make them interesting research targets, not only from a pharmaceutical and medical perspective, but also from a pest management perspective. Nuclear receptors are good drug targets as their ligands can act as pure agonists or antagonists, in addition they can bind as partial agonists and antagonists, all which can produce different conformational changes in the receptors that can result in different and selective biological outcomes (103). Nuclear receptors have been used as targets for insecticides, where mimics of the natural ligand 20-hydroxyecdysone (20E) is used to inhibit the ecdysone receptor complex to disrupt molting in insects that pose a threat to agriculture (104, 105). Interestingly, different ecdysone insecticides display species specificity, even in insects of the same order (105). From a sea lice perspective, species specificity of ecdysteroid antagonists and agonist is encouraging as it would be possible to develop drugs that selectively targets the ecdysone receptor or other nuclear receptors in the salmon louse.

1.5 Ecdysteroids

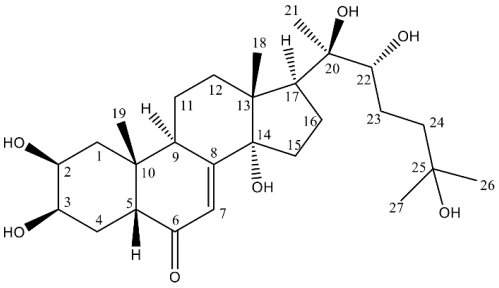
1.5.1 Ecdysteroid biosynthesis

Ecdysteroids regulate several facets of arthropod physiology such as development, molting, and reproduction (106-109). Ecdysteroids are steroid hormones synthesized in ecdysozoan organisms from cholesterol, a sterol lipid consisting of 4 linked

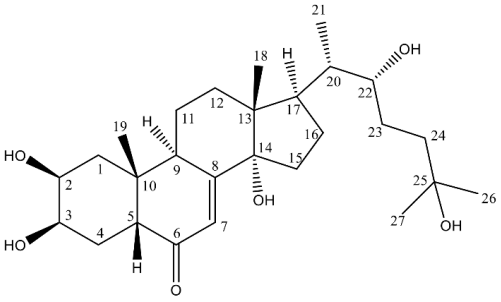
hydrocarbon rings with a hydrocarbon tail at one end and a hydroxyl group at the other end (Fig. 4). Unlike vertebrates, arthropods and other ecdysozoans lack the enzymes required to synthesize cholesterol *de novo* and are dependent on the dietary intake of cholesterol or plant sterols for substrates for ecdysteroid synthesis (110, 111). Unlike the vertebrate style steroids, ecdysteroids maintain the side chain of the cholesterol. There are a multitude of different ecdysteroids found in ecdysozoans (112, 113), and the differences lie predominantly on sites located on the cholesterol side chain (114). Most of the knowledge about the ecdysone biosynthetic pathway and the enzymes involved comes from studies on insects and decapod crustaceans.

In the insect model organism, the fruit fly (*Drosophila melanogaster*), the insect molting prohormone ecdysone (E) is made in the prothoracic gland (PG) before being released to peripheral tissue for conversion into the biologically active 20-hydroxyecdysone (20E). The first step of ecdysone biosynthesis is the conversion of cholesterol into 7-dehydrocholesterol (7-dC) by the Rieske-domain enzyme named neverland (nvd) (115). 7-dC is then converted to 5β-ketodiol in several steps fittingly labelled “the black box”, as the precise pathways are still unclear (116). A few enzymes in the black box have been identified, two of them named Spook and Spookier, which belong to a group of genes called Halloween genes (117). The Halloween genes are mono-oxygenases of the Cytochrome P450 (CYP) superfamily of enzymes and are responsible for the production of ecdysteroids (118). In the last steps of the biosynthesis of 20E, 5β-ketodiol is sequentially hydroxylated at carbon 25, 22, 2 and lastly carbon 20 by the Halloween gene enzymes Phantom (Phm), Disembodied (Dib), Shadow (Sad) and Shade (Shd), respectively (119). Ecdysteroid biosynthesis in decapod crustaceans occurs in the Y-Organ (YO), a gland analogous to the PG in the fruit fly (120). The biosynthesis is similar to the pathway in insects, but the crustacean YO produces a larger diversity of ecdysteroids (Fig. 5).

20-hydroxyecdysone



Ecdysone



Ponasterone A

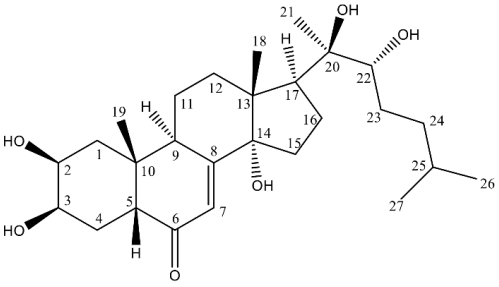


Figure 4. Structures of the common arthropod ecdysteroids ecdysone (E), 20-hydroxyecdysone (20E) and Ponasterone A (PonA). Drawn with ChemDraw 20.1.1.

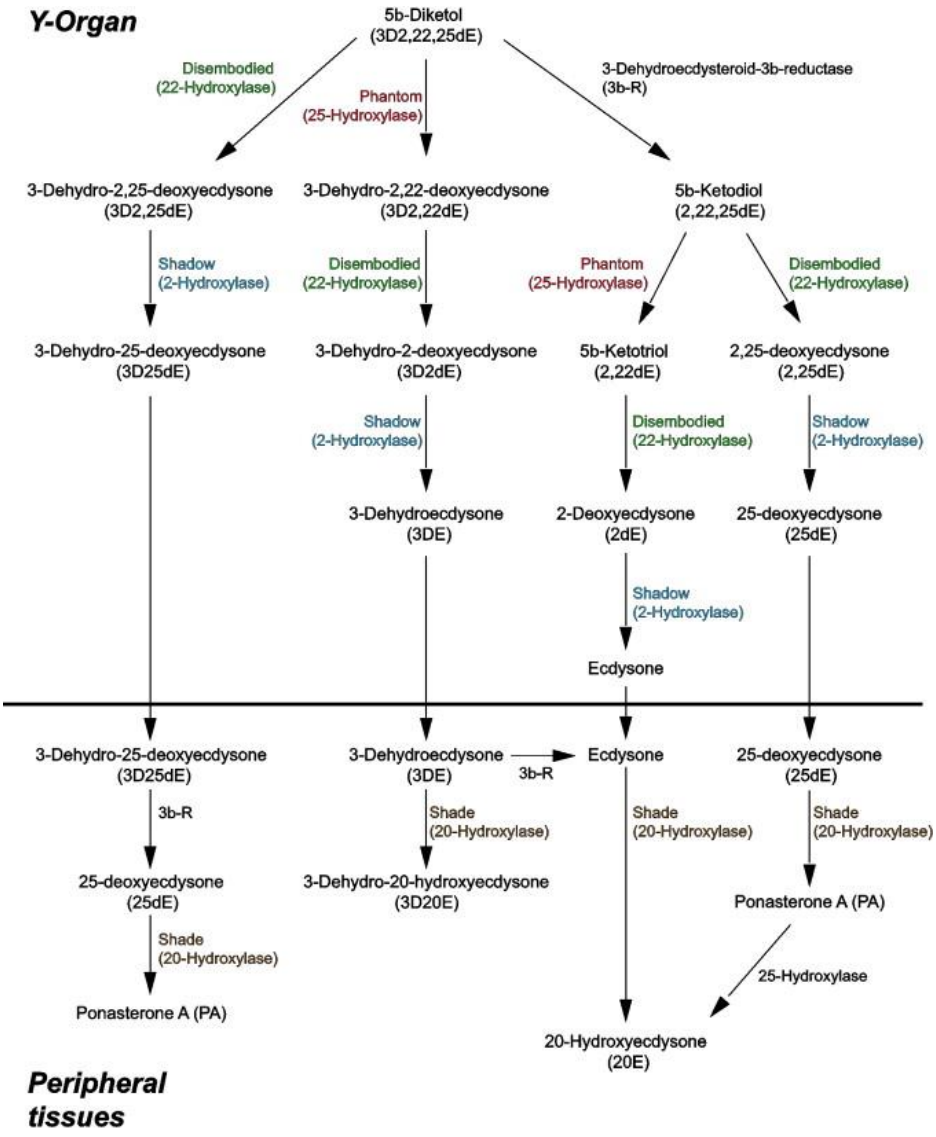


Figure 5. Overview of ecdysteroid biosynthesis in the Y-organ (YO) and peripheral tissues of decapod crustaceans. Used with permission from (113).

The intermediates 5β-diketol and 5β-ketodiol acts as two branching points to produce four potential secretory products from the YO, which after secretion are converted by 20-hydroxylase and 3β-reductase enzymes to their predominant forms (113). The predominant ecdysteroids found in the haemolymph of decapod crustaceans are E, 20E, PonA and 3-dehydro-20-hydroxyecdysone (3D20E), but the composition and the respective levels of each vary between species, developmental stages and seasons (113). In the YO, the intermediate 5β-diketol can be converted into 5β-ketodiol, 3-dehydro-2,25-deoxyecdysone (3D2,25dE) or 3-dehydro-2,22-deoxyecdysone (3D2,22dE) (120). 5β-ketodiol acts as a second branching point and can be either converted to 5β-ketotriol to secrete ecdysone which is converted to 20-E, or to 2,25-deoxyecdysone (2,25dE) to secrete 25dE, which can be converted to PonA and subsequently converted to 20E (121) (Fig. 5). At the first branching point, 5β-diketol can otherwise be hydroxylated at C25 to produce 3D2,22dE, followed by sequential hydroxylation of C22 and C2 to produce secreted 3DE, which is in peripheral tissue converted to 3-dehydro-20-hydroxyecdysone (3D20E) by 20-hydroxylation (122). Another potential path is hydroxylation at C22 to 3D2,25dE, followed by hydroxylation at C2 to produce the secreted 3-dehydro-25-deoxyecdysone (3D25dE), which is reduced at C3 followed by a hydroxylation at C20 to produce PonA (123). The Halloween genes are conserved in crustaceans and are believed to play similar roles in crustaceans as in insects (102, 124). Due to the different branching points found in decapod crustaceans, it is believed that the enzymes coded by the Halloween genes can bind multiple substrates, but as in insects the enzymes can only hydroxylate in the specific order of C25, C22, C2 and C20 (125). For example, Phm cannot hydroxylate C25 if C2 has already been hydroxylated by Sad. Which ecdysteroids are secreted from the YO vary depending on the species and developmental stage, but usually no more than two ecdysteroids are secreted concurrently (113). The biologically active forms 20E and PonA have been shown to bind the ecdysone receptor complex in crustaceans *in vitro* (126-128), but less is known about the biological roles of PonA compared to 20E, which is known to play roles in molting and reproduction in branchiopod and decapod crustaceans (109, 129-131). Exogenous PonA induces similar physiological responses as exogenous 20E (131), and shows a stronger affinity to the ecdysone

receptor compared to 20E *in vitro* (131, 132). Insects do not synthetize PonA, but similarly to findings in crustaceans, the insect ecdysone receptor is still induced by lower concentrations of PonA compared to 20E *in vitro* (133). Little is known about ecdysteroid synthesis in crustaceans outside of decapods. A key difference is that of the organisms with investigated ecdysteroid synthesis, the water flea and salmon louse, which do not appear to have a molting gland in which ecdysteroid prohormones are synthesized and subsequently released to peripheral tissue for conversion to its biologically active form (120, 134). The exact manner of how ecdysteroid synthesis is compartmentalized in copepods remains unknown, but the intestine and ovaries have been suggested as main sites of ecdysteroid prohormone synthesis prior to dispersion into the hemolymph (134).

1.5.2 Ecdysteroids in arthropods

As more arthropod and ecdysozoan genomes have become available, it has become possible to investigate how well genes in the ecdysone biosynthetic pathway and ecdysone signalling pathways are conserved. A wide analysis on arthropod genomes revealed that the genes encoding for EcR and Usp are present in all arthropod subphylum (102). EcR and Usp have not been found in the genomes of free-living nematodes, but orthologues are present in the genome of the parasitic nematode *Dirofilaria immitis* (135). As for the ecdysteroids, E and 20E have been identified in the parasitic nematode and annelids, suggesting that ecdysone regulated molting predates the origin of the ecdysozoans (136, 137). Most Halloween genes are conserved in all arthropods, however *Phm* has not been identified in any Chelicerata species to date (102). *Shd*, the 20-hydroxylase responsible for conversion of E to 20E, was also notably missing among decapod crustaceans. However, another CYP enzyme with little phylogenetic similarities to *Shd*, named *Shed*, was found to catalyse the same reaction (138). The absence of *Phantom* in Chelicerata genomes have led to the hypothesis that PonA is the regulator of molting in this group (102). A study on the spider mite *Panonychus citri* supports this hypothesis, as it was shown that molting could be rescued by the supplementation of PonA and not 20E in mites where mRNA of the Halloween gene *Spook* was knocked down (139). However, 20E has been detected as the predominant ecdysteroid in other chelicerates such as sea spiders (140),

spiders (141) and scorpions (142), suggestive of the presence of an unknown *Phm*-like CYP hydroxylase in Chelicerata, perhaps analogous to the functional orthologues *Shed* and *Shd*.

1.6 Molting

1.6.1 Overview of molting and its connection to metamorphosis

The first step in arthropod molting is apolysis, the detachment of the old cuticle from the epidermis. The epidermal cells lay down a new cuticulin layer, which makes out the outermost layer of the epicuticle, and fill the gap between the old and new cuticle with a molting fluid (143). The molting fluid consists of a multitude of proteins, among them enzymes such as chitinases, chitin deacetylases and proteases, which will degrade the old cuticle and allow the break down products to be recycled to form the new cuticle (144). Once the new procuticle is laid down, the animal will undergo ecdysis, the process where the old exoskeleton is shed. Ecdysis is a sequence of behavioural events controlled by the nervous system, which through neuropeptide hormone signalling activates muscle contractions and convulsions to loosen, break up and finally shed the old cuticle (145). Once the old exoskeleton is shed, the cuticle will expand to make room for potential future growth. In insects, this is done by swallowing air, while crustaceans take in water to expand their cuticle following ecdysis (146). After ecdysis, the epidermal cells will deposit chitin, cuticular proteins and minerals to strengthen and harden the newly synthesized cuticle (146, 147). The period of growth between molts is called the intermolt period and varies in length between different arthropod species and between individuals of the same species depending on environmental factors and the developmental stage (148, 149).

Molting in arthropods, depending on the species and developmental stage, can be coupled with metamorphosis. Insects can be divided into separate categories based on the nature in the way they molt (150). Ametabolous insects undergo little to no physical change and appear to just grow for each successive molt until the reproductive adult stage. Hemimetabolous insects gradually acquire adult features between each successive molt, with several immature nymph stages prior to development to

reproductive adults. Holometabolous insects have larval forms which are very different from the reproductive adults. The larvae feed and successively molt to facilitate growth in size, but they do not acquire adult features like the hemimetabolous nymphs. When the larva is fully grown, it enters an immobile pupal stage and undergoes a complete and drawn-out metamorphosis to the adult stage. Crustaceans also have diversity of life cycles and strategies, however, the differences and similarities associated with insect metamorphosis are mostly observed on the surface level as there is little knowledge concerning the molecular mechanisms in crustaceans compared to insects (151). In insects, molting and metamorphosis are regulated predominantly by three types of hormones; peptide hormones, ecdysteroids and the sesquiterpenoid known as juvenile hormone (JH) (152). While ecdysteroids and peptide hormones regulate the timing and execution of the molt, JH determines the nature of the molt (153). In the fruit fly and the moth *Manduca sexta*, the presence of JH during larval stages prevents metamorphosis, resulting in larval-to-larval molts. In the absence of JH, the larvae will initiate transition to the pupal stage in response to the next surge of ecdysteroid hormone (154). The sesquiterpenoid methyl farnesoate (MF) has been identified in crustaceans and chelicerates, and is believed to be the analogue of JH (155). However, the exact molecular function of MF signalling remains unknown in crustaceans (156). In insects, the intra-nuclear Basic Helix-loop-Helix/Per-ARNT-SIM (bHLH-PAS) protein Methoprene-tolerant (Met) has been shown to bind JH (157, 158), and its role as a mediator of JH signalling was demonstrated in the beetle *Tribolium castaneum*, where knockdown of Met resulted in the premature onset of metamorphosis (159). Metamorphosis and molting appears to be linked in insects. The JH receptor Met has been shown to regulate the expression of the ecdysteroid-inducible gene *E75A* in the absence of 20E by interacting with the ecdysone induced nuclear receptor beta isoform of Fushi-tarazu Factor-1 (βFTZ-F1) (160). In addition, JH signalling has been demonstrated to repress ecdysone biosynthesis in the prothoracic gland (PG) by regulating the expression of the Halloween gene *Spook* (161). The interaction goes both ways, as 20E signalling has been shown to regulate JH synthesis in insects and vice versa (162, 163).

1.6.2 How nuclear receptors regulate ecdysteroid titers

An important aspect of the regulation of molting is the timing of ecdysteroid biosynthesis and its dispersal to peripheral tissue. In insects, synthesis of ecdysone in the PG is influenced by extracellular and autocrine signals to ensure correct timing of molting (164) (Fig. 6). The predominant regulator is the neuropeptide prothoracicotropic hormone (PTTH), which when released in the nervous system binds the receptor tyrosine kinase Torso on PG cells to active an extracellular signal regulated kinase (ERK) pathway (165). Hormone receptor 4 (HR4), an ecdysone inducible nuclear receptor, oscillates between the nucleus and cytoplasm of PG cells. Torso activated signalling promotes this oscillation, resulting in the accumulation of HR4 in the cytoplasm when ecdysteroid levels are low, preventing HR4 from blocking the transcription of the *Cyp6t3*, a member of the ecdysteroid biosynthetic "black box" (166). PTTH is believed to regulate other ecdysteroid inducible transcription factors in the PG, as expression of the Halloween genes *Phm*, *Dib* and *Sad* have been shown to be affected by PTTH signalling (167, 168). However, only the nuclear receptor HR4 has to date been shown to be a transcriptional mediator of PTTH signalling in the PG (166). PTTH expression and secretion occurs in a rhythmical pattern which is regulated by the circadian clock in the insect brain, the biological internal clock (169).

Although expression of ecdysteroid inducible nuclear receptors is regulated by ecdysteroids from the PG, the nuclear receptors regulate the biosynthesis of ecdysteroids themselves through negative feedback loops. For example, high levels of 20E in the hemolymph in larvae of the moth *Manduca sexta* initiates the expression and phosphorylation of a specific Usp isoform in the PG, that in complex with EcR downregulates ecdysteroid synthesis even in the presence of PTTH (170). Ecdysteroid signalling has also been implicated in the maintenance of the circadian rhythm in insects, as the nuclear receptor Ecdysone-inducible protein 75 (E75) has been shown to negatively regulate the expression of CLOCK (CLK) in the fruit fly (171), a conserved regulator of the circadian rhythm in animals (172). E75 is also a mediator of signalling from the diatomic gas nitric oxide (NO), a short-range neurotransmitter, which is produced by nitric oxide synthase (NOS) in the PG (173, 174). E75 functions as a negative regulator of hormone receptor 3 (HR3), but binding of NO to the heme

ligand within the LBD of E75 interferes with the ability of E75 to dimerize with HR3. Without inhibition by E75, HR3 can induce transcription of the nuclear receptor βFTZ-F1, which in the fruit fly regulates the expression of the Halloween genes *Phm* and *Did* in the PG (175).

Input from the nutritional condition of the animal also plays a role in regulating ecdysone production in the PG through an insulin related pathway (176). During well fed conditions insulin-like peptides will bind the Insulin Receptors on PG cells and through a phosphokinase cascade phosphorylate the Forkhead Box Class O transcription factor (FoxO), a negative regulator of growth in the fruit fly (177). FoxO in its unphosphorylated state localize to the nucleus where it forms a complex with Usp that inhibits the transcription of *Phm* and *Dib*, delaying molting and metamorphosis. Once the appropriate weight and nutritional conditions have been met, insulin signalling results in the phosphorylation of FoxO, sequestering it in the cytoplasm away from its Usp partner, enabling ecdysteroid synthesis and eventually molting and metamorphosis (176). Other extracellular signals released in response to nutrition, such as TGFβ/Activin signalling and signalling from serotonergic neurons, also regulates ecdysone biosynthesis in coordination with PTTH and insulin (178, 179). Additionally, the PG has its own nutritional sensor through the Target of Rapamycin (TOR) kinase pathway, which when activated by nutritional markers such as ATP and amino acids, promote ecdysteroid production in the PG through pathways that crosslinks with PTTH signalling (180, 181). PTTH and insulin signalling is also dependent on signalling through the β3-octopamine receptor, a G-protein coupled receptor, which is activated in an autocrine manner by synthesis of its ligands octopamine and tyramine in the PG (182). The insect PG has to interpret and integrate a myriad of signals before committing to the production of surges of ecdysteroid hormones, and the analogy of the insect PG acting like a “CPU-like decision-making centre” is a fitting one (169).

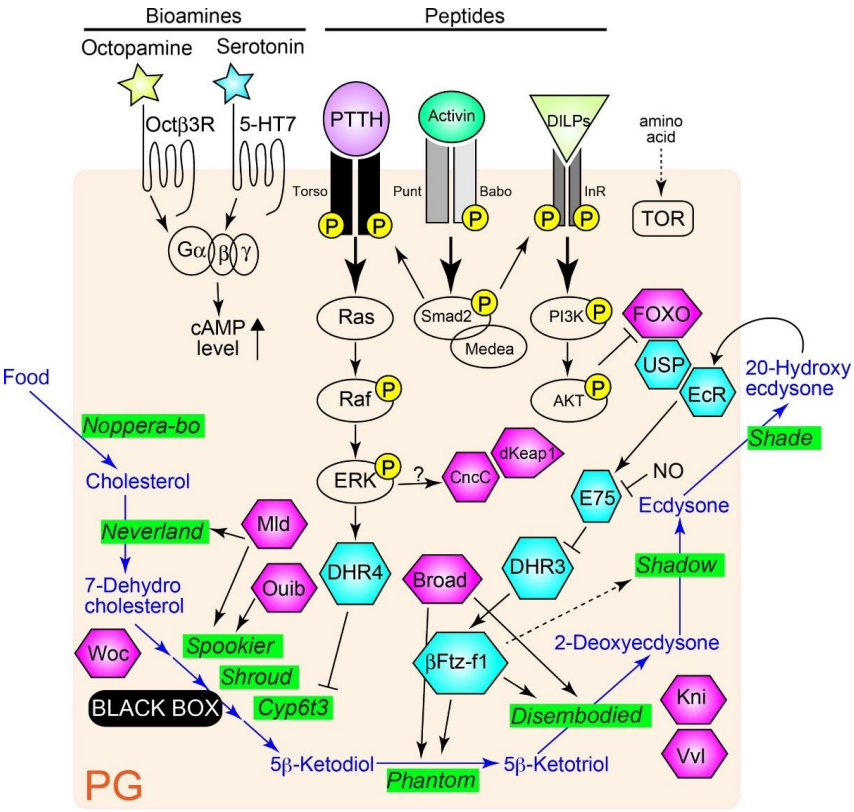


Figure 6. Overview of the regulatory mechanisms controlling ecdysteroid biosynthesis in the prothoracic gland (PG) of the fruit fly, Drosophila melanogaster. The ecdysteroids in the biosynthesis pathway are written in blue. The ecdysteroid biosynthesis enzymes are highlighted in green. Hexagons represent transcription factors, with nuclear receptors coloured in sky blue. DHR4: Hormone receptor 4 (HR4), DHR3: Hormone receptor 3 (HR3), Usp: Ultraspiracle, EcR: Ecdysone receptor, FTZ-F1: Fushi-tarazu Factor 1, E75: Ecdysone-induced protein 75. Figure re-used with permission from (164).

The current knowledge concerning the molecular regulation of ecdysone biosynthesis in crustaceans is predominantly based on studies from decapod crustaceans and the neuropeptides molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). Analogous to PTTH in insects, MIH and CHH are produced by neuronal cells which in decapod crustaceans are located in the eyestalks. But unlike PTTH, MIH and CHH are negative regulators of ecdysteroid biosynthesis in the crustacean YO (183), with MIH being the more potent regulator of the two (184). Removal of the eyestalks of shrimp results in elevated levels of circulating ecdysteroids and a shortened molt

cycle (185). Binding of CHH and MIH to their respective receptors, a membrane bound guanylyl cyclase and a G-protein coupled receptor (GPCR) (186, 187), results in elevated levels of cGMP and cAMP that inhibits ecdysteroid synthesis (188). The current model of crustacean MIH signalling describes a triggering and a summation phase (146). In the triggering phase, elevated levels of cAMP following MIH receptor binding results in the phosphorylated activation of a Ca^{2+} -channel by cAMP dependent protein kinase A. Influx of Ca^{2+} subsequently activates Calmodulin (CaM) and the Ca^{2+}/CaM dependent phosphatase Calcineurin (CaN). In the summation phase Nitric Oxide Synthase is activated through dephosphorylation by CaN and prolongs the response to MIH by activating a guanylyl cyclase that in turn activates a cGMP-dependent protein kinase that inhibits ecdysteroid synthesis. Signalling pathways operating in the insect PG, such as TOR and TGFβ/Activin, appear to be conserved regulators of ecdysteroid levels in the crustacean YO as well (189, 190). However, the exact transcriptional regulators and which ecdysone biosynthetic target genes are affected by the pathways described above remain unknown. It is also unknown how high ecdysteroid levels represses YO ecdysteroid synthesis to return to its pre-induced state during ecdysis and post-molt, and how the ecdysone receptor complex and other ecdysteroid inducible factors tie into this (191).

1.6.3 Molting is regulated by an ecdysone induced transcriptional cascade

Most of what is known about molecular regulation of molting comes from research on insects, and in particular the fruit fly. A good illustration of how ecdysteroids, the ecdysone receptor complex, and other nuclear receptors regulate molting in insects comes from studies by Clever (192) and by Ashburner (193-195), and how 20E controls a sequence of “puffing” of polytene chromosomes in the fruit fly. Polytene chromosomes, found in cells in the salivary gland of fruit flies, are large chromosomes produced from repeated rounds of replication without cell division. The bands of the polytene chromosomes can at certain time swell, which are called puffs, and these puffs indicate sites of the chromosome where active gene expression is taking place. When Ashburner treated isolated *in vitro* cultured salivary glands with 20E, he observed within 10 minutes a small number of early puffs, and after some hours an additional of

100 more puffs were observed. It was subsequently revealed that inhibition of protein synthesis did not prevent the early puffs immediately following 20E treatment but did prevent the late puffs. It appeared that the early puffs were under direct control of 20E, while the late puffs were dependent on a critical concentration of expression of the genes from the early puffs. In addition, removal of 20E at different times after exposure could cause regression of the early puffs and premature induction of the late puffs. These observations gave rise to what is known as the Ashburner model (Fig. 7a). 20E and its receptor induces expression of early response genes, which in turn induces the expression of late genes while also repressing their own expression. The expression of the late genes is also prevented by the ecdysone receptor and their expression is dependent on the decrease of 20E concentrations in order to be expressed.

As years have passed, the transcription factors involved in mediating the ecdysteroid signal have been identified, many of them belonging to the nuclear receptor superfamily (196). Many of the complexities and intricacies of the ecdysteroid signalling pathway in insects have been unveiled as well. Some examples include: isoforms of EcR and other ecdysteroid inducible factors can produce tissue and stage specific responses to 20E (197-200), amount and duration of ecdysone pulses can produce different responses (166, 201), ecdysone induced factors provide competence for stage specific responses during the next ecdysone pulse (202-204) and interaction with JH signalling pathways (160, 205). After decades of research on insect molting the principle of the Ashburner model still holds true. Molting is regulated by a transcriptional cascade, mostly consisting of other nuclear receptors, initiated by the ecdysone receptor dimer complex after binding to its hormone ligand 20E (Fig. 7b) (99). The ecdysone receptor and the ecdysteroid inducible factors through interconnected regulation ensures that the members of the transcriptional cascade are expressed at specific times and in a specific order (154, 196). This ensures that the many downstream genes, such as proteases, chitinases and ecdysis triggering peptide hormones, are also expressed at a correct time and order for proper execution of molting. Two early response genes are the ETS transcription factor *Ecdysone-inducible protein 74* (*E74*) and the nuclear receptor *Ecdysone-inducible protein 75* (*E75)*. *E74* encodes two isoforms, *E74A* and *E74B*, which have distinct functional roles in the

ecdysone signal transduction hierarchy (206). E74B is a negative regulator of late gene expression and is itself induced at low ecdysone levels and repressed at high ecdysone levels. E74A, however, is induced at higher levels of 20E and is an inducer of late gene expression. This switch in E74 isoform usage ensures correct timing of expression of downstream targets in the transcriptional cascade. *E75* also encodes several isoforms with different functions (207). A key role of E75 is to ensure that the nuclear receptor βFTZ-F1 is expressed at the correct time, this is achieved by binding and inhibiting the function of HR3, an inducer of *βFTZ-F1* expression (207, 208). HR3 and HR4, together with the early gene products themselves, inhibit the continued expression of early genes (206, 208-210). Although *HR3* and *HR4* are induced by 20E expression, their accumulation and ability to induce expression of *βFTZ-F1* requires a drop in 20E levels (211). βFTZ-F1 expression is required for all stage transitions and molt, and its mutants are associated with cuticular abnormalities and the inability to initiate the ecdysis sequence (211-213).

The members of the ecdysteroid induced transcriptional cascade appear well conserved between insects and crustaceans (214). Some nuclear receptors in the ecdysteroid signalling pathway have been identified and characterized in a variety of crustaceans, including the salmon louse, such as EcR and Usp (108, 126, 128, 215-220), E75 (221-224), HR3 (223, 224) and FTZ-F1 (225, 226). However, little is known about the functional roles nuclear receptors play in crustacean molting and how their expression is correlated with ecdysteroid levels, reviewed in (151). The combined knockdown of *EcR* and *Usp* in nauplius II salmon louse larvae resulted in molting arrest, revealing that ecdysteroid signalling regulates molting in the salmon louse (227). A knockdown study of *EcR* and *Usp* in the fiddler crab, *Uca pugilator*, also resulted in molting failure (228). Knockdown of *E75* in the crab *Fenneropenaeus chinensis* caused molting arrest (222), and knockdown of *Usp* resulted in the downregulation of *E75* (229). *In vivo* injections of 20E in the shrimp *Litopenaeus vannamei* and the crayfish *Procambarus clarkii* revealed that the expression of *EcR*, *Usp* and *E75* are all regulated by levels of 20E (230, 231). A study in the water flea, *Daphnia magna*, showed that *HR3* and *HR4* expression were induced by soaking the animals in solutions containing 20E (224).

These findings strongly suggest that putative members of the ecdysone hierarchy may play conserved roles as regulators of molting in crustaceans similar to their roles insects.

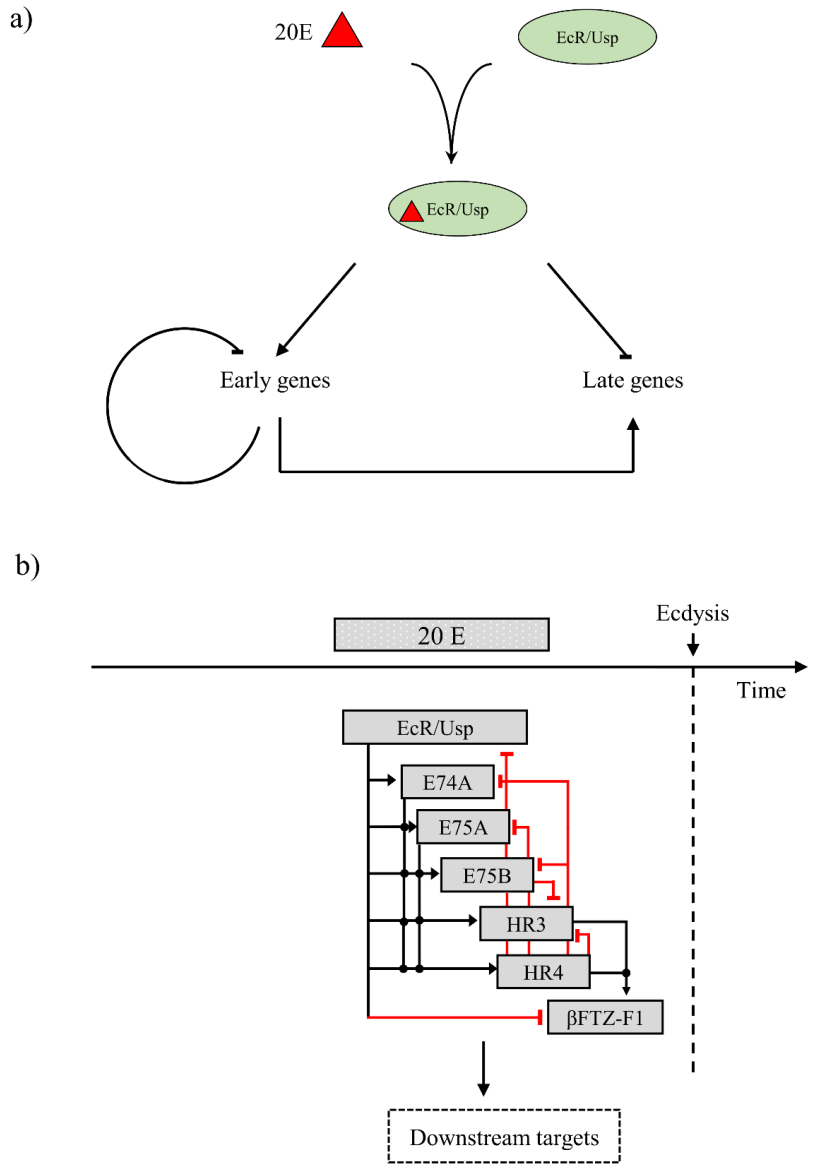


Figure 7. The Ashburner model illustrating the regulation of molting and metamorphosis during larval and pre-pupal development in the fruit fly, Drosophila melanogaster. ***a)*** *Model proposed by Ashburner et al (193-195) to explain the control of the puffing sequence of polytene chromosomes in D. melanogaster in response to pulses of the ecdysteroid 20E.* ***b)*** *A diagram illustrating how expression of ecdysone inducible transcription factors is regulated in a temporal and sequential order in the ecdysone transcriptional hierarchy regulating molting and metamorphosis in D. melanogaster (154, 196, 232).*

2. Aims of the study

The main goal of the study is to elucidate molecular mechanism regulating molting in the salmon louse. Based on previous studies in the salmon louse and what is known about the regulation of molting in insects, it is suspected that molting in the salmon louse is controlled by a similar ecdysteroid regulated transcriptional hierarchy. In order to elucidate mechanisms behind the regulation of molting in the salmon louse, the following aims were set:

- Verification of whether 20E or PonA is the molting hormone in the salmon louse through *in vivo* and *in vitro* studies.

- Investigate the correlation between ecdysteroid levels and the expression of putative ecdysteroid regulated factors by obtaining high resolution measurements of ecdysteroids and selected mRNA levels in the pre-adult I stage.

- Investigate if a drop in ecdysteroid levels prior to ecdysis following an initial pulse is required for molting in the salmon louse.

- Elucidate the molecular structure of the nuclear receptors *HR3*, *HR4*, *E75* and *FTZ-F1*, and their potential functions through RNA interference in different stages of salmon louse development.

3. Summary of papers

3.1 Paper I

"The ecdysteroid Ponasterone A serves as the major regulator of molting in the salmon louse, an ectoparasitic crustacean (*Lepeophtheirus salmonis*)"

In this project, the goal was to learn more about the ecdysone signaling pathway that regulates molting in the salmon louse. In insects, molting is controlled by pulses of the ecdysteroid 20-hydroxyecdysone (20E). The ecdysone receptor complex, which consists of the nuclear receptors ecdysone receptor (EcR) and Ultraspiracle (Usp), is activated by the ligand 20E. Once activated, the receptor complex initiates a transcriptional cascade consisting of a small set of ecdysone inducible transcription factors. Through feed-forward and feedback loops, the members of the transcriptional cascade are expressed in specific temporal profiles. This ensures that the genes regulated downstream of the cascade are expressed at the correct time and place during the molt cycle. Knowledge of the molecular mechanisms of ecdysone signaling is limited in crustaceans compared to insects. Through the implementation of a two-hybrid reporter assay, it was found that the ecdysteroid Ponasterone A (PonA) is the ligand for the salmon louse ecdysone receptor complex. This was verified by treatment of nauplii II lice with different ecdysteroids. Treatment also revealed that the studied transcription factors have specific responses to increases in PonA levels. Measurements of ecdysteroid levels and gene expression of the putative ecdysteroid regulated transcription factors *HR3*, *E74*, *E75*, *HR4*, and *FTZ-F1* in high-resolution across the salmon louse pre-adult instar, revealed that the transcription factors display distinct expression profiles correlating with ecdysteroid levels. Additionally, treatment with PonA in the middle of the nauplius II stage resulted in molting arrest, indicating that salmon louse molting is dependent on a drop in ecdysteroid levels. Combined, these results suggest that molting in the salmon louse is regulated by a PonA induced transcriptional cascade, similar to the ecdysone signaling hierarchy described in insects.

3.2 Paper II

"The *FTZ-F1* gene encodes two functionally distinct nuclear receptor isoforms in the ectoparasitic copepod salmon louse (*Lepeophtheirus salmonis*)"

The objective of this study was to functionally characterize the salmon louse gene encoding the nuclear receptor Fushi Tarazu Factor-1 (FTZ-F1). Study of FTZ-F1 orthologues in insects and nematodes have found that the nuclear receptor is a crucial regulator of molting in both subphyla. In the fruit fly, disruption of βFTZ-F1 isoform function results in abnormal cuticle development, developmental arrest, and failure to initiate ecdysis at the time of molting. The role of FTZ-F1 in molting has not been characterized in crustaceans. The potentially conserved function across the ecdysozoan phylum of FTZ-F1 as a regulator of molting makes it an interesting target in order expand the knowledge how molting is regulated in the salmon louse. Molecular characterization of the salmon louse *FTZ-F1* revealed that the gene encodes two isoforms with unique N-terminal domains, αFTZ-F1 and βFTZ-F1, which are generated by alternative promotor usage and splicing. Re-analysis of available FTZ-F1 gene sequences in other ecdysozoan showed that the FTZ-F1 gene structure observed in the salmon louse and fruit fly is conserved in all ecdysozoans apart from nematodes. The salmon louse *FTZ-F1* isoforms display similar temporal expression profiles, with *βFTZ-F1* being the predominant isoform. Analysis of tissue specific expression in adults revealed that *αFTZ-F1* is the predominant isoform in brain tissue, while *βFTZ-F1* is the predominant form in ovaries and eggs. Depletion of *βFTZ-F1* in adult females resulted in the destruction of oocytes at the vitellogenic stage. Knockdown of *βFTZ-F1* in nauplii and pre-adult I lice resulted in molting arrest to the copepodid and adult stage, respectively. Transcriptome sequencing of *βFTZ-F1* knockdown nauplius II lice indicate that *βFTZ-F1* regulates genes involved in cuticle recycling, cuticle detachment, and ecdysis. The potential functions of *αFTZ-F1* remain unknown.

4. Discussion

Ecdysteroid hormones regulate a diverse range of biological processes in arthropods, including molting. In insects, molting is controlled by pulses of the ecdysteroid 20E. Binding of 20E to EcR/Usp activates the receptor complex, which initiates a transcriptional cascade of a small set of response genes, mostly consisting of other nuclear receptors (196). Interconnected regulation between the different factors, and a decrease in levels of 20E after the initial pulse, ensures that the factors and their target genes are expressed at the correct order and time during molting (166, 200, 206, 208-213, 232-238). Comparably little is known about the molecular mechanism of how the ecdysteroid signal is transduced to regulate molting in crustaceans (151). Most studies in crustaceans on the orthologues of transcription factors in the insect ecdysone transcriptional hierarchy are limited to sequence isolation, ecdysteroid responsiveness, and expression profiles (224, 230, 231). A handful of functional studies through RNAi have been performed on crustacean orthologues of the nuclear receptors E75 (173, 222, 239), EcR (228), and Usp (229), including in the salmon louse (227), demonstrating a role in the regulation of molting.

Study of the salmon louse ecdysteroid signalling hierarchy will provide further insight into how molting is regulated in the salmon louse and other crustaceans. Here, the ligand of the salmon louse ecdysone receptor complex was verified both *in vitro* and *in vivo* (**Paper I**). In addition, the relationship between ecdysteroid levels and gene expression of the probable ecdysteroid regulated genes *HR3*, *HR4*, *E74*, *E75*, and *FTZ-F1*, were characterized in high-resolution across the salmon louse pre-adult I and nauplius II stage (**Paper I**). To further understand the potential functions of ecdysteroid regulated factors in the salmon louse, the gene encoding the nuclear receptor FTZ-F1 was identified and functionally characterized (**Paper II**).

4.1 Ponasterone A is the molting hormone in the salmon louse

Crustaceans produce a larger diversity of ecdysteroids compared to insects, with the two predominant forms being 20E and PonA (113). Both ecdysteroids are known to have biological functions, but there has mainly been a focus on studying the *in vivo* effects of 20E (230, 231, 240). Interestingly, *in vitro* ligand binding assays of various crustacean ecdysone receptors revealed a strong binding affinity for PonA (127, 241, 242). In species where it was studied, the ecdysone receptor also showed a stronger affinity to PonA compared to 20E (126, 132). This was also the case in the salmon louse (**Paper I**). Only PonA was able to induce dimerization and activation of the salmon louse ecdysone receptor complex. Molting arrest was only induced with PonA when treating salmon louse nauplii with different concentrations of E, 20E, and PonA. In addition, treatment with E and 20E had little effect on the expression of ecdysteroid regulated factors in comparison to PonA. ESI-LC-MS/MS analysis of pre-adult I lice of both sexes revealed that both PonA and 20E were present, with PonA being the predominant ecdysone derivate (**Paper I**). PonA has been shown to be present in the nauplius II and copepodid stage (134), but quantification through a molt cycle had not been performed before now. In summation, the *in vivo* and *in vitro* data show that PonA is the molting hormone in the salmon louse.

In the majority of decapod species studied, several isoforms of EcR have been identified with differences in the LBD (218, 241-247), which is unusual as most isoforms in insects have differences only in the A/B domain (243). This is interesting as it has been speculated that the ratio of 20E/PonA might have functionality in decapod crustaceans (113, 248), and these isoforms could potentially exhibit different binding affinities to 20E and PonA. The salmon louse, however, only produce *EcR* mRNA isoforms with differences in the 5' untranslated region (UTR) (108). In addition, the salmon louse ecdysone receptor appears to have a lower affinity to 20E compared to other crustaceans (126, 132). The possible functions of 20E in the salmon louse remain unknown, but 20E appears to have less functionality in the salmon louse compared to the decapod and branchiopod species studied thus far.

4.2 Putative members of the ecdysone signalling cascade display molt specific expression profiles in the salmon louse

In insect larval and pupal development, ecdysone regulated factors display specific temporal expression profiles in response to pulses of 20E hormone (154, 232). These factors, as well as many ecdysone biosynthetic genes, are conserved among arthropods (214). If the factors are conserved as regulators of molting and mediators of ecdysone signalling in other arthropods, such as the salmon louse, they would likely display specific and repeatable expression profiles across different developmental stages.

As suspected, measurements of the expression of the probable ecdysteroid regulated factors *HR3*, *HR4*, *E74*, *E75*, and *FTZ-F1* revealed that these genes exhibit specific temporal expression profiles in both the pre-adult I and nauplius II stage (**Paper I)**. The expression profiles of the genes were similar in both stages, suggesting that their expression profiles are associated with molting. Such cyclical expression profiles of *E75*, *HR3* and *FTZ-F1* was also found in the nematode model organism *Caenorhabditis elegans* (249). RNAi mediated knockdown of the *HR3* (*NHR-23*) and *FTZ-F1* (*NHR-25*) orthologues in *C. elegans* resulted in molting defects (250-252). These repeatable expression profiles for the different salmon louse ecdysteroid regulated factors were also found in a transcriptome sequencing time-series of chalimus and pre-adult lice, divided into instars of different instar ages (46). However, measurements were done on samples comprising of pooled animals of similar instar age at lower resolution (3-4 sampling points per stage). In the current study, high-resolution expression timelines were produced from single individuals through the pre-adult I molt cycle (**Paper I**). There were several notable differences between the individual pre-adult data and the transcriptome sequencing data when comparing the expression profiles of the genes studied. In the case of *HR3* and *HR4*, the steep drop in expression observed in the middle of the pre-adult I stage was not seen in the transcriptome sequencing. This demonstrates the importance of measuring gene expression in a high-resolution across the molt cycle. The expression of certain factors can change very quickly within a short

developmental time frame, changes which can be hard to detect when dividing the animals into instars of different instar ages in low resolution.

A notable difference between the expression profile in the nauplius II and pre-adult I stage was the upregulation of *HR3* and *HR4* and the downregulation of *E74* and *FTZ-F1* at the end of the pre-adult I molt cycle (**Paper I)**. This was likely due to the different nature of the next developmental stages, and not caused by the lower resolution of the nauplius II timeline. The copepodites enter an intermolt period until a suitable host is found, while the pre-adult II lice have continuous access to nutrition from the host. This is supported by observations in the transcriptome sequencing timeline done on salmon louse from planktonic copepodites to pre-adult I, which showed that *HR3* and *HR4* expression remains low while *E74* and *FTZ-F1* expression remains high in planktonic copepodites (46). These findings show that the expression of *HR3* and *HR4* is negatively correlated with *E74* and *FTZ-F1* in both the nauplius I and pre-adult molt cycle, indicating that their regulation is connected.

There is only data on the expression of these factors in a few other crustaceans (224, 239, 253). A comparison to the transcriptome sequencing of *HR3*, *HR4*, *E74*, and *E75* through the molt cycle of the shrimp *L. vannamei* (253), reveals that the expression profiles of *HR4*, *E74*, and *E75* are somewhat similar in both species (**Paper I**). The expression profile of *HR3* on the other hand appear to be the opposite in *L. vannamei*. *E75* expression in the black tiger shrimp, *Penaeus monodon*, also showed a similar profile to the salmon louse and *L. vannamei*. The expression of *E74* in *L. vannamei* appear to increase prior to *HR3*, *HR4* and *E75*, but the expression profiles of the latter genes were indistinguishable from each other due to the low-resolution sampling.

4.3 The Ponasterone A induced transcriptional hierarchy in the salmon louse

In the insect ecdysone induced transcriptional hierarchy, genes have different responses to different levels of the ecdysteroid 20E. The factors BR-C, E75A, E74A, and E74B are considered early response factors as their expression is primarily induced by

elevated levels of 20E through the activity of the ecdysone receptor complex (201, 254, 255). The expression of the early late genes *HR3* and *HR4* is dependent on the activity of both the ecdysone receptor complex and early response factors (209, 210). The expression of FTZ-F1 is a secondary response to 20E signalling, requiring the withdrawal of 20E and early response factors, before expression is induced by HR3 and HR4 (209-211, 256).

Measurements of ecdysteroid titers and gene expression through the salmon louse pre-adult molt cycle showed that high levels of ecdysteroids were correlated with high levels of *HR3* and *HR4* expression and low levels of *E74*, *E75*, and *FTZ-F1* expression (**Paper I**). To more accurately determine what effect PonA levels had on the expression of the different genes, nauplii II were treated with PonA at two different time points during the molt cycle. The results revealed that *HR3*, *HR4*, and *E75* expression was upregulated in response to PonA, while increases in *E74* and *FTZ-F1* expression was repressed by the introduction of PonA (**Paper I**). This indicates that the expression profiles of the probable ecdysteroid regulated factors during the pre-adult I molt cycle are connected to levels of PonA. Studies on other crustaceans also show that these genes are regulated by ecdysteroid hormones and mimics (224, 230, 231, 239, 257), indicating that crustaceans and insects likely have similar mechanisms of mediating ecdysteroid signals during molting.

Expression of *HR3* and *HR4* in the salmon louse appears to be primarily regulated by levels of PonA (**Paper I**). Their expression levels were strongly increased by the introduction of exogenous PonA. In addition, treatment with PonA at time points of low *HR3* and *HR4* expression increased the expression of both back to similar peak levels as observed at the start of the molt cycle when PonA levels were likely at their highest (**Paper I**). In insects, *HR3* and *HR4* expression requires both 20E and expression of preceding early response factor proteins, but in the salmon louse, *HR3* and *HR4* appear to be early response factors in response to PonA (**Paper I**). *HR3* expression in the water flea, *D. magna*, was also induced by ecdysteroid treatment and its expression in the adult molt cycle coincided with a pulse of ecdysteroid hormone (224).

Increases in the expression of *E74* and *FTZ-F1* were repressed by high levels of PonA in the salmon louse (**Paper I)**. *E74* and *FTZ-F1* expression was induced after *HR3* and *HR4* in the salmon louse molt cycle and their peak expression levels coincided with low levels of *HR3* and *HR4* expression. The repression of *E74* expression in response to ecdysteroids was unlike in other crustaceans. Injection of 20E in the prawn, *Procambarus clarkii*, resulted in the upregulation of *E74* expression (231). Comparing the transcriptome sequencing timeline in *L. vannamei* (253) to a study measuring ecdysteroid levels through the *L. vannamei* molt cycle (258) seem to suggest that *E74* is upregulated in response to ecdysteroid titers in this species as well. Unlike in the salmon louse, *E74* expression in *L. vannamei* also appears to precede that of the other ecdysteroid regulated factors in response to ecdysteroid hormones. The expression of *E75* was induced by PonA in the salmon louse (**Paper I)**. This has also been reported in several other crustaceans (224, 230, 231, 239). Although *E75A + E75B* levels were induced by PonA, peak expression levels occurred after increased expression of *E74* and the *FTZ-F1* isoforms, at a timepoint when ecdysteroid titers were low (**Paper I**). The reported *E75* expression in the current study was measured targeting two *E75* paralogs. Their independent expression profiles and PonA responses might be different, confounding the results. Measuring the paralogs independently would be necessary to more clearly define what potential role the two paralogs play in the ecdysone signalling hierarchy.

There are no comparable studies done on any other crustaceans. The timeline studies of hormone levels and gene expression in the *L. vannamei* molt cycle provides some insight (253, 258), but the compratively lower sampling resolution in the two mentioned studies makes a more detailed comparison of expression profiles difficult. In other species, the specific responses of different genes to treatment with exogenous ecdysteroids have been investigated (224, 230, 231), but not studied in the context of an expression and hormone level timeline as in the current study (**Paper I**). The expression profiles of the ecdysteroid regulated factors in the current study are suggestive of similar regulatory mechanisms as observed in the well-studied fruit fly

molting model. However, the sequential order of expression and the genes individual response to ecdysteroids appear to vary between different arthropod species.

A limitation of the current study is that the expression profiles of the studied genes were only measured at an mRNA level (**Paper I/II**). Protein profiles have not been compared to the transcript profiles. Another limitation is that all genes were measured in whole salmon lice or pooled samples of salmon lice. It is not known in which tissues the ecdysone transcriptional cascade occurs, or if the genes are expressed at similar times in different tissues. The genes studied are likely also regulated by other molecular pathways than ecdysteroid signalling. This is observed for *E75* and *E74* during fruit fly development, as they are expressed in a sequential manner in the absence of ecdysteroid pulses (232). This might be a confounding factor when measuring expression levels in the whole animal and relating it to hormone levels.

4.4 A decrease in Ponasterone A levels is a prerequisite for ecdysis

A drop in molting hormone levels before ecdysis appears to be a conserved feature of arthropod molting. This has been shown in decapods (248, 258, 259), branchiopods (130), as well as in insects (154, 260). In the fruit fly, the expression of *βFTZ-F1* is dependent on a drop in ecdysteroid levels. βFTZ-F1 is an important regulator of ecdysis in the fruit fly, and βFTZ-F1 mutants enter molting arrest (211, 236). Analysis of ecdysteroid levels in individual pre-adult I lice show that a drop in ecdysteroid levels is also a feature in the salmon louse molt cycle (**Paper I**). The level of 20E and PonA was high at the start of the molt cycle and gradually decreased towards the end of the stage. Treatment with PonA approximately in the middle of the molt cycle also resulted in molting arrest at the nauplius II to copepodid molt (**Paper I**), indicating that elevating PonA levels late in the molt cycle inhibits ecdysis. Curiously, treatment with PonA earlier in the nauplius II stage did not result in molting arrest. Why treatment at the two timepoints had different outcomes on molting success is unknown. PonA treatment earlier in the stage possibly gave the lice enough time to reduce PonA by metabolism to sufficiently low levels prior to ecdysis. Measuring hormone levels at

different times after PonA treatment and comparing it to untreated lice can confirm whether the lice are able to metabolize the exogenous PonA in time before ecdysis, or if the difference in outcome might be related to varying vulnerabilities to exogenous PonA at different times in the instar.

4.5 The *βFTZ-F1* isoform is required for ecdysis

In the fruit fly, the *FTZ-F1* gene encodes two nuclear receptor isoforms, αFTZ-F1 and βFTZ-F1, produced through different promotor usage and splicing (236). αFTZ-F1 is responsible for regulating pair-rule segmentation during early embryogenesis (261), while βFTZ-F1 is expressed later in development and is required for each stage transition and molt during fruit fly development (211-213). FTZ-F1 has been shown to be an important regulator of molting in nematodes as well, suggesting that its function as a regulator of molting is conserved among ecdysozoans (250, 251). The function of FTZ-F1 has not been studied in crustaceans before. Due to its potentially conserved role as a universal regulator of molting in ecdysozoans, *FTZ-F1* gene function was characterized in the salmon louse (**Paper II**).

Like in the fruit fly, the salmon louse *FTZ-F1* encodes two isoforms with unique N-terminal A/B domains, which are generated by transcription of the *FTZ-F1* gene from two alternative promotors (**Paper II**). By re-analysing available *FTZ-F1* gene sequences in other ecdysozoans, it could be shown that the structural organization of the *FTZ-F1* gene was a conserved feature in all analysed ecdysozoans apart from nematodes (**Paper II)**. Knockdown of *βFTZ-F1* in nauplius larvae resulted in molting arrest during the molt to the copepodid stage. Histology revealed that nauplii had developed a new cuticle in addition to copepodid limbs and segments, but the animals failed to undergo ecdysis and remained trapped in the old cuticle at the time of molting (**Paper II**). To check if RNAi of *βFTZ-F1* would cause molting arrest in other stages as well, RNAi knockdown was performed in pre-adult II females. Knockdown in pre-adult II females did not cause molting arrest but caused the destruction of oocytes during the vitellogenic stage of the emerging adult louse (**Paper II**). A similar phenotype was also observed in a study where *EcR* was knocked down in pre-adult II

lice, indicating that the ecdysone signalling hierarchy is crucial for oocyte development in the salmon louse (108). The expression profile of *βFTZ-F1* through the salmon louse molt cycle shows that expression of the gene is upregulated in the middle of the stage (**Paper II**). The RNAi effect in the pre-adult II lice potentially occurred after expression of βFTZ-F1 proteins required for the next molt. To avoid this, RNAi knockdown of *βFTZ-F1* was performed in the pre-adult I lice instead. This ensured that the lice had to pass through a full molt cycle under the effect of the RNAi. As suspected, the pre-adult I lice were able to molt to the pre-adult II stage but were unable to molt to adults (**Paper II**).

Transcriptome sequencing of *βFTZ-F1* knockdown lice revealed that genes associated with proteolysis, chitin metabolic process, and chitin binding were enriched among the genes differentially expressed between the knockdown and control lice (**Paper II**). This suggests that *βFTZ-F1* regulates processes involving cuticle recycling and detachment. These findings fit well with the observed phenotype in the molting arrested *βFTZ-F1* knockdown animals (**Paper II**). Among the downregulated genes following *βFTZ-F1* knockdown was also a homolog of the ecdysis triggering hormone receptor (ETHR), a G-protein coupled receptor (**Paper II**). This indicates that the molting arrest could also have been caused by a failure to initiate the ecdysis sequence. Downregulation of *ETHR* was also observed in RNAi knockdown of *βFTZ-F1* in the Colorado potato beetle, *Leptinotarsa decemlineata*, which entered molting arrest when ETHR was depleted (262). In insects, a critical function of βFTZ-F1 is to confer secretory competence to specific neurons that release ecdysis-triggering hormone (ETH), a neuropeptide that initiates the ecdysis sequence (212). Once released, the ETH activates the ETHR of other neurons, initiating a sequential release of neuropeptides required for ecdysis behaviour, cuticle tanning and sclerotization (263).

A comparison of the differentially expressed genes in *βFTZ-F1* knockdown lice to genes classified as up or downregulated in young, middle, or old lice based on their cyclical expression during a molt cycle (46), showed that depletion of *βFTZ-F1* systematically disrupted the expression of genes at the transition between the middle and late stage of the molt cycle (**Paper II**). Genes categorized as “up in middle” and

"down in old" were systematically upregulated, while genes categorized as "up in old" were downregulated. This fits well with the idea of βFTZ-F1 regulating a network of downstream genes that are expressed in specific temporal profiles during the molt cycle. The transcriptome sequencing and qPCR measurements also revealed that the two *E75* paralogs were downregulated in *βFTZ-F1* knockdown lice compared to control. This indicates that the upregulation of *E75* levels observed at the end of the molt cycle after peak expression of *βFTZ-F1*, *αFTZ-F1*, and *E74* is regulated either directly or indirectly by *βFTZ-F1* (**Paper II**).

The function of the *αFTZ-F1* isoform remains unknown. The isoform was not as highly expressed as *βFTZ-F1* but displayed a similar cyclical expression pattern. Knockdown of *αFTZ-F1* did not produce any visible phenotype in the current study across several RNAi experiments. Transcriptome sequencing of *αFTZ-F1* knockdown nauplii revealed that there were only a few differentially expressed genes compared to the control, but these were determined not to be associated with the *αFTZ-F1* knockdown (**Paper II**). The lack of phenotype and effect on overall gene expression suggests that the experimental depletion of *aFTZ-F1* was insufficient to cause adverse effects, or that *aFTZ-F1* had no critical roles during the salmon louse development studied here.

4.6 A proposed framework for the regulation of molting in the salmon louse

The findings in the current study indicate that molting in the salmon louse is regulated in a manner reminiscent of the Ashburner model in fruit flies (Fig. 7a). A model describing the current understanding of the ecdysone signalling hierarchy in the salmon louse is presented in Figure 8.

Measurements of *EcR* mRNA in high-resolution across a molt cycle (**Paper I**), and transcriptome sequencing data (46), show that expression of *EcR* and *Usp* across a molt cycle is more stable compared to the other nuclear receptors in the ecdysone signalling hierarchy in the salmon louse. In *L. vannamei* and *P. clarkii*, expression of *EcR* and *Usp* fluctuates across the molt cycle and increase in response to elevated levels of

ecdysteroid hormones (230, 231, 253). In the salmon louse, EcR/Usp appear to function solely as a sensor to ecdysteroid levels, and not as initial response factors. The nuclear receptors *HR3* and *HR4* appear to function as early response factors to increases in PonA levels and activation of the ecdysone receptor complex (**Paper I**). The rapid decrease in *HR3* and *HR4* expression towards the middle of the molt cycle prior to increases in *E74* and *FTZ-F1* expression, suggests that *HR3* and *HR4* and other early response genes might inhibit their own expression through negative feedback. In the fruit fly, HR3 has been shown to negatively regulate its own expression and that of HR4, which in turn negatively regulates the expression of HR3 (209, 233). Such potential negative feedback loops are yet to be investigated in the salmon louse.

Increases in *E74* and *FTZ*-F1 expression appear to be a secondary response to increases in PonA levels in the salmon louse (**Paper I**). Their expression increases after the expression levels of *HR3* and *HR4* have dropped, which is also accompanied by a decline in ecdysteroid levels. *HR3* and *HR4* are regulators of *FTZ-F1* expression in insects (209, 256, 264). Future work will need to investigate whether this regulatory connection is conserved in the salmon louse. However, it is probable that the early response factors are driving the expression of the secondary response factors in combination with the gradual removal of the repressing effect from PonA and the ecdysone receptor complex.

Knockdown of *βFTZ-F1* in nauplii and pre-adult I resulted in molting arrest at the time of ecdysis (**Paper II**). The transcriptome sequencing of RNAi knockdown nauplii revealed that genes associated with proteolysis and chitin binding were enriched among genes differentially expressed between the knockdown lice and control lice. A homolog of the ETHR was also downregulated in knockdown lice. This suggests that βFTZ-F1 might play a role in regulating processes associated with cuticle recycling, cuticle detachment, and the ecdysis sequence. Although levels of *E75A + E75B* were induced by exogenous PonA, their expression levels did not correlate with ecdysteroid titers in pre-adult I lice (**Paper I)**. *E75* expression increased at a time point in the molt cycle when natural ecdysteroid levels are low. Transcriptome sequencing and qPCR analysis of the *βFTZ-F1* knockdown lice revealed that the increase of *E75* levels at the end of

the molt cycle was affected by depletion of *βFTZ-F1*. The functions of *HR4*, *HR3*, *E74*, and *E75* and the genes they regulate remain unknown in the salmon louse.

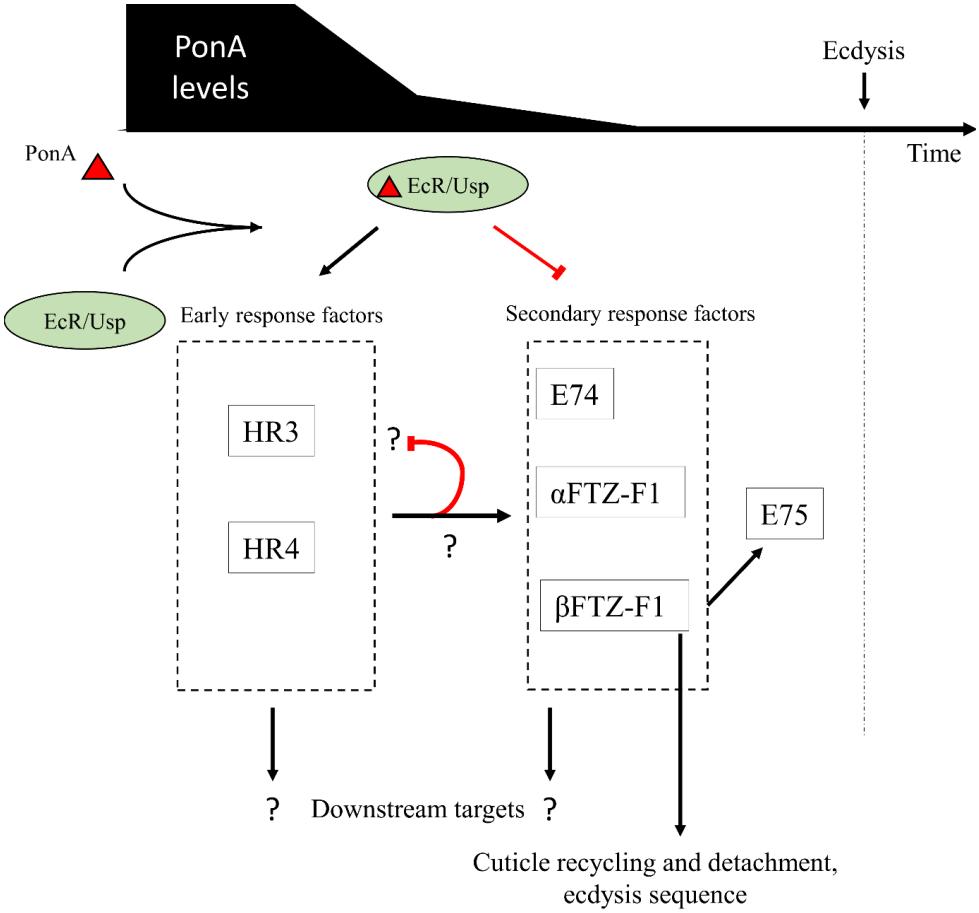


Figure 8. Model for the molecular regulation of molting in the salmon louse based on current data. See text for discussion.

4.7 Challenges with RNA interference targeting molting related genes

Functional characterization of the ecdysteroid regulated factors with RNAi proved challenging due to two main factors; 1) the large fluctuations in the expression profiles

of ecdysteroid regulated genes, and 2) individual differences in the developmental rate of lice (31). These two factors impacted the determination of knockdown efficacy and the phenotypic outcome of the RNAi.

When performing RNAi on nauplii it is often required to pool larvae from several eggstring pairs from different adult females to have enough material for both qPCR analysis and for assessing phenotype by following animal development. From experience, the more synchronized the nauplii from the different eggstrings were regarding time of hatching, the easier it was to accurately assess differences in mRNA levels between the knockdown and control group. The knockdown efficacy following RNAi of *FTZ-F1* in nauplii was between 40-50% for both isoforms (**Paper II**). The reduction in 40-50% was only reliably detected when nauplii were pooled from eggstrings initiating hatching with no more than 5 hours between each other. This meant a difference in hatching time of approximately 10 hours between the first and last egg. Larger differences in age between pooled nauplii introduced more variability in gene expression between samples, making it difficult to accurately assess the knockdown efficacy. The strategy of synchronizing the nauplii was also implemented for RNAi of *HR3* and *HR4*, but no knockdown was detected. It was suspected that the mRNA levels by RNAi could not fall below the lowest naturally occurring levels observed within the nauplius II stage for both *HR3* and *HR4* (**Paper I**). No abnormal phenotypes were observed following RNAi of either transcript after the nauplii had developed to planktonic copepodids. Due to the naturally occurring low levels of *HR3* and *HR4* expression from mid nauplius II to planktonic copepodids, it is suspected that any silencing effect would not manifest until the next naturally occurring rise in expression, which would not happen before lice are placed on host fish. Transcriptome sequencing data of parasitic copepodids show that *HR3* and *HR4* expression is not upregulated again until 3 days after host infection (265). A pilot experiment placing lice treated with *HR3* dsRNA on host fish resulted in the lice falling off at the time of chalimus molt. In order to assess the knockdown effect, copepodites would likely need to be sampled 3-5 days after infection but prior to the onset of a lethal phenotype, since knockdown cannot be detected in nauplii or planktonic copepodids due to the natural low levels of *HR3* expression in that developmental period.

In the established protocol for RNAi in pre-adult II females, the lice are left to develop for approximately 35 days after injection of dsRNA until they produce their 2nd pair of eggstrings. Unlike in nauplii, a knockdown effect of *αFTZ-F1* was not detected in RNAi adult females 35 days after injection. The amount of knockdown of some genes are readily detectable in most cases 35 days after injection (50, 220, 266-268), but in some cases the knockdown effect can only be measured at certain timepoints after injection (108). The knockdown efficacy of RNAi of the *EcR* gene was only detectable 12 days after injection, but not after 35 days when a phenotype similar to RNAi of *βFTZ-F1* had manifested (108). No knockdown was detected for *βFTZ-F1* in RNAi adult lice 35 days after infection (**Paper II**). It can be argued that knockdown efficacy should be measured prior to development of phenotypes as severe as those produced by *EcR* and *βFTZ-F1* RNAi, as knockdown lice and control lice are no longer comparable. However, this was not done for *FTZ-F1* RNAi knockdown lice in experiments where pre-adult II female lice were injected with dsRNA.

RNAi can be performed on pre-adult I lice as well. As previously described, this can be done to ensure that genes with potential functions in molting can be followed through a full molt cycle under the effect of RNAi. With this strategy, *βFTZ-F1* RNAi was shown to prevent molting from pre-adult II to adult, similar to knockdown in nauplius I where molting to the copepodid stage was blocked. The same strategy was also employed in a study on chitin synthases in the salmon louse, which similarly demonstrated that RNAi in pre-adult I did not affect molting until the pre-adult II to adult molt (269). Determining the knockdown efficacy of *βFTZ-F1* proved more challenging in pre-adult II compared to nauplii. The individual differences of the development rate of lice led to lice of different instar age at a given sampling time point. This entails that the injection of dsRNA was done in pre-adult I individuals which were at a different point in the molt cycle, spread from early in the molt cycle (before *βFTZ-F1* peak expression) to later in the molt cycle (during or after *βFTZ-F1* peak expression). This also means that the sampled lice in the subsequent pre-adult II stage were at different points in their molting cycle and expressed naturally highly variable levels of *βFTZ-F1* mRNA (**Paper II**). Also control lice can have low levels of

expression depending on their progress in the molt cycle. This can explain the high variability of *βFTZ-F1* expression in control group. In both nauplii II and pre-adult II lice, the level of *βFTZ-F1* expression in both RNAi and control lice were higher than that of naturally occurring levels observed at the start of the respective stage. As expected, this indicates that the *βFTZ-F1* expression levels was following the natural profile through the molt cycle in both RNAi and control lice, but at a slightly reduced level. This means that determining RNAi knockdown efficacy of molting related genes with highly variable expression profiles, without accounting for instar age, can result in both false positives and negatives. Pre-adult lice can be sorted by age using the CT/TL method, but this method is not perfect as lice with similar ratios can display large differences in expression (**Paper I**). Sampling of lice after dsRNA injection is done at a given time point and lice can be uneven in their development. In the current study, the CT/TL ratio of the sampled *βFTZ-F1* pre-adult II knockdown lice for knockdown assessment were spread across the middle part of the stage when expression levels of *βFTZ-F1* were increasing. A significant knockdown effect was detected in one of two experiments, with a more variable but on average higher expression of *βFTZ-F1* in the control lice compared to knockdown lice (**Paper II**). However, this was assessed with the knowledge that *βFTZ-F1* dsRNA was demonstrated to reduce *βFTZ-F1* levels in nauplii and caused molting arrest in both nauplii and pre-adult II. A larger number of pre-adult lice sorted by instar age than used in this study would likely be required to get a more reliable estimate of knockdown with dsRNA probes with no previously documented knockdown effect or phenotype (**Paper I**). A recommendation would be to always elucidate the expression profiles of molting related genes prior to the design of RNAi experiments. In addition, it would be recommended to assess knockdown efficacy of dsRNA probes in nauplii or copepodites before committing to RNAi experiments on pre-adult, as it is easier to synchronize nauplii by age compared to pre-adults.

5. Concluding remarks and future directions

In the present work, knowledge of the molecular mechanism regulating molting in the ectoparasitic salmon louse was expanded. The ecdysteroid Ponasterone A was identified as the functional molting hormone. It was demonstrated that Ponasterone A regulates the expression of the conserved ecdysteroid regulated transcription factors HR3, HR4, FTZ-F1, E74, and E75. In response to Ponasterone A, these transcription factors display distinct repeatable expression profiles in salmon louse molt cycles. Through functional studies using RNAi it was shown that the nuclear receptor βFTZ-F1 is a regulator of molting in the salmon louse. The findings of the current study indicate that molting in the salmon louse is regulated by a Ponasterone A initiated transcriptional cascade similar to the ecdysone signaling hierarchy in the well-studied fruit fly molting model. The regulatory functions of HR3, HR4, E74 and E75 remain unknown. Increased knowledge of the endocrine systems controlling molting in the salmon louse can provide novel targets for the development of chemotherapeutants to combat sea lice infestations.

Future studies should continue to elucidate the functions of the ecdysone receptor complex and other members of the ecdysone signalling hierarchy through RNAi in combination with transcriptome sequencing. This can identify the regulatory connections between the members of the ecdysone signalling hierarchy and identify the gene networks that control molting in the salmon louse. There is currently a lack of knowledge of how ecdysteroid biosynthesis is organized in the salmon louse. So far, only three genes of the ecdysone biosynthetic pathway have been studied in the salmon louse, but this was performed in stages not actively undergoing molts (134). We do not know where or when these genes are expressed in stages actively undergoing molts. Future work should aim to study the tissue specific expression of ecdysone biosynthetic enzymes and ecdysteroid regulated factors across the salmon louse molt cycle. This can be done by performing *in situ* hybridization on pre-adult lice sorted by intra-instar age. From experience, the variable mRNA levels of the ecdysteroid regulated factors can make them difficult to detect with *in situ* hybridization. Immunohistochemistry, which is already an established method in salmon louse, is much more sensitive. Future

work should aim to acquire specific antibodies targeting the different ecdysteroid regulated factors. Fruit fly antibodies targeting conserved areas of the nuclear receptors might work against the salmon louse orthologues. Antibodies synthesized on demand is also an option, but expensive. With antibodies, changes in protein levels following RNAi knockdown can also be estimated alongside mRNA as an alternative tool to determine knockdown efficacy. Immunoprecipitation (IP) and chromatin immunoprecipitation sequencing (ChIP-Seq) methods could also be developed for the salmon louse. ChIP-Seq of untreated lice in combination with transcriptome sequencing of RNAi knockdown lice, can help identify genes possibly directly regulated by the different members of the ecdysone transcriptional hierarchy.

From a pest management perspective, further studies can be done on the ligand-binding properties of the salmon louse ecdysone receptor. Current pesticides such as teflubenzuron and diflubenzuron target crustaceans indiscriminately. *In vitro* binding assays on crustaceans have shown that the ecdysone receptor of different species display different affinities to 20E and PonA (126, 132). Insecticides mimicking the insect molting hormone 20E have also been shown to display different specificities to insect species within the same order (105). Natural and synthetic ligands can be screened against relevant crustacean ecdysone receptors using a two-hybrid reporter assay to find compounds with selective affinity for the salmon louse ecdysone receptor. A computational approach could also be pursued. Using nuclear magnetic resonance (NMR), the structure of the salmon louse EcR ligand-binding domain can be determined. Chemical libraries could then be virtually screened against the EcR ligand-binding pocket through molecular docking studies to identify potential candidates for further studies.

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II

PLOS ONE

RESEARCH ARTICLE

The *FTZ-F1* gene encodes two functionally distinct nuclear receptor isoforms in the ectoparasitic copepod salmon louse (*Lepeophtheirus salmonis*)

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Abstract

The salmon louse, *Lepeophtheirus salmonis*, is an ectoparasitic crustacean that annually inflicts substantial losses to the aquaculture industry in the northern hemisphere and poses a threat to the wild populations of salmonids. The salmon louse life cycle consists of eight developmental stages each separated by a molt. *Fushi Tarazu Factor-1 (FTZ-F1)* is an ecdysteroid-regulated gene that encodes a member of the NR5A family of nuclear receptors that is shown to play a crucial regulatory role in molting in insects and nematodes. Characterization of an *FTZ-F1* orthologue in the salmon louse gave two isoforms named *αFTZ-F1* and *βFTZ-F1*, which are identical except for the presence of a unique N-terminal domain (A/B domain). A comparison suggest conservation of the *FTZ-F1* gene structure among ecdysozoans, with the exception of nematodes, to produce isoforms with unique N-terminal domains through alternative transcription start and splicing. The two isoforms of the salmon louse *FTZ-F1* were expressed in different amounts in the same tissues and showed a distinct cyclical expression pattern through the molting cycle with *βFTZ-F1* being the highest expressed isoform. While RNA interference knockdown of *βFTZ-F1* in nauplius larvae and in pre-adult males lead to molting arrest, knockdown of *βFTZ-F1* in pre-adult II female lice caused disruption of oocyte maturation at the vitellogenic stage. No apparent phenotype could be observed in *αFTZ-F1* knockdown larvae, or in their development to adults, and no genes were found to be differentially expressed in the nauplii larvae following *αFTZ-F1* knockdown. *βFTZ-F1* knockdown in nauplii larvae caused both down and upregulation of genes associated with proteolysis and chitin binding and affected a large number of genes which are in normal salmon louse development expressed in a cyclical pattern. This is the first description of *FTZ-F1* gene function in copepod crustaceans and provides a foundation to expand the understanding of the molecular mechanisms of molting in the salmon louse and other copepods.

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Introduction

The salmon louse, *Lepeophtheirus salmonis*, is an ectoparasitic copepod that lives of salmonids by feeding on mucus, skin, and blood. The salmon louse life cycle consists of eight developmental stages each separated by a molt: two planktonic nauplius stages followed by an infective copepodid stage, two immobile chalimus stages, two mobile pre-adult stages and finally the mobile reproductive adult stage [1–3]. The parasite is a threat to the welfare of both wild and farmed salmonids [4], and commercially important as it is responsible for significant financial losses in the salmon farming industry [5]. Better knowledge of the ecdysone pathway in salmon lice will help in understanding the initiation and regulation of the complex process of molting. To combat the parasite with new drugs, knowledge of endocrine regulatory mechanisms is indispensable with molt and reproduction as the main target processes. Molting is necessary for growth and development of the parasite and may represent a step where it is specifically vulnerable towards attack.

Ecdysteroid hormones are used to regulate various aspects of arthropod development, including molting and reproduction [6]. The hormone signals are typically mediated by a nuclear receptor dimer, the most prominent example is the ecdysteroid receptor which consists of a dimer of the ecdysone receptor (EcR) and Ultraspiracle (Usp) [7–9]. In the well-studied *Drosophila melanogaster* molting model, the liganded EcR/Usp dimer controls a transcriptional cascade of ecdysteroid regulated genes, consisting mainly of other nuclear receptors that subsequently regulate metamorphosis and timing of molting [10]. The specific temporal and spatial expression of ecdysteroid inducible nuclear receptors such as ecdysone inducible factor 75 (E75), hormone receptor 3 (HR3), hormone receptor 4 (HR4) and Fushi-Tarazu Factor 1 (FTZ-F1) are crucial for the correct transition through a molt cycle [11].

The ecdysone receptor has been identified in crustaceans belonging to the decapod and branchiopod order, reviewed in Nakagawa and Henrich [12]. However, the functional role of the ecdysone receptor and other putative members of the ecdysone regulatory cascade are not as explored in crustaceans compared to insects, with only a few recorded knockdown studies conducted on crustaceans [13–15]. In the salmon louse, separate knockdown of EcR [16, 17] and Usp [18] affected growth and reproduction in larvae and pre-adult females, while dual knockdown of EcR/Usp resulted in molting arrest at the second nauplius stage [17].

FTZ-F1, a member of the NR5A class of the nuclear receptor superfamily, is an ecdysteroid regulated nuclear receptor. In *D. melanogaster*, alternative transcription starts and splicing in the *FTZ-F1* gene generates two protein isoforms named αFTZ-F1 and βFTZ-F1. They contain identical DNA and ligand-binding domains, but unique N-terminal A/B domains [19]. In other investigated insect species, only one FTZ-F1 isoform has been reported [20–25]. However, a similar mechanism of alternative transcription, as seen in *Drosophila FTZ-F1*, has been reported in the beetle *Leptinotarsa decemlineata* [26] and the branchiopod crustacean *Daphnia magna* [14]. In *Drosophila*, *αFTZ-F1* transcript is maternally deposited and the protein is required for pair-rule segmentation in early embryogenesis by interacting with the homeobox domain protein FTZ and activating the transcription of *Engrailed* [27]. βFTZ-F1 is required for all stage transitions, and its expression is induced mid-to-late embryogenesis, prior to all larval-to-larval and larval-to-pupa ecdysis, and eclosion from pupa to adult [28]. *βFTZ-F1* mutants display cuticular abnormalities and fail to shed cuticle during molting throughout *Drosophila* development [29, 30].

The importance of the *FTZ-F1* gene in molting has been shown in different ecdysozoans. In holometabolous [11], hemimetabolous insects (*Blattella germanica*) [23], and the nematode *Caenorhabditis elegans* [31, 32], depletion of FTZ-F1 led to abnormal cuticle development and molting arrest. There are only few reports on FTZ-F1 orthologues in crustaceans. In the crab,

Eriocheir sinensis, FTZ-F1 is involved in the regulation of vitellogenin expression [33], and in *Daphnia magna*, knockdown of *FTZ-F1* transcripts in embryos resulted in hatching failure.

The aim of the present study was (I) to identify *FTZ-F1* orthologues in the salmon louse, (II) to elucidate its molecular structure, and (III) to functionally characterize its isoforms through gene expression and knockdown studies.

Materials and methods

Animal culture

A laboratory strain (LsGulen) of salmon louse was propagated on Atlantic salmon (*Salmo salar*) as described in Hamre et al. [34]. The salmon were fed a commercial diet and kept in sea water with a salinity of 34.5 ppt and temperature of 10˚C. Adult gravid females were collected with forceps from salmon anesthetized with a mixture of metomidate (5 mg/l) and benzocaine (60 mg/l). Eggstrings were removed with forceps and laid for hatching in flow-through hatching wells as described in Hamre *et al.* [34]. Hatched larvae were kept in the same hatching wells. Fish infection was done as described in Hamre *et al.* [34]. All experiments were performed according to Norwegian animal welfare regulations with the approval of the governmental Norwegian Animal Research Authority (ID7704, no 2010/245410).

Sampling of sea lice and dissection of tissue

For qPCR measurements of embryonal development, egg string pairs from individual lice were divided, one egg string was put on RNA later, while the other eggstring was incubated further to observe the hatching time point. For time series of nauplii I and nauplii II larvae development, the time point for hatching was registered.

Individual differences between lice in developmental pace have been explored in Eichner *et al.* [35]. Lice are not of exact comparable instar age, even when sampled at same time point after infection. Therefore, preadult lice were photographed at sampling and sorted based on the ratio between the length of the cephalothorax (CT) and the total length (TL) of the animal. Since the growth pattern of the cephalothorax and abdominal segment are different, with more growth of the abdominal segment compared to the cephalothorax within the same stage, this ratio can be used as a proxy for age [35], with the range going from younger (higher ratio) to older (lower ratio).

Tissues were extracted from adult lice. Dissection of brain, testis, spermatophore, oocytes and ovaries were performed by removing the ventral exoskeleton with a scalpel and removing organs with as little surrounding tissue as possible. Subcuticular tissue was obtained by cutting the outer sides of the animals containing just subcuticular tissue and cuticular glands. Intestine was obtained by pulling it out of the animal. All samples with the exclusion of sexual organs contained a mixture of female and male tissue.

RNA extraction and cDNA synthesis

Salmon louse larvae. Larvae (15–25 larvae per sample) were homogenized (30s x 4 at 6.0 m/s) with a FastPrep™ machine in 300 μl TRI Reagent® (Sigma) using 1.4 mm zirconium oxide beads (Bertin). RNA was extracted using Direct-Zol RNA micro kit (Zymo Research) with on-column DNase digestion according to manufacturer's protocol.

Pre-adult and adult salmon lice. Individual pre-adult and adult salmon lice were homogenized in 1 ml TRI Reagent® (Sigma) using a 5 mm steel bead (Qiagen). RNA was extracted according to suppliers' protocol with the following change: for phase separation 200 μl 24:1 chloroform/isoamylalcohol was added. The RNA was dissolved in 20–40 μl of RNase free

water, treated with DNase I of Amplification Grade (Invitrogen) as described by the manufacturer. RNA was either used directly in cDNA synthesis or stored at -80˚C until use.

Reverse transcription

AffinityScript qPCR cDNA synthesis kit (Agilent Technologies) was used according to suppliers' protocol with 300 ng total RNA from larvae or 200 ng of total RNA from pre-adult salmon lice in a 10 μl reaction. The cDNA was diluted ten-fold in nuclease free water and stored at -20˚C until use.

Molecular cloning and sequencing

Full-length sequence of *FTZ-F1* was obtained with SMARTer™ RACE cDNA Amplification Kit (TaKaRa Bio). Reverse transcription of DNase treated total RNA from larvae or adult female was done using SMARTscribe according to the manufacturer's protocol. 5' and 3' RACE on larval and adult female RACE-ready cDNA was done with universal and gene specific primers (S1 Table) in a first and nested PCR reaction using conditions specified by the manufacturer. PCR products were purified from agarose gels using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), and cloned in a pCR®4-TOPO TA® vector (Invitrogen) in TOP10 *Escherichia coli* cells. Colony PCR was performed using MOD M13-primers with the following reaction conditions; 5 min denaturation at 95˚C, 30 cycles of 30 seconds denaturation at 95˚C, 30 seconds annealing at 55˚C, and elongation at 72˚C for 1 minute. Clones were grown o/n in 5 ml LB medium containing 100 μg/ml ampicillin and purified using NucleoSpin® Plasmid Purification kit (Macherey-Nagel). Clones were sequenced using a BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems). Sequences were analyzed and assembled using Gap4 from the Staden Package [36].

Gene structure comparison

Orthologous sequences were found using the "orthologues" function in Ensembl Metazoa (Metazoa.ensembl.org), and through default pBLAST search against the ecdysozoa superphylum (hexapoda, chelicerata, crustacea, myriapoda, tardigrada, nematoda, priapulida) with FTZ-F1 as a query sequence (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For predicted *αFTZ-F1* and *βFTZ-F1* sequences, Splign [37] was used for comparison to the genomic sequence (taken from NCBI genome or Ensembl Metazoa) and predict gene structure. All sequences found were verified by blasting the sequence against the Sequence Read Archive (SRA) at NCBI at default settings. The accession numbers for *FTZ-F1* for all species investigated are listed in Table 1.

Gene expression measurements

Expression of *FTZ-F1* was quantified by RT-qPCR carried out on a LightCycler 480® using LightCycler 480® SYBR Green 1 Master kit (Roche Diagnostics) with the following reaction settings: Pre-incubation at 95˚C for 10 min, 42 cycles of amplification (95˚C, 10s, ramp rate 4.4˚C/s; 55˚C, 10s, ramp rate 2.2˚C/s; 72˚C, 20s, 4.4˚C/s). Each sample was measured in triplicates in 20 μl reactions according to manufacturer's protocol using 2 μl cDNA and a primer concentration of 0.5 μM. Melting point analysis was performed after the final amplification cycle. Primer sequences are shown in S1 Table, and the position of the FTZ-F1 primers are highlighted in Fig 1. The primer efficiencies were determined by creating a standard curve using 1:10 serial dilutions of the PCR product as template. The housekeeping gene *Elongation Factor 1α* was used as the internal reference [38].

Table 1. Sequences used in gene structure comparison.

	Accession no.		Comments
Species	***αFTZ-F1***	***βFTZ-F1***	
Insects			
D. melanogaster	NM_079419	NM_168775	
A. mellifera	XP_016766345.1 + XM_006557392.2	XM_006557392.2	
B. mori	AF426830.1	AB649122.1	
S. litura	HQ260326.1	XM_022976553.1	
M. sexta	XM_030168105.1	AF288089.1	
B. germanica	FM163377.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
C. felis	XM_026619028.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
A. pisum	XP_029344120.1		*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
F. occidentalis	XM_026421392.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
T. castaneum	XM_008193153.2	XM_008193151.2	
Myriapoda			
S. maritima			*αFTZ-F1*: Constructed from two Ensembl genes (SMAR006163 + SMAR006161), *αFTZ-F1* start missing *βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
Chelicerata			
T. urticae	XM_015929659.2	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
P. tepidarorum	XM_016053025.2	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
C. sculpturatus	XM_023378248.1	XM_023378247.1	
V. destructor	XM_022789294.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
Crustacea			
H. azteca	XM_018152684.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
L. salmonis	MT150277	MT150276	
D. magna	LC105700.1	LC105701.1	Ishak *et al.* [14]
Tardigrada			
H. dujardini	OQV18443.1	-	*βFTZ-F1*: Start of ORF from genomic sequence upstream of DBD encoding exon in *αFTZ-F1*
Nematoda	***NHR-25***		
C. elegans	NM_001029379/ WBGene00003623		Gissendanner and Sluder [31]
S. carpocapsae	TKR80278.1		
T. trichiura	CDW52832		

https://doi.org/10.1371/journal.pone.0251575.t001

Measurements of expression following RNA interference experiments were analyzed using the $2^{\wedge\text{-}\Delta\Delta Ct}$ method [39]. T-test was used to determine significant difference between experimental groups with a p-value of <0.05 as threshold. Expression profiles in both egg, larvae and pre-adults, expression levels are presented as $E^{-\Delta Ct}$.

RNA sequencing data from a former study were re-analyzed based on the sequences obtained in this study [40]. The time series data contain samples of chalimus I and II as well as pre-adult I, divided into different instar ages called young (directly after molt), middle, and old (directly before molt) using the ratio between the cephalothorax and total length as a proxy for age.

RNA interference

Synthesis of double stranded RNA probes. PCR fragments for both *FTZ-F1* isoforms, and a control fragment from the trypsin gene in Atlantic cod (XM_030370867.1) [41],

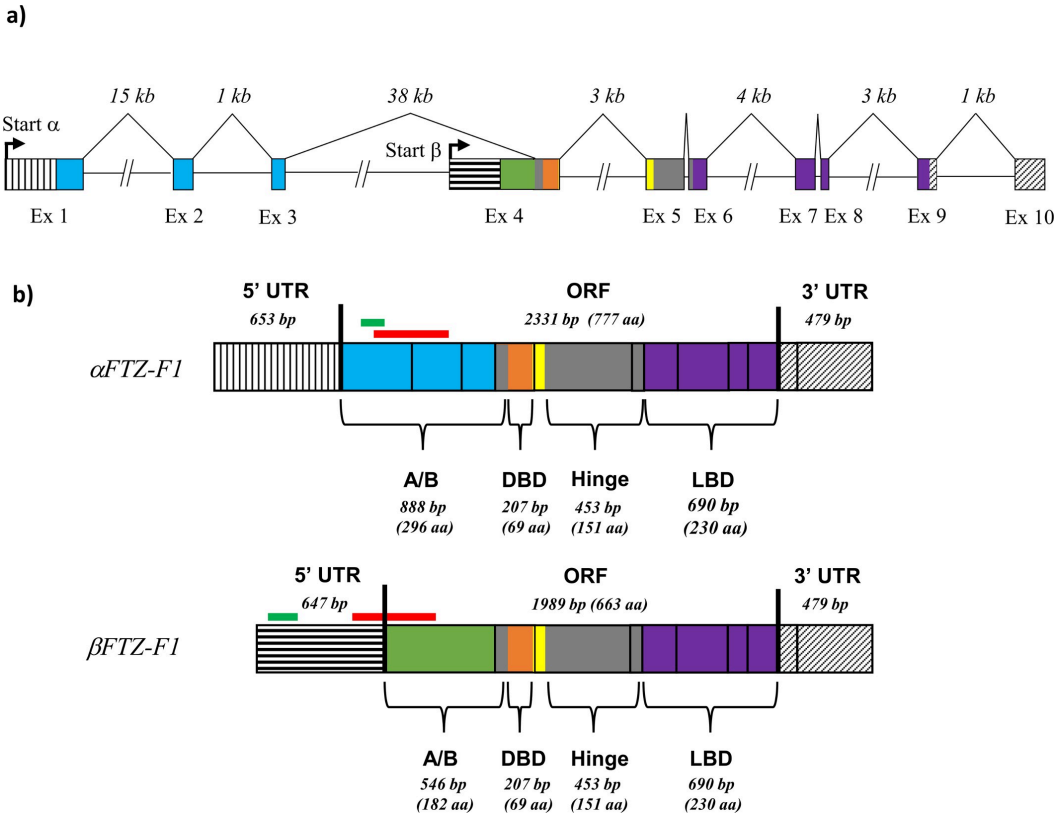

Fig 1. Genomic structure of the *FTZ-F1* gene, and the transcripts produced through alternative transcription. a) Schematic representation of the *FTZ-F1* gene in the salmon louse. Two transcript variants of *FTZ-F1* were found. The start of transcription for the α-isoform and β-isoform are highlighted with black arrows, with the 5' UTR for the two variants highlighted with vertical and horizontal lines, respectively. The shared 3' UTR is highlighted with diagonal lines. Exons and short introns up to 1 kb are drawn in scale. **b)** Schematic representations of the mRNA of *αFTZ-F1* and *βFTZ-F1*. Black lines represent exon borders, while the open reading frame (ORF) of the transcripts are marked between two thick extended lines. The DBD, LBD and N-terminal A/B domain are highlighted, as are the unique N-terminal domains of the *α* and β-isoform. The position of the dsRNA probes for RNA interference is highlighted by a red bar above the transcripts, and the qPCR amplicons are highlighted by green bars.

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were generated using 5x FirePol® 7.5 mM $MgCl_2$ Ready to Load PCR Master Mix (Solis Biodyne) with primers flanked by promotor sites for T7 RNA polymerase (S1 Table). The length of the resulting products were 405 and 468 bp for *αFTZ-F1* and *βFTZ-F1*, respectively (Fig 1) and 849 bp for the control fragment. Position of the fragments were in the unique 5' regions, covering residue number 109–244 for αFTZ-F1 and the last 180 bp for the 5' UTR and residues 1–93 for βFTZ-F1. The following amplification program was run using plasmid DNA as template in a 50 μl reaction: initial denaturation at 94˚C for 5 min, 30 cycles (94˚C for 30 s; 60˚C for 30 s; 72˚C for 1 min) and a final elongation step of 72˚C for 7 min. The PCR products were run on an ethidium bromide stained 1% agarose gel, and the PCR product was purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) using DEPC treated water to elute the PCR template. The dsRNA was

generated using the MegaScript RNAi kit (Ambion) according to manufacturer's protocol with 1 μg of DNA template.

RNAi of *βFTZ-F1* and *αFTZ-F1* mRNA in salmon louse larvae. RNA interference in larvae was performed as described in Eichner *et al*. [42]. Approximately 300 nauplius I larvae were treated in 150 μl sea water containing 1500 ng dsRNA per fragment, including the control group, which was treated with the control fragment CPY185 [41]. After overnight incubation with dsRNA, larvae were transferred to flow-through incubators and incubated at 8.8 ± 0.1˚C for 48 hours before half of the larvae were sampled, placed in RNAlater™ (Ambion) and stored at -20. The remaining larvae were kept in flow through wells to observe further development until the lice from the control group had molted to the copepodid stage. Larvae were followed in the microscope (Olympus SZX 0.5 and 1.6x Olympus objective) and photographed (Canon EOS 600D camera). The larvae were collected and stored in Karnovsky's fixative for histology. Copepodids from the control and *αFTZ-F1* group were used to infect 3 fish each and kept in single fish tanks. Approximately 50 copepodids were used per fish. Larvae were left to develop to reproductive adults until the females had produced their second eggstring. The lice were removed with forceps from anesthetized salmon. The untreated females were screened for attached spermatophores and eggstrings were placed into hatching wells to investigate hatching success.

RNAi of βFTZ-*F1* in pre-adult 1 males. dsRNA was diluted to 600 ng/μl and 1% saturated bromophenol blue was added. Pre-adult 1 males were removed with forceps from anesthetized salmon and placed in a petri dish lined with wet Whatman-paper. dsRNA was injected in the animals as described in Dalvin *et al*. [41], with a borosilicate glass needle pulled using the P-2000 laser-based micropipette puller system (Sutter Instrument). The dsRNA solution was added to the needle using a microloader tip, and then coupled to a HI-7 injection holder (Narishige). After the injection, the lice were kept in sea water for 2–3 hours at 10˚C to recover. The injected males were placed upside-down on wet paper together with untreated females in a 2:1 ratio, the paper was subsequently placed on the anaesthetized salmon to attach animals to the host. Untreated females were co-infected with the knockdown male lice, as a lack of females might encourage male lice to abandon the host. 60 males in the pre-adult I stage, were injected with *βFTZ-F1* dsRNA and placed out on 3 fish kept in individual tanks with seawater. 120 males were injected with control fragment and distributed on 6 fish kept in individual tanks. The lice from 1 fish from the *βFTZ-F1* knockdown group and 3 fish from the control group were collected 6 days after injection to assess gene expression and phenotype in the pre-adult II stage. The remaining lice were collected 35 days post infection (2 fish with βFTZ-F1 knockdown lice and 3 fish with control lice) when lice from control group had progressed to the adult stage. The experiment was repeated, but each group contained 60 injected pre-adult I males divided on 5 fish kept in a common tank. 6 days post infection, lice in the pre-adult II stage were collected from 3 fish from each tank to assess downregulation. Male lice were imaged as described in section 2.8.2 and fixed in RNAlater™ (Ambion) for qPCR analysis or in Karnovsky's fixative for histology. Females were also imaged and investigated for attached spermatophores.

RNAi of *αFTZ-F1* and *βFTZ-F1* in pre-adult 2 females. The experiment was performed as described for pre-adult I males in the above section. 30 dsRNA injected pre-adult II females were divided to 3 fish with untreated males in a 2:1 ratio. The fish were kept in individual tanks. The animals were collected from the fish 35 days post injection. The adult female lice were imaged as previously described and placed in RNAlater™ (Ambion) for qPCR analysis, or Karnovsky's fixative for histology.

Histology

Salmon lice fixed in Karnovsky's fixative at 4˚C were washed in phosphate buffered saline (PBS) before being dehydrated in a graded ethanol series. The animals were subsequently pre-infiltrated for two hours in 50/50 Technovit/ethanol (Technovit 7100, Heraeus Kulzer Technique), and then infiltrated with Technovit and hardener overnight prior to embedding. Animals were cut in two-micrometer sections using a Leica RM 2165 microtome and stained with toluidine blue (1% in 2% borax). Sections were mounted using DPX Mountant for histology (Sigma). Microscopy and imaging were done using an Axio Scope A1 light microscope connected to an Axiocam 105 color camera (Zeiss International).

RNA sequencing

Nauplii I larvae originating from the same pair of eggstrings were divided into three groups and treated with 2500 ng dsRNA of either *αFTZ-F1*, *βFTZ-F1* or *CPY185* (control). About 50 larvae from each group were transferred to RNAlater™ (Ambion) 48 hours after molting from nauplius I to nauplius II, and the remaining larvae were left to molt again and develop to copepodids. This experiment was repeated three times, producing three biological parallels per dsRNA treatment. Total RNA extracted from dsRNA treated larvae were sequenced at the Genomics Core Facility, University of Bergen. Sequencing libraries were prepared from 400 ng total RNA using Illumina® TruSeq® mRNA Stranded Sample Preparation kit. Samples were barcoded, pooled together and sequenced in a single lane using the Illumina HiSeq4000 (Illumina, Inc., San Diego, CA, USA), producing 2x75 base pair (bp) paired end reads. Image analysis and base calling were performed using Illumina's RTA software version 2.4.11, and the data was converted to fastQ format using bcl2fastq version 2.1.7.1.14.

Data processing of RNA sequencing

The sequences were quality controlled using FastQC v.0.11.9 and summarized using MultiQC v.1.7. The reference genome utilized was a combination of the Ensembl Metazoa reference assembly of the nuclear genome (LSalAtl2s, *Lepeophtheirus salmonis*) and the mitochondrial genome RefSeq sequence NC_007215 [43]. The gene models from Ensembl Metazoa were further enhanced with the inclusion of gene models of *FTZ-F1* isoforms derived from sequences obtained through rapid amplification of cDNA ends (RACE). RNA-seq reads were aligned using RNA STAR with default settings in Galaxy under the Norwegian e-infrastructure for Life sciences (NeLs) platform [44, 45]. Differential expression analysis was done with DESeq2 [46] on counts produced from the FeatureCounts tool. All genes with a p-adjusted value of < 0.05 were included in the list of differentially expressed genes. GO annotation enrichment analysis was performed using the GOEnrichment tool in the public European Galaxy server [44, 47]. In order to get accurate counts for the two *FTZ-F1* isoforms, we performed a Kallisto quantification using the database of all salmon louse cDNA from Ensembl Metazoa edited to contain the correct full-length transcript of the *FTZ-F1* isoforms [48]. A DESeq2 analysis was performed on the Kallisto quantification in the same manner as described above. The hierarchical clustering was made with data from the time series study from Eichner *et al.* [40] using J-Express [49].

Results

FTZ-F1 alternative transcription start sites and splicing are conserved among ecdysozoans

pBLAST search in LiceBase.org against all salmon louse protein entries gave a hit with the salmon louse gene EMLSAG00000008902. The 5' and 3' Rapid Amplification of cDNA Ends

(RACE) on RNA from nauplius II and adult female produced two cDNAs, later identified as *αFTZ-F1* (accession number: MT150277) and *βFTZ-F1* (accession number: MT150276) extended over 3460 and 3112 nucleotides (nt), respectively. The two *FTZ-F1* transcripts share sequence with the exception of a unique 5' end of 1431 (*α*) and 1022 (*β*) nt. The two predicted ORFs of 777 and 663 aa, includes unique N-terminal ends of 259 and 145 aa, and share DNA-binding domain (DBD) and ligand-binding domain (LBD). The predicted genome organization of the two *FTZ-F1* transcripts are shown in Fig 1. The two transcripts are generated through an alternative transcription start and splicing. The isoforms were named *αFTZ-F1* and *βFTZ-F1* due to the similarity in gene structure to the *Drosophila melanogaster* orthologues. The 3460-nucleotide long *αFTZ-F1* stretches over exon 1–9, while the transcription of the 3112 base pair long *βFTZ-F1* starts in an alternative start exon that extends into exon 4 of *αFTZ-F1*.

FTZ-F1 in *Drosophila melanogaster* and *Lepeophtheirus salmonis* have similar structural organization, as do *Daphnia magna* [14], where *αFTZ-F1* and *βFTZ-F1* are generated through alternative transcription start and splicing (S1 Fig). In all three species, *βFTZ-F1* transcription originates in an intron of *αFTZ-F1* upstream of the DBD encoding exon, generating a transcript with an alternative 5' end. Available gene sequences of *FTZ-F1* in organisms from the different subphyla of the ecdysozoan superphylum (Table 1) were investigated to explore potential conservation of the FTZ-F1 gene structure. A selection of 8 hexapod, 2 crustacea, 4 chelicerate, 1 myriapod, 1 tardigrade and 1 priapulid species were investigated, and showed a potential upstream ORF in the extension of the DBD coding exon that may include an alternative transcription start to generate a putative *βFTZ-F1*. It appears to be a general feature that the arthropod *FTZ-F1* gene encodes two transcripts that generates isoforms with unique N-terminal parts. The size of the predicted N-terminal within the α and β isoforms varied substantially between species, as well as the length of the unique part of the α and β N-terminal parts within the same species. A comparison of the predicted gene structures based on cDNA sequences from representative species selected from different subphyla is shown in Fig 2. The *FTZ-F1* gene of three nematode species investigated showed a structure that differed from the other ecdysozoan species, with the conserved DBD being split into three or two exons. The two isoforms reported in C. elegans are generated through an alternative splicing that results in a new initiation of translation that produces an isoform lacking a DBD [31, 32]. All transcript structures are depicted in S1 File along with the corresponding sequences, in addition to reads discovered in the NCBI sequence read archive (SRA).

Expression of *αFTZ-F1* and *βFTZ-F1* mRNA

RT-qPCR quantification of both *FTZ-F1* isoforms revealed that *αFTZ-F1* and *βFTZ-F1* have a similar expression pattern during early larval development until hatching, and from hatching to second molting, but are present at significantly different levels (Fig 3). In eggs shortly after fertilization, both transcripts are expressed at their lowest levels, with *αFTZ-F1* levels close to the detection limit, roughly 40 times lower compared to *βFTZ-F1*. 7 days before hatching, the expression of both transcripts increase, followed by another marked increase 4 days before hatching. Expression of *βFTZ-F1* decreases steadily through the nauplius I stage and increases again at 11 hours old nauplius II with the highest rise between 11 and 23 hours. *βFTZ-F1* levels are reduced by 40% from the nauplius II peak to the last measurement prior to molt. The *αFTZ-F1* mRNA expression also decreases at the beginning of the nauplius I stage, but appears to start increasing late in nauplius I and continues to rise at a steady rate throughout the nauplius II stage with a 95-fold increase from the lowest expression level in nauplius I to the last measurements in nauplius II prior to molt.

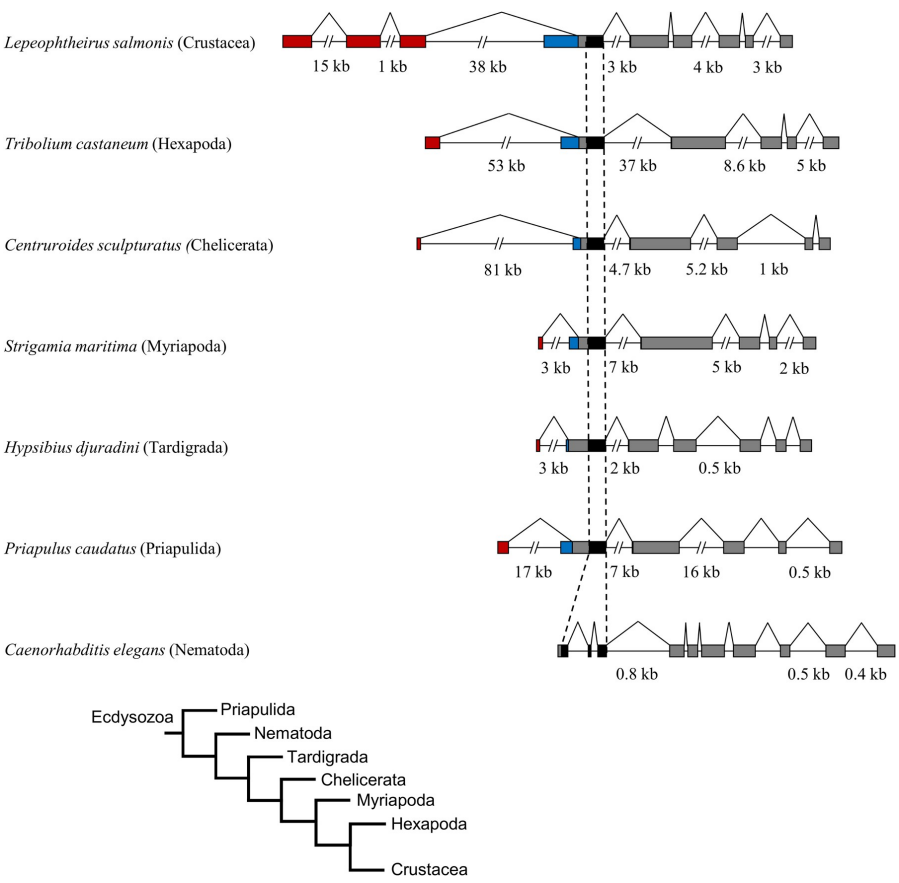

Fig 2. Comparison of *FTZ-F1* gene structure between ecdysozoans. The open reading frame of *FTZ-F1* genes from different ecdysozoans organisms are aligned through the area of the gene coding for the DNA-binding domain (black), with the stippled lines highlighting this alignment. The area coding for the isoform specific parts of the N-terminal domain is highlighted by red (*αFTZ-F1*) and blue (*βFTZ-F1*). Exons are represented by boxes, and introns and splicing patterns are shown with lines. Exons and introns of 1kb or lower are in scale, with sizes of selected introns given in kilobases (kb). The evolutionary relationship between the different ecdysozoans are shown in the schematic tree chart in the bottom [50, 51]. For accession IDs see Table 1. All sequences and structure are available in S1 File.

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The overall expression patterns of *αFTZ-F1* and *βFTZ-F1* during the pre-adult I stage for both sexes are similar to the patterns observed during the nauplius II stage and egg development (Fig 4A). However, unlike during egg development and the nauplius stages, the two isoforms are expressed at similar levels at the start of the pre-adult I stage for both sexes. *βFTZ-F1* expression then increases 13-fold for males and 22-fold for females, while the increase of *αFTZ-F1* is slightly delayed and lower compared to *βFTZ-F1* in both sexes. The expression of *αFTZ-F1* remains at a constant level throughout the pre-adult I stage, while peak *βFTZ-F1* expression is followed by a decline of 80–90% in both sexes at the end of the instar. A similar shift in the expression levels of *αFTZ-F1* and *βFTZ-F1*, with

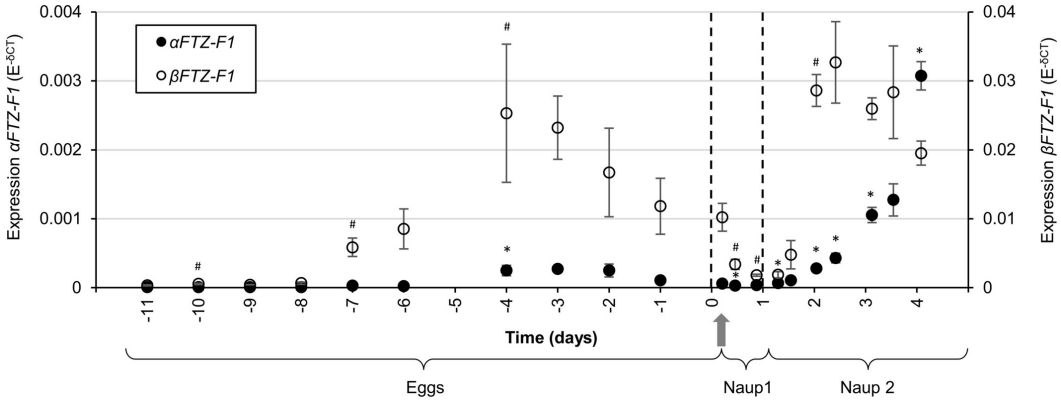

Fig 3. Expression of *αFTZ-F1* and *βFTZ* from early fertilized egg developmental to late nauplius II. The expression of *αFTZ-F1* and *βFTZ-F1* was measured by RT-qPCR against Elongation Factor 1α as internal reference in eggs, nauplii (Naup) 1 and 2. Every point represents the mean of three biological parallels (eggs = 1 eggstring each, larvae = 10–25 individuals each). Error bars indicate standard deviation. The stippled lines highlight stage transitions and the gray arrow indicates time of hatching. * marks groups significantly different (T-test: p value < 0.05) in *βFTZ-F1* expression compared to sample time point before.

https://doi.org/10.1371/journal.pone.0251575.g003

βFTZ-F1 earlier upregulated than *αFTZ-F1* can also be seen in RNA sequencing data from a time series study done on chalimus and pre-adult lice divided into different intra-instar ages (Fig 4B).

Expression of *αFTZ-F1* and *βFTZ-F1* was also measured in different tissues dissected from adult individuals of both sexes (Fig 5). Both isoforms were expressed in all tissues. In the brain and testis, *αFTZ-F1* expression was approximately 2-fold higher compared to *βFTZ-F1*, while *βFTZ-F1* expression was approximately 7-fold higher compared to *αFTZ-F1* in ovaries and 4-fold higher in oocytes. In the other tissues as well as in whole lice the two isoforms were not expressed significantly different from each other. The expression of *αFTZ-F1* and *βFTZ-F1* (individual part only) was also investigated in available RNA sequencing data. Expression of each isoform in different tissues is shown in Fig 5B and of larvae of different instar age in Fig 5C. For ovaries, oocytes and testis a similar trend can be seen with *αFTZ-F1* lowly expressed in ovaries and oocytes and *βFTZ-F1* lowly expressed in testis. In adult male lice, *αFTZ-F1* was much higher expressed than *βFTZ-F1*. The lowest *αFTZ-F1* to *βFTZ-F1* ratio was found in larvae of middle instar age, the highest in young larvae. Nauplii 1 shows a different trend with a low ratio in young larvae comparable with measurements shown in Fig 3. Male and female larvae do not differentiate from each other.

RNAi mediated knockdown of *βFTZ-F1* in salmon louse larvae and pre-adult male caused molting arrest

Knockdown efficacy of the two transcript isoforms in nauplius I larvae 48 hours after treatment was 42% and 49% for *αFTZ-F1* and *βFTZ-F1*, respectively, and no significant difference was detected between the control group and the non-targeted isoform in their respective experimental group (Fig 6A and 6D). When the animals in both the control group and the two *FTZ-F1* knockdown groups approached molting from the nauplius II to copepodid stage, they appeared normal and sank to the bottom of their incubation well. The *αFTZ-F1* knockdown animals and the *CPY185* control group molted successfully to the copepodid stage (Fig 6B), however animals treated with dsRNA targeting *βFTZ-F1*

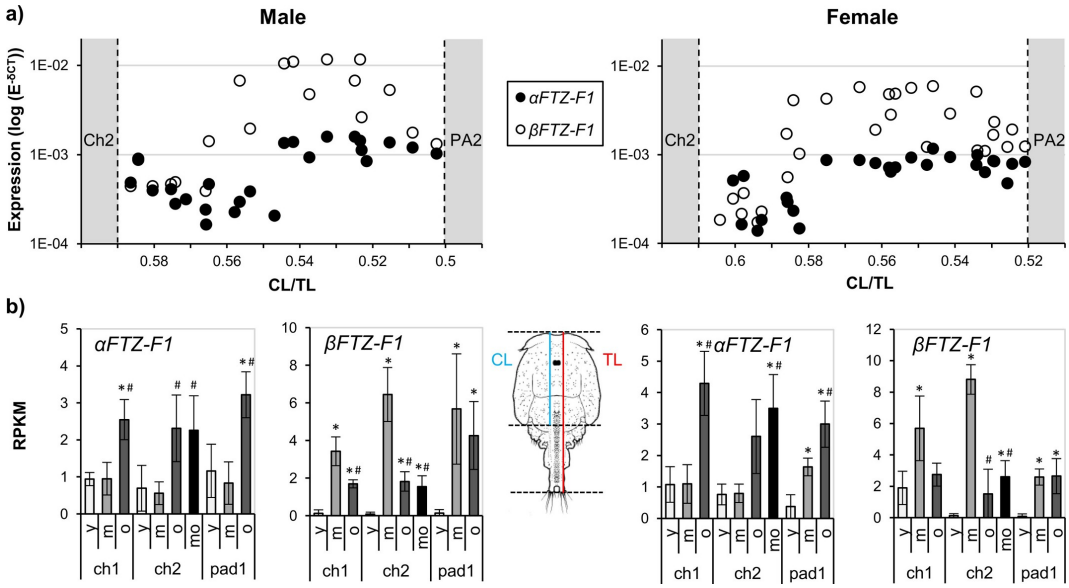

Fig 4. *αFTZ-F1* and *βFTZ-F1* expression during the molt cycle of male and female salmon lice. a) Expression of *αFTZ-F1* and *βFTZ-F1* during the molt cycle of pre adult 1 male and female lice. Each point represents the expression of *αFTZ-F1* or *βFTZ-F1* in one individual. The lice have been sorted by their individual instar age based on the ratio between the length of the cephalothorax (CL) and the total length (TL) of the animal (See materials and methods) with the youngest lice having the highest CL/TL. The stippled lines represent boundaries to the previous life stage, chalimus 2 (Ch2), and the next life stage, pre-adult 2 (PA2). **b)** Expression of *FTZ-F1* transcripts during the molt cycle in chalimus (Ch) 1 and 2 and pre-adult 1 (Pad). Expression data are taken from a time series study measured by RNA sequencing [40] for alpha and beta unique transcript parts only. Values are shown as reads per kilo base per million mapped reads (RPKM). For each stage the gene expression in lice of different instar age of the 3 to 4 categories; young (y), middle (m), old (o) and molting (mo) sorted by use of CL and TL ratios is shown. Significantly different sample groups (T-test <0.05) are marked with * for difference to young and with # for difference to middle. A significant difference (T-test <0.05) between male and female expression of the different sample groups could be only seen for Ch1 y. For details of the composition of lice see [40].

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remained at the bottom of the incubator motionless and alive, but unable to molt (Fig 6E). Closer inspection of these animals revealed the presence of limbs and segments like in the copepodid, also seen in the histological sections (Fig 6F). Histology also revealed the presence of two cuticular layers, showing the synthesis of the new cuticle and breakdown/recycling of the old cuticle. After settlement on its host, the *αFTZ-F1* dsRNA treated larvae developed to reproductively functional adults, and their offspring molted successfully to copepodids with no apparent phenotype.

Knockdown of *βFTZ-F1* in pre-adult I males also caused developmental defects. Initially, the pre-adult I knockdown males developed normally to the pre-adult II stage without any apparent phenotype, but 35 days after injection when terminating the experiment, no adult males were found on the fish remaining in the *βFTZ-F1* knockdown group (Fig 7). It appeared that no *βFTZ-F1* knockdown males had been able to molt from the pre-adult II stage to the adult stage, and subsequently were fallen from the host either to be flushed out of the tank or eaten by fish. Untreated females that were co-infected with *βFTZ-F1* knockdown males did not have any spermatophores attached to the genital segment at the end of the experiment, supporting the idea that the *βFTZ-F1* knockdown males failed to develop past the pre-adult II stage. The knockdown efficacy was only significant in the second

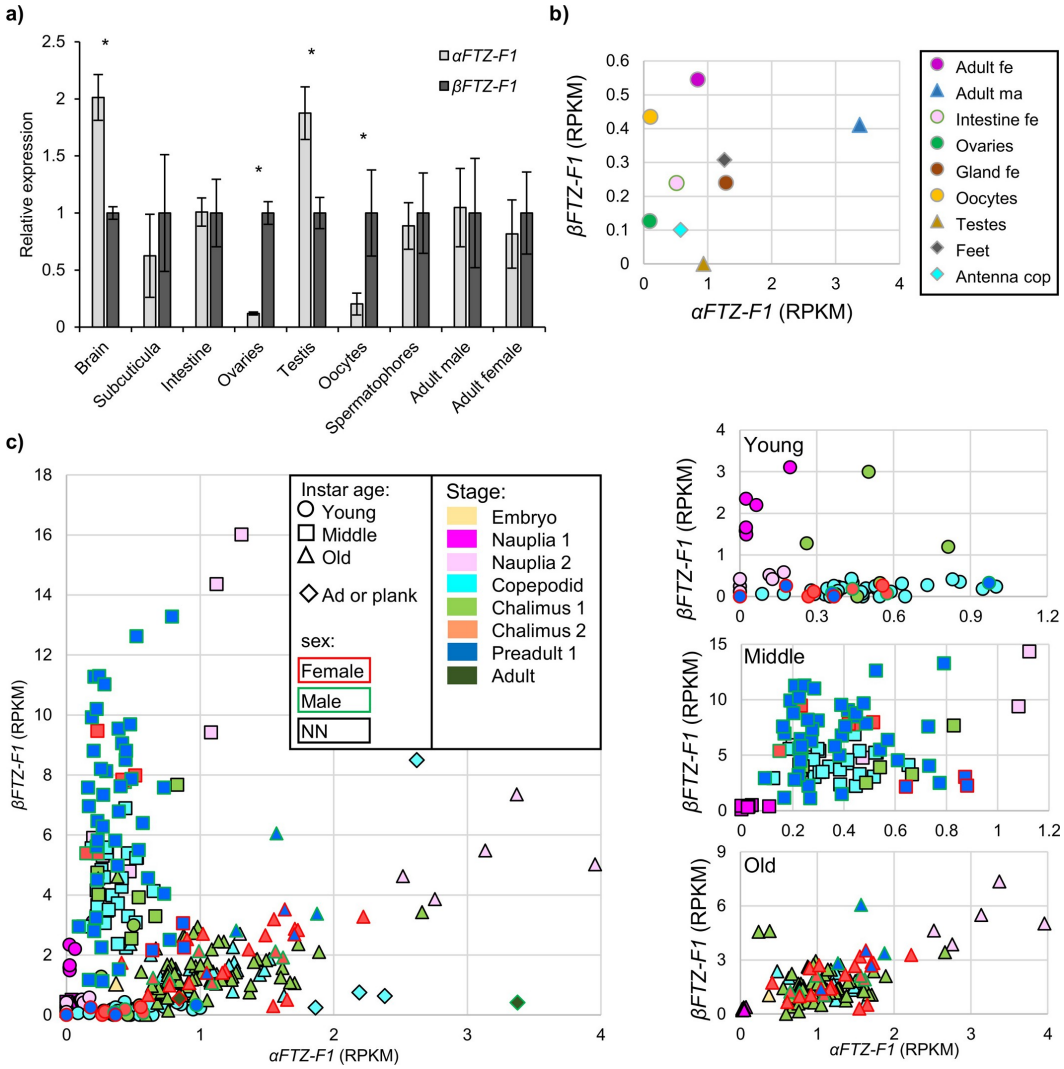

Fig 5. Relative expression of *αFTZ-F1* and *βFTZ-F1* in different adult salmon louse tissues and stages. a) The relative expression of the two *FTZ-F1* isoforms was measured in various adult salmon louse tissues by RT-qPCR. Samples from the intestine, brain and subcuticular contains tissue from both sexes. The expression of *βFTZ-F1* was used as a calibrator, with expression set to 1. Error bars show the standard deviation. N = 3 for each tissue type. * shows significant difference (T-test p value<0.05) between the two isoforms. The expression of the two isoforms measured by RNA sequencing is shown in b) and c). Data are taken from LiceBase.org and from [40] and were analysed for alpha and beta unique transcript parts only. Values are shown as reads per kilo base per million mapped reads (RPKM). b) Expression in different tissues as well as in adult lice; c) different stages and instar ages. For better clarity are data for the different instar ages (young, middle, old) shown separately in the right panel.

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experiment, due to the high variability in expression of *βFTZ-F1* between lice in the control groups (Fig 7).

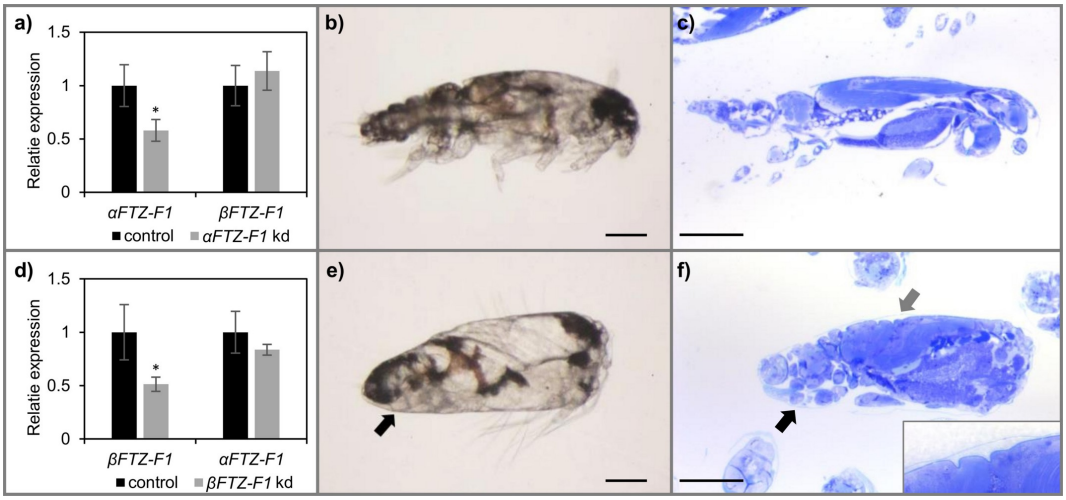

Fig 6. RNAi mediated knockdown of *βFTZ-F1* in nauplius I causes molting arrest at the nauplius II stage. Nauplius I larvae were treated with either dsRNA for *βFTZ-F1*, *αFTZ-F1* or control fragment. **a)** Relative expression of *αFTZ-F1* and *βFTZ-F1* in control (N = 6) and *αFTZ-F1* dsRNA (N = 6) in nauplius II larvae 48 hours after treatment. * indicates significant differences between control and knockdown group (T-test p-value < 0.05) **b)** Larvae treated with dsRNA targeting *αFTZ-F1* molted to the copepodid stage as control **c)** Also, histological sections of *αFTZ-F1* knockdown copepodids showed apparent normal phenotype as the control. **d)** Relative expression of *αFTZ-F1* and *βFTZ-F1* in control (N = 6) and *βFTZ-F1* nauplius II larvae 48 hours after treatment (N = 4). **e)** At the time of molting from nauplius II to copepodid, *βFTZ-F1* knockdown larvae stayed in the bottom of the incubation well and remained trapped in the cuticle. **f)** Sections of molting arrested larvae treated with *βFTZ-F1* dsRNA revealed the development of limbs and segments typical for copepodids (black arrow), and the separation of the new and old cuticle (gray arrow). Scale = 0.1mm.

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RNAi mediated knockdown of *βFTZ-F1* in pre-adult II females disrupts oogenesis during the vitellogenic stage in adults

We attempted to selectively knock down both *αFTZ-F1* and *βFTZ-F1* in pre-adult II females to investigate the isoforms potential role during sexual maturation and reproduction (Fig 8). After undergoing sexual maturity, both the control and *αFTZ-F1* knockdown were able to produce viable eggstrings and offspring, and there was no visible phenotype in *αFTZ-F1* knockdown lice. Measurement of eggstring length did not show a significant difference between *αFTZ-F1* knockdown and control group. Females treated with *βFTZ-F1* dsRNA were able to molt to adults but had little intestinal blood and produced no eggstrings (Fig 8A). Oocytes were disorganized in the vitellogenic stage in the genital segment or appeared to be broken apart (Fig 8B and 8C). Ovaries and oocytes in the oviduct did not vary from control lice. The subcuticular tissue of the cephalothorax in the *βFTZ-F1* knockdown lice contained less and smaller cells than that of the control lice (Fig 8D) while the subcuticular tissue of the *αFTZ-F1* knockdown lice seemed to be unchanged. The experiment was performed three times producing the same result. No significant knockdown was detected for either isoform in their respective group.

Transcriptome sequencing

The effect of RNAi knockdown of *αFTZ-F1* and *βFTZ-F1* on the salmon louse transcriptome was investigated by Illumina® mRNA sequencing of dsRNA treated nauplius II larvae. The sequencing of each sample produced on average 39.5 million reads. Out of all the reads, 92.1%

a)

	# Pre-adult I males injected		# Pre-adult II males collected		# Adult males collected	
Group	**Exp. 1**	**Exp. 2**	**Exp. 1**	**Exp. 2**	**Exp. 1**	**Exp. 2**
Control	120 (6 fish)	60 (5 fish)	24 (3 fish)	15 (2 fish)	9 (3 fish)	12 (3 fish)
βFTZ-F1 kd	60 (3 fish)	60 (5 fish)	11 (1 fish)	14 (2 fish)	0 (2 fish)	0 (3 fish)

b)

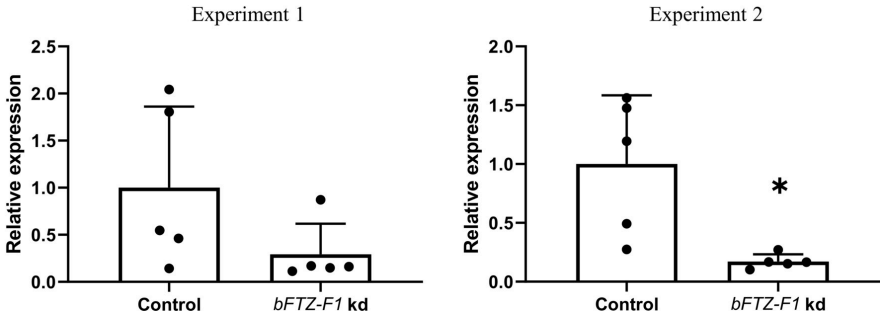

Fig 7. RNAi mediated knockdown of *βFTZ-F1* in pre-adult I salmon louse inhibits development into adults. a) Collective number of lice injected with *βFTZ-F1* dsRNA and collected at the two sampling time points. The number of fish at start and terminated at each time point is listed as well. Pre-adult I male lice injected with *βFTZ-F1* dsRNA developed into pre-adult II lice but failed to molt to adult lice across identical experiments. **b)** The relative expression of *βFTZ-F1* in control (n = 5) and knockdown (kd) (n = 5) group across two parallel RNAi experiments. Each dot represents a pre-adult II male louse. * indicates statistically significant knockdown of *βFTZ-F1* in knockdown group compared to control (T-test: P-value ≤ 0.05).

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mapped uniquely to the salmon louse reference genome, and 4.1% mapped to multiple loci. On average between all samples, 76.2% of all reads overlapped with exon regions in the annotated salmon louse gene models. Sequencing data are available at the NCBI SRA read archive (BioProject: PRJNA687532).

***βFTZ-F1* knockdown exhibits strong effects on overall gene expression compared to *αFTZ-F1* knockdown.** Knockdown of *αFTZ-F1* and *βFTZ-F1* was verified by RT-qPCR prior to mRNA sequencing and amounted to 42% and 51%, respectively (Fig 9A). The principle component analysis (PCA) revealed that larvae treated with *αFTZ-F1* dsRNA displayed little difference in overall gene expression compared to the control samples, while larvae treated with *βFTZ-F1* dsRNA displayed a large difference compared to the same control samples (Fig 9B). Differential gene expression (DGE) analysis identified a total of 217 upregulated and 278 downregulated genes following *βFTZ-F1* knockdown (Fig 9C). DGE analysis on *αFTZ-F1* knockdown samples identified only 10 differentially expressed genes compared to control, with differences (log2 fold change) ranging from only 0.17 to 0.25 (Fig 9C). The results from both DE analysis as well as the normalized gene counts are listed in S2 Table. In an attempt to verify the findings from the DESeq2 analysis of the *αFTZ-F1* knockdown, we performed RT-qPCR on 4 of these 10 differentially expressed genes on the same samples as well as on samples from a previous *αFTZ-F1* RNAi experiment (S3 Fig). RT-qPCR measurements on the same samples confirmed a similar fold change in all four genes, but we did not observe any difference in expression of these genes between control and knockdown group in the samples from the previous RNAi experiment shown in Fig 6.

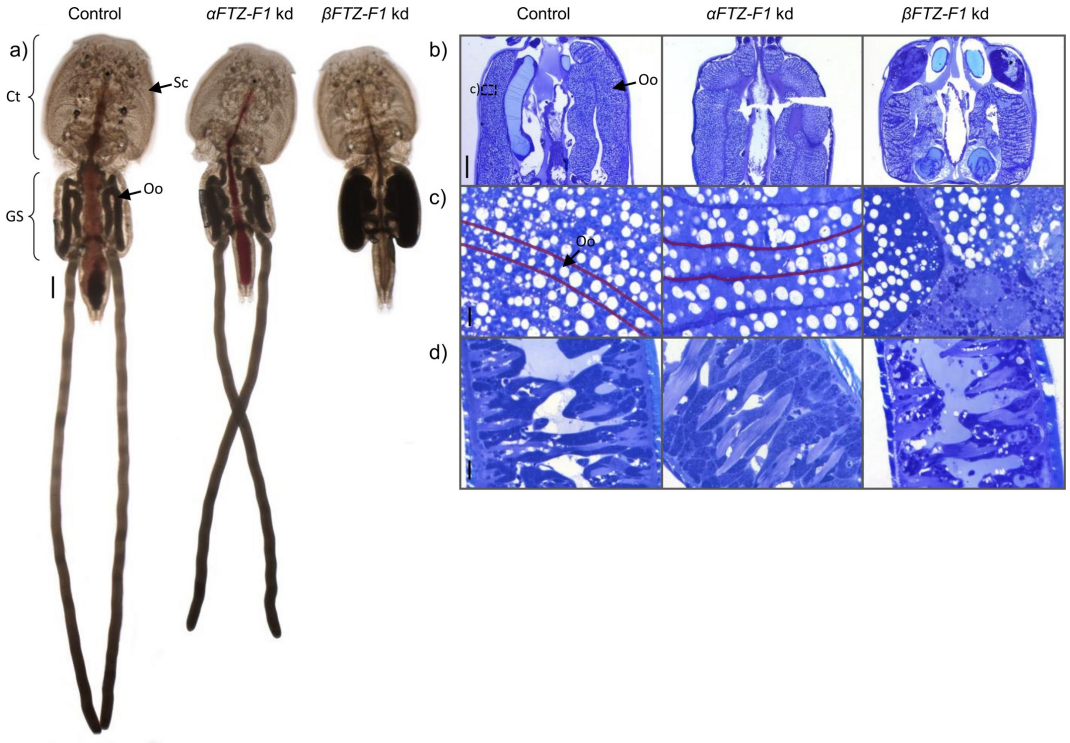

Fig 8. RNAi mediated knockdown effect of *αFTZ-F1* and *βFTZ-F1* in adult female lice. a) photographs of typical adult female lice at sampling for the three groups: control lice, αFTZ-F1 and knock-down of βFTZ-F1. Ct = Cephalothorax, GS = Genital segment, Sc = subcuticular tissue, Oo = oocytes, scale bar = 1mm. Toluidine blue dyed sections of lice from the same groups are shown in b-c. b) genital segment, scale bar = 500μm; c) magnification of the oocytes marked by a square in b). The outline of one oocyte in the genital segment is drawn in red in c), scale bar = 10μm; d) subcuticular tissue of the cephalothorax, scale bar = 10μm.

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***βFTZ-F1* downregulation disrupts the cyclical expression of genes, and affects genes associated with proteolysis and chitin-binding.** There was a high number of differentially expressed genes following *βFTZ-F1* knockdown with no annotations regarding potential functions or homology to other genes. Of the 217 upregulated genes, 109 (50%) were completely without annotations, as the genes had no BLAST hits, or matched hypothetical/uncharacterized genes in other species, and had no known Pfam domains. 75 of the 278 downregulated genes (27%) were also without any annotations. Gene ontology enrichment analysis revealed only 2 enriched molecular function (MF) and biological process (BP) terms among the upregulated genes (S4 Fig). The MF terms enriched were inward rectifier potassium channel activity and chitin binding. Among the downregulated genes, the terms with the highest significant enrichment across both MF and BP categories were associated with proteolysis, organo-nitrogen compound metabolic process and chitin binding (S4b Fig). No terms in the cellular compartment (CP) category was enriched among the differentially expressed genes. Enrichment analysis data is shown in S3 Table.

In a time-series study by Eichner *et al.* [40], the expression of 707 cyclically expressed genes were divided into 6 categories based on their expression pattern within an instar in

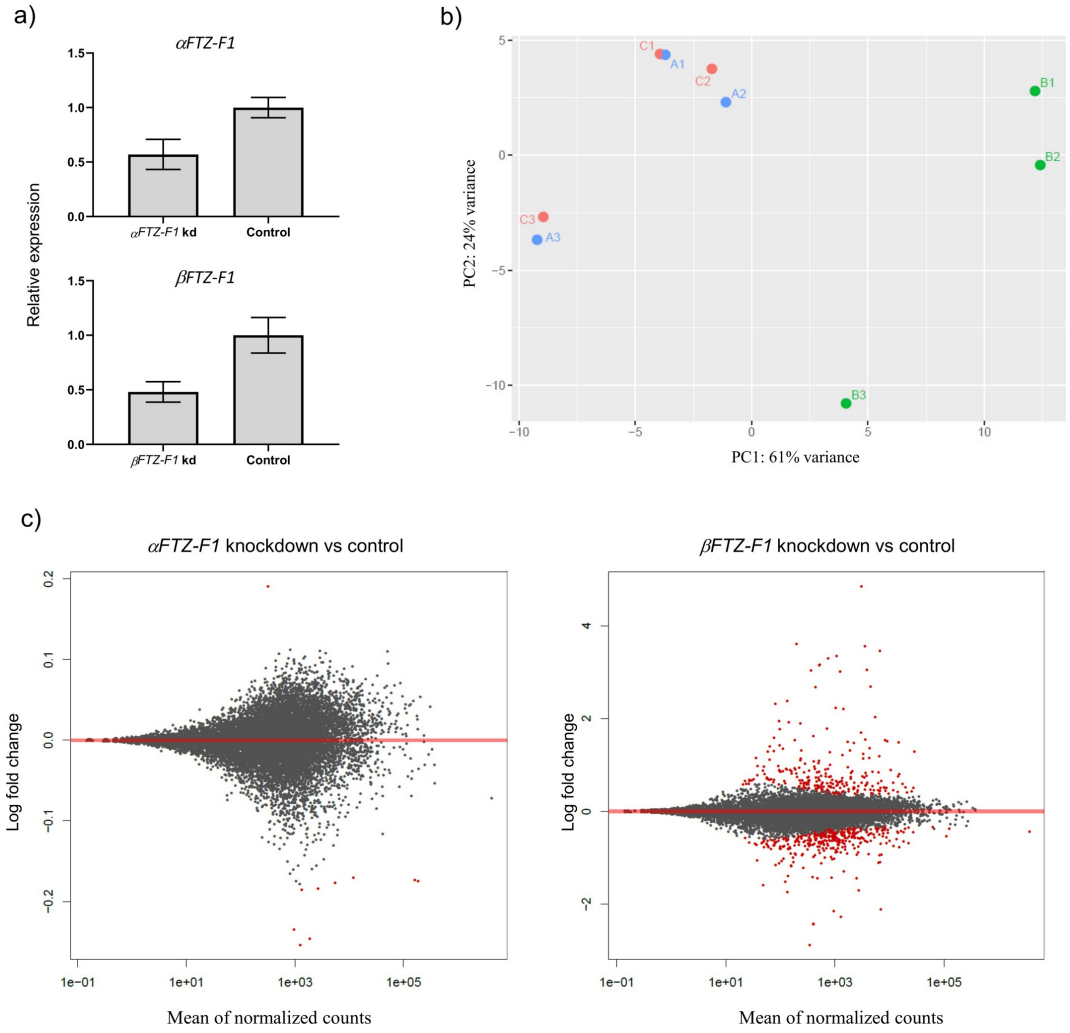

Fig 9. Principal Component Analysis (PCA) and MA plot of the RNAi sample transcriptomes. a) RT-qPCR measurements of *αFTZ*-F1 and *βFTZ-F1* in the mRNA sequencing samples, N = 3. b) PC1 and PC2 represent the two top dimensions of the differentially expressed genes among the three dsRNA treated groups and their three parallels. A = *αFTZ-F1* dsRNA treated larvae, B = *βFTZ-F1* dsRNA treated larvae, C = control dsRNA treated larvae. **c)** MA-plots for DESeq2 comparing the expression of genes in *αFTZ-F1* and *βFTZ-F1* knockdown samples compared to control. The average binary logarithm of the expression across all samples is shown on the x-axis and the binary logarithm of fold change is shown on the y-axis (note different y-axis). Red dots indicate differentially expressed genes (P-adjusted ≤ 0.05), while grey dots represent genes that are not differentially expressed between the two groups.

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chalimus and pre-adult I lice. Of the 495 differentially expressed genes following β*FTZ-F1* knockdown, 178 belonged to cyclically expressed genes, 116 upregulated and 62 under the downregulated category. The DE genes and whether or not it belongs to one of the genes described as cyclically expressed during development through chalimus and pre-adult I

stages are shown in Fig 10 [40]. Strikingly many of the genes (43%) upregulated following β*FTZ-F1* knockdown belong to the genes that were found to be cyclically upregulated in the middle of the stage (pink marked). A small cluster, comprising unknown genes, however, shows the opposite pattern with lowest expression in larvae in the middle of the stage. Under the genes downregulated following *βFTZ-F1* knockdown, less cyclically expressed genes were generally found and most of these were upregulated in larvae prior to next molt (old up). The genes strongest upregulated after knock-down of *βFTZ-F1* (at least 5 times on average, 29 genes) are mainly unknown genes (10 genes without any annotation or Pfam domain as well as 15 uncharacterized or hypothetical proteins). All but 2 of these are upregulated in the middle of the stage compared to expression during normal development.

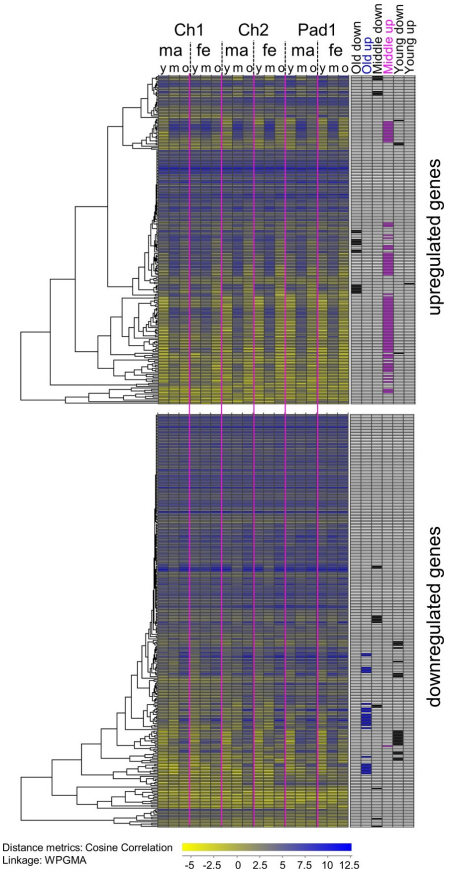

Fig 10. Gene expression up and downregulated after *βFTZ-F1* knockdown during the development of chalimus larvae into the preadult 1 stage. Data are taken from Eichner *et al.* [40] and show the expression profiles of the differentially expressed genes in chalimus stage 1 and 2, and preadult 1 lice (mean values) divided into different instar age with respect to molting ((young (y): directly after molting, middle (m): in the middle of the stage and old (o): directly before the molt to next stage)). The cyclically expressed genes (old, middle and young up or down respectively) described in Eichner *et al.* [40] are marked.

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Under the strongest downregulated genes (at least 5 times, 8 genes) are proteases, a cuticle protein-encoding gene and two unknown genes without annotations.

Discussion

The revealed structure of the *FTZ-F1* gene of *L. salmonis* with different N-terminal isoforms generated through alternative promoter usage and splicing are not uncommon among nuclear receptors, and have been described in human ROR-alpha [52], human HNF4-alpha [53], fruit fly E75 [54], fruit fly EcR [55], and murine thyroid hormone receptor beta [56]. FTZ-F1 has been characterized in several ecdysozoan species, and N-terminal isoforms have been reported in *D. melanogaster* [19], *L. Decemlineata* [26], and *D. magna* [14] (Fig 2). FTZ-F1 has also been described in the arthropods *Metapenaeus ensis* [57], *Eriocheir sinensis* [33], *Aedes aegypti* [25], *Tribolium castaneum* [21], *Blattella germanica* [23], *Manduca sexta* [24], *Bombyx mori* [58], *Spodoptera litura* [22], and *Apis mellifera* [20], but in these studies, only one isoform of FTZ-F1 was reported. That isoforms were not detected in the former studies could have technical reasons (e.g. that rapid amplification of cDNA ends (RACE) and northern blots were performed on only one developmental stage, tissue or cell type, or that the antibodies and RT-PCR primers were specific to the N-terminal domain of only one potential isoform). To discover if the conserved organization of the *FTZ-F1* gene found in *D. melanogaster*, *L. salmonis* and *D. magna* could also be found in the different subphyla of the ecdysozoa we investigated available sequences of ecdysozoan organisms with only a predicted *αFTZ-F1* sequence. By re-analyzing the sequences in light of this study we could show that this structure is a conserved feature among most ecdysozoans, but not among nematodes. The nematode sequences currently available display a different splicing pattern of the DBD domain encoding exon compared to the other ecdysozoans to produce a DBD lacking isoform [31]. No isoforms with unique N-terminal domains has been described in nematodes thus far (Fig 3). The number of species investigated outside of the hexapods were limited, mainly due to few available sequenced genomes from these subphyla. However, summary of available data favor that the animals of the group called panarthropoda have two FTZ-F1 isoforms.

In *D. melanogaster*, the two *FTZ-F1* isoforms are expressed at different stages during development, with *αFTZ-F1* being expressed only during early embryogenesis, prior to any *βFTZ-F1* expression [19]. In the salmon louse however, expression of *βFTZ-F1* and *αFTZ-F1* is not spatially or stage separated as in the fruit fly, but both isoforms can be found in all stages and are found in all investigated tissues. Expression height varies within the stage and also the ratio between the isoforms. The ratios vary also between different tissues. (Figs 3–5). Interestingly, in the study by Yussa *et al.* [59], it was revealed that pair-rule segmentation in *Drosophila αFTZ-F1* knockouts could be rescued with βFTZ-F1 protein expression. The variable N-terminal domain did not affect the function of FTZ-F1, which was further demonstrated by the fact that the mouse orthologue SF-1, which does not have an N-terminal domain, was also able to rescue the fruit fly αFTZ-F1 knockouts. A study by Ohno *et al.* [60] also demonstrated that the two fruit fly isoforms compete for the same response elements when co-transfected into mammalian cells. The ability of the two *Drosophila FTZ-F1* isoforms to interact with the same transcriptional partners and bind the same DNA binding sites when regulating gene expression, raises interesting questions in regards to isoform function and specificity since the two isoforms are expressed in the same tissues at the same time in the salmon louse (Fig 5). A similar observation has also been reported in *L. decemlineata* and *D. magna* [14, 26]. The mRNA knockdown efficacy in larvae for *αFTZ-F1* was similar to the efficacy for *βFTZ-F1* (Figs 6 and 9A), no apparent phenotype was detected in the *αFTZ-F1* knockdown group. The exact function of *αFTZ-F1* thus remains unknown, as the knockdown might not have been sufficient to

reduce the number of αFTZ-F1 proteins below the threshold for function. However, the expression of both isoforms concurrently in the same tissues raises the possibility that the different N-terminal domains of the two salmon louse *FTZ-F1* receptors confer a specificity in function, which could explain the difference in phenotype we observed when the two transcript variants were subjected to selective knockdown through RNA interference (Fig 6). There are no studies on the function of the N-terminal A/B domain in the class V (NR5A) family of nuclear receptors. A major reason for this is likely the lack of an A/B domain in the mammalian NR5A orthologues SF-1 and LRH-1. Studies on N-terminal function in other nuclear receptor families provide some ideas as to how the N-terminal isoforms could provide specificity of function. N-terminal isoforms have been shown to have different transcriptional output when regulating the same target genes [61], and a different response to ligand activation [62]. However, since the two FTZ-F1 isoforms in the salmon louse are expressed in the same tissues, it is possible that the isoforms have different affinities to DNA response elements and regulate different gene targets [52, 63]. The different N-terminals could also have different affinities to transcriptional partners and cofactors [64]. These affinities shown to be affected by intra domain interactions between the A/B domain and the LBD [65, 66], which in turn can confer specificity in regulation of downstream gene targets. Knockdown of *βFTZ-F1* caused downregulation of three G-protein-coupled inwardly-rectifying potassium channel (S4 Fig), proteins commonly found in the nervous system [67]. Interestingly, *αFTZ-F1* is the predominant isoform in brain tissue (Fig 5). This viewed in context with the potential competitive nature of the two receptor isoforms mentioned previously, raises the possibility that the ratio between the two receptor isoforms could be what is necessary for proper regulation of gene targets, and that a shift in dosage between the two receptors could affect the normal function of one or both receptors. The molecular mechanics of N-terminal function in FTZ-F1 are areas of interest for future in-vitro studies.

mRNA sequencing on larvae 48 hours after treatment with dsRNA, but prior to development of a lethal phenotype, revealed that 495 genes were differentially expressed as a result of *βFTZ-F1* downregulation (Fig 9). In particular, a significant number of genes associated with proteolysis and chitin binding were affected by *βFTZ-F1* knockdown (S4 Fig). This together with the presence of two cuticular layers and the late onset of phenotype at the moment of stage transition (Fig 6), suggests that the molting arrest is due to complications with complete breakdown and detachment of the old cuticle, leaving the nauplii trapped in their own cuticle and unable to proceed with ecdysis. Many of the genes upregulated after β*FTZ-F1* knockdown belonged to a group which during normal development are higher expressed in the middle of the stage, while the ones downregulated after knock-down are usually upregulated directly before the molt (Fig 10). This also confirms that these genes are important for the molting process itself. Involvement in the molt process was also evident when pre-adult I males were subjected to *βFTZ-F1* knockdown. The lice were able to develop to the pre-adult II stage but were unable to molt to the adult stage (Fig 7). No molt arrest occurred at the pre-adult I to pre-adult II molt, which was expected as injection of *βFTZ-F1* dsRNA likely happened at a time point where most of the lice had already developed past the point of peak *βFTZ-F1* expression in the pre-adult I stage. However, the effect of *βFTZ-F1* knockdown on the molt cycle became clear when the lice had to undergo a full molt cycle following the pre-adult I to pre-adult II molt, as no adult males were found 35 days post infection. Our findings provide the first description in crustaceans of FTZ-F1 gene function in molting, and together with known observations of FTZ-F1 function in insects and nematodes, this supports the already established idea of FTZ-F1 as a conserved regulator of molting across the ecdysozoan superphylum. There is high variability in expression of *βFTZ-F1* between individuals in the control group (Fig 7), which is likely due to differences in the instar age (See Fig 4), caused by individual differences in the

development rate [35, 68]. Therefore, it was difficult to document the knockdown efficacy in pre-adult II lice.

Injection of dsRNA selectively targeting *βFTZ-F1* in pre-adult II lice as expected did not inhibit molting to the next developmental stage, similar to the observations made when injecting pre-adult I lice. However, the adult females failed to produce viable oocytes with treatment resulting in disorganized and ruptured oocytes in the genital segment during the vitellogenic stage (Fig 8). Also, the cells of the subcuticular tissue seemed to be less and smaller compared to control lice. The subcuticular tissue was shown to be the production area for vitellogenins [69] and the yolk-associated protein, LsYAP [41]. A similar phenotype was observed in a study by Sandlund *et al*. [16] when knocking down EcR transcripts in the same developmental stage, indicating that the putative ecdysone regulatory cascade, like in insects, play a crucial role in the reproductive development in crustaceans. FTZ-F1 appears to be a conserved regulator of oocyte maturation and reproduction among ecdysozoans as in addition to our findings it also plays a vital role in vitellogenesis in crabs [33], mosquitos [25] and honey bees [70], and is shown to be crucial for somatic gonad development in C. elegans [31, 32]. In the aforementioned species, with the exception of nematodes, only one *FTZ-F1* transcript was studied. In the current study, we demonstrated that the *βFTZ-F1* isoform is crucial for oocyte maturation, while injection of dsRNA targeting the *αFTZ-F1* isoform had no effect on oocyte maturation or embryonal development. This observation fits well with the tissue expression analysis of the isoforms, with *βFTZ-F1* being the most predominant isoform in both ovaries and oocytes (Fig 5). No downregulation compared to control lice was detected for either isoform 35 days post injection. This was also seen in the study by Sandlund *et al*. [16], where *EcR* mRNA knockdown was only detectable 2- and 4-days post injection. However, *FTZ-F1* isoform expression fluctuates within a molt cycle (see Fig 4), which makes it difficult to accurately determine downregulation in the pre-adult stages. Downregulation of *αFTZ-F1* was detectable at the larval stage when soaking the lice with dsRNA during the first molt, showing that the fragment is effective. The injection of both dsRNA in pre-adult lice was done with the same dosage and the experiment repeated three times producing the same outcome. Based on this, it appears that only βFTZ-F1 plays an important role during oocyte maturation and female sexual development in the salmon louse. However, if downregulation of *βFTZ-F1* would have an impact on male development, could not be answered by these experiments, since the defect in molting interfered with development to adult male lice.

Knockdown of *αFTZ-F1* did not produce many changes on the overall gene expression (Fig 9B). Analyzing differential expression between the control and the *αFTZ-F1* knockdown larvae revealed only 9 genes, all with a minor fold change (Fig 9C). To verify the results for 4 of the highest differentially expressed genes, RT-qPCR were run for both the sequenced samples and samples from another RNAi experiment. The minor difference in expression could only be verified in the samples subjected to mRNA-sequencing, and not in a repeated RNAi experiment (S2 Fig), and is therefore probably caused by individual biological differences of single animals. We measured similar knockdown efficacy of both *FTZ-F1* isoforms with RT-qPCR (Fig 9A), however, mRNA sequencing did not show the same amount of downregulation. Only reads representing the different N-termini could be counted, resulting in low numbers of reads, and uncertain quantification [71]. A different quantification method (Kallisto) improved the counts for both isoforms compared to FeatureCounts (S3 Fig). However, only *βFTZ-F1* showed a significant difference this way. RT-qPCR is a more accurate method compared to mRNA sequencing when quantifying isoform expression. For *αFTZ-F1*, RNAi in larvae resulted in no visible phenotype and no effect on the transcriptome. There is an uncertainty over how persistent the RNAi effect is during development to adults following soaking at the larval stage. For two other salmon louse genes knocked down, the knockdown

effect wore off as the animals grew [17, 72], likely influenced by factors pertaining to mRNA stability, protein turnover and dilution of the dsRNA probe. *αFTZ-F1* knockdown lice placed on hosts developed normally to reproductive adults, suggesting that the downregulation in larvae was insufficient to reduce the amount of *αFTZ-F1* proteins below the threshold for function in the larval stage and in the subsequent stages, or that it has no vital functions during development from larvae to adults. The higher ratio of *αFTZ-F1* expression in the male gonads could suggest a potential role in male reproduction. The investigation of the role of *αFTZ-F1* in male reproduction would have been strengthened by treatment at the pre-adult II stage. RNAseq analysis was only performed on knockdown larvae, so any changes in the transcriptome passed the nauplius II stage was not investigated. In the fruit fly, *αFTZ-F1* is required for pair-rule segmentation during early embryogenesis. A significant rise of transcript levels was observed in late embryo development of *L. salmonis* at 4 days up until hatching (Fig 3). Since there is no method to knock down genes in the eggstrings themselves, we are not able to investigate the effect of *αFTZ-F1* knockdown on the embryonal development.

Conclusion

Here we propose the hypothesis of a structural conservation of the *FTZ-F1* gene in ecdysozoans to produce two isoforms with different N-terminal domains, a feature not found in nematodes. Our findings raise questions around the mechanism of specificity in function provided by N-terminal domains in class V nuclear receptors, and other nuclear receptors in general. We demonstrated that *βFTZ-F1* is a major regulator of molting and oocyte maturation in the salmon louse. This is the first description of *FTZ-F1* gene function in copepod crustaceans. Our findings provide a foundation to expand the understanding of the molecular mechanisms of molting in the salmon louse and other copepods.

Supporting information

S1 Fig. The generation of two isoforms of FTZ-F1 through alternative transcription is conserved in the fruit fly, salmon louse and water flea. A comparison of the FTZ-F1 gene structure of the fruit fly (*Drosophila melanogaster*), the salmon louse (*Lepeophtheirus salmonis*) and the water flea (*Daphnia magna*). The sizes of the primary transcripts and some introns are given in kilobases (kb). Exons are represented as boxes, and introns and splicing patterns as lines. The starts of transcription for α*FTZ-F1* (α) and β*FTZ-F1* (β) are marked with a curved arrow. The 5' UTR of the α*FTZ-F1* and β*FTZ-F1* transcripts are highlighted yellow and green, respectively. The area of the gene coding for the N-terminal is highlighted red for α*FTZ-F1*, and blue for β*FTZ-F*. The area coding for the shared DBD domain for both transcript variants is colored black. The 3' UTR is colored light gray. Exons and short introns are in scale.
(DOCX)

S2 Fig. RT-qPCR verification of four DESeq2 differentially expressed genes following *LsαFTZ-F1* knockdown. a) RT-qPCr measurements of the genes EMLSAG00000008331, EMLSAG00000011833, EMLSAG00000010679, and EMLSAG00000007107 in the samples analysed with mRNA sequencing from both *LsαFTZ-F1* knockdown (kd) and control. **b)** RT-qPCR measurements of the same genes in samples from a previous RNAi experiments, n = 3.
(DOCX)

S3 Fig. Counts of *LsFTZ-F1* transcripts from mRNA sequencing with FeatureCounts and Kallisto quantification. a) Normalized counts from FeatureCounts of *FTZ-F1* regions in genome annotation file; whole transcript (*αFTZ-F1* + common region), *LsαFTZ-F1* specific region, *LsβFTZ-F1* specific region. **b)** Counts of *LsαFTZ-F1* and *LsβFTZ-F1* full-length

transcripts from Kallisto quantification. Star indicates significant difference compared to control (P-adjusted value ≤ 0.05).
(DOCX)

S4 Fig. Gene ontology enrichment analysis of *βFTZ-F1* knockdown animals. GO terms enriched under the genes downregulated (a) or upregulated (b) after *βFTZ-F1* knockdown. Shown are all terms with a q-value <0.05 for the categories biological process (BP) and molecular function (MF). No terms for cellular compartment (CC) met these conditions. Bars show the frequency of the given GO term under the selected genes (study frequency) and in the whole genome (population frequency). The enrichment compared to occurrence in the whole genome is shown as a number on top of each bar. Terms are sorted according to its q-values for each category.
(DOCX)

S1 File. Overview of structures, sequences and accession numbers of FTZ-F1 sequences in ecdysozoan species investigated.
(DOCX)

S1 Table. RT-qPCR primers used in this study and their application.
(DOCX)

S2 Table. Differential expression analysis (DeSeq2) of salmon louse genes.
(XLSX)

S3 Table. Data from GOEnrichment analysis.
(XLSX)

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Errata for
Molecular regulation of molting in the salmon louse (*Lepeophtheirus salmonis*)

Joakim Brunet

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at the University of Bergen

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(date and sign. of candidate) (date and sign. of faculty)

Errata

Page 19 Misspelling: “Oogenia differentiates” – Corrected to “Oogenia differentiate”

Page 21 Misspelling: “length of the domain vary” – Corrected to “length of the domain varies”

Page 23 Misspelling: “Retinoic X receptor” – Corrected to “Retinoid X receptor“

Page 38 Misspelling: “puffs indicates” – Corrected to “puffs indicate”

Page 94 Misspelling: “DBD” used incorrectly in two places, should be “LBD”: “The DBD encoding fragments were ligated with T4 DNA ligase into pM-vector (Clontech) and the DBD + LBD into pVP16-vector (Clontech), fusing the DBD to the GAL4 DNA binding domain and the DBD and LBD to the VP16 activation domain.” – Corrected to “The LBD encoding fragments were ligated with T4 DNA ligase into pM-vector (Clontech) and the DBD + LBD into pVP16-vector (Clontech), fusing the LBD to the GAL4 DNA binding domain and the DBD and LBD to the VP16 activation domain.”

Page 111 Misspelling: “Hydroxyecdsyone – Corrected to “Hydroxyecdysone”

Page 111 Wrong word: “red lines” should read “black lines” and vice versa in figure legend

Page 111 Misspelling: “Ponasteron” – Corrected to “Ponasterone”

Page 112 Misspelling: “Ponasteron” – Corrected to “Ponasterone”

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