Lipid transporters and receptor in salmon louse (*Lepeophtheirus salmonis*)

Effect of RNAi Knockdown on oogenesis, embryonal development and larval maturation

Muhammad Tanveer Khan

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

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Date of defense: 23.04.2021

Year: 2021

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Name: Muhammad Tanveer Khan

Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

The research work for this thesis was conducted at the Sea Lice Research Centre (SLRC), Department of Biological Sciences (BIO), University of Bergen, Norway, during the period from September 2012 to March 2017. The research project was financed by the Research Council of Norway: Centre for Research-Based Innovation (SFI) under grant number 203513/O30. The PhD education and training was formally offered by the Department of Biological Sciences (BIO), University of Bergen.

Dedication

This thesis is dedicated to my mother and late father for their endless love, support, prayers and advices.

Acknowledgements

First and foremost, I would like to thanks my supervisor, Rune Male, for his continuous support, patience and guidance throughout my PhD education. Thank you so much for your availability whenever I needed your input to plan experiments and to complete my manuscripts and PhD thesis. I would like to thank my co-supervisor, Sussie Dalvin, for your inputs on technical aspects of my experiments, correcting my grammatical mistakes and other technical contents again and again in my manuscripts and in my PhD thesis. I would also like to thank Frank Nilsen being a great co-supervisor and also providing me with the opportunity to pursue my PhD education at Sea Lice Research Centre. You were always available for long discussions and your particular expertise in Sea Lice, excellent advice and inspiring attitude made it very easy to undertake this research work.

My special thanks to Ingunn Wergeland for all of your kind help regarding my administrative matters. I am extremely thankful to Heidi Kongshaug for all of your help during RNAi experiments and in the well-organized lab. I humbly thank Wenche Telle for all of your grateful help during my lab work on the 5th floor. I would also like to thanks Lars Hamre and Per Gunnar Espedal for their excellent help and support in the animal facility.

Moreover, it is my pleasure to thank all of my colleagues (Aina Øvergård, Ewa Harasimczuk, Liv Sandlund) at SLCR for creating a friendly work environment and being willing to help and give advice. I would also like to thank Christiane Eichner for Q-PCR help and Anna Komisarczuk for her enormous support with my experiments and manuscripts. Thanks to my friends, Waqas Azeem, Qaiser Waheed and Hanif Khan, for their excellent support, encouragement and for sharing pleasant time at coffee and lunch breaks.

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Paper II: <u>Khan MT</u>, Dalvin S, Waheed Q, Nilsen F, Male R (2018) Molecular characterization of the lipophorin receptor in the crustacean ectoparasite *Lepeophtheirus salmonis*. PLoS ONE 13(4): e0195783. https://doi.org/10.1371/journal.pone.0195783.

Paper III: <u>Khan MT</u>, Dalvin S, Nilsen F and Male R (2020). Apolipoprotein 1 (LsLp1) knock down reduces reproductive capacity in the salmon louse (*Lepeophtheirus salmonis*). **(Manuscript)**

Abbreviations

AKH: adipokinetic hormone

Apo: apolipoprotein

B. germanica: Blattella germanica

C.elegans: Caenorhabditis elegans

CE: cholesteryl ester

CM: chylomicron

DAG: diacylglycerol

EGF: epidermal growth factor

ER: endoplasmic reticulum

HDL: high density lipoprotein

IDL: intermediate density lipoprotein

LDL: low density lipoprotein

LDLR: low density lipoprotein receptor

LsLp: *L. salmonis* apolipoprotein

Lp: Lipophorin

LpR: Lipophorin receptor

L. salmonis: *Lepeophtheirus salmonis*

LsYAP: yolk associated protein

LBD: ligand binding domain

LLTP: large lipid transfer protein

MTP: Microsomal triglyceride transfer protein

OLSD: *O*-linked sugar domain

RNAi: RNA interference

TAG: triacylglyceride

UTR: untranslated region

Vg: vitellogenin

VLDL: very low density lipoprotein

UTR: untranslated region

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Abstract

The salmon louse, *Lepeophtheirus salmonis*, is a marine ectoparasite of salmonids in the Northern Hemisphere. At present, salmon louse infestation is considered as one of the biggest challenges in the salmon farming industry, causing huge economic losses, and also considered a threat to wild populations of salmonids. Control of salmon lice on farmed salmon has mainly depended on the use of chemotherapeutants. However, over the past few years, the salmon louse has developed resistance against most available chemicals. As a consequence, non-chemical treatment methods such as cleaner fish have been introduced in salmon farming, but the production, health and welfare in the cleaner fish have been challenging. It is, therefore, evident that new treatment methods are needed to control this parasite. For this purpose, further understanding of the biology of this parasite is crucial to identify new principles or drug targets.

Lipids are an important source of energy for the growth and reproduction of animals. Other functions include their role in cellular signalling and as structural components in the cell membranes. In oviparous animals, females deposit lipids to maturing eggs to be utilized during embryogenesis and larval development. Transport of lipids through the circulation of animals to developing oocytes is facilitated by lipoproteins, which consist of lipids and protein components known as apolipoproteins. Lipoproteins carry lipids from the site of synthesis or storage to the site of utilization/storage while lipoprotein receptors facilitate uptake of lipoproteins. Previous studies in vertebrates and some insects showed that maturation of these lipoproteins is under the control of another protein known as microsomal triglyceride transfer protein (MTP). Female salmon lice produce large numbers of lipid-enriched eggs throughout its life span. Similar to other oviparous animals, female louse accumulates a large amount of lipids in developing eggs during vitellogenesis. In female salmon lice, transport of maternal lipids to growing oocytes of female lice has

not been addressed before. Presence of genes encoding MTP, apolipoproteins (apoLps) and lipophorin receptor (LpR) may suggest a similar mechanism of lipid metabolism/transport as found in other organisms.

Lipoproteins require for extracellular transport of lipids to different tissues of animals and assembly, as well as secretion of these lipoproteins depend upon MTP. In oviparous species, female supply enough lipids to oocytes to secure successful embryogenesis and early larval development. It is likely that female salmon lice use similar lipoprotein based mechanism to supply maternal lipids to growing oocytes. Therefore it is important to study the role of MTP in the supply of lipids to growing oocytes. Three transcript variants of *MTP* were found in the salmon louse and all variants transcribed differently in different tissues of an adult female. Functional studies conducted through RNAi induced transcript knock down confirmed that female lice produce offspring with very low lipid contents and survival rate of 10-30% compare to control group animals. The present study suggests that MTP has an important function in reproduction and lipid metabolism in salmon louse and may be considered in the development of a new anti-parasitic treatment method.

Protein components of lipoproteins, apoLPs, are essential in the transport of lipids to different tissues of animals through their interaction with cell surface lipoprotein receptors. Similar to other oviparous animals, it is possible that female salmon louse use lipoproteins for the transport of maternal lipids to growing oocytes where apoLps of lipoproteins bind with lipoprotein receptors and release lipids to the oocytes. In salmon lice, two apoLps encoding genes (*LsLp1 and LsLp2*) were identified. Expression of both genes were found in the intestine and sub-cuticular tissue of adult female louse. RNAi mediated-knockdown of both genes in female louse confirmed significant reduction of transcripts levels. Female lice injected with *LsLp1* double-stranded RNA produced short egg-strings as well as significantly fewer offspring compared to control lice. Knockdown of *LsLp2* did not show any effect on the

eggstring production and numbers of offspring compared with control lice. Functional studies were conducted through RNAi suggested that *LsLp1* play an important role in reproduction of female lice.

Previous studies in different organisms show that members of low-density lipoprotein receptor (LDLR) superfamily mediate the endocytosis of lipoproteins. In salmon louse genome database, single gene homologous to insect lipophorin receptor was identified and named as *L. salmonis* lipophorin receptor (*LsLpR*). The *LsLpR* consists of 16 exons and encodes a protein of 952 amino acids. Structural analysis showed that the predicted structure of LsLpR contains five functional domains similar to LpR of insects. Phylogenetic analysis placed *LsLpR* together with *LpR* of insects. The highest abundance of *LsLR* transcripts was found in copepodids and adult females. In the adult females, receptor transcripts and proteins were found in the ovary and vitellogenic oocytes. While in larvae, the *LsLpR* transcripts were found in the neuronal somata of the brain and in the intestine. Possible functions of *LsLpR* in reproduction and lipid metabolism were investigated through RNA interference. Knockdown in larvae decreased the transcription of *LsLpR* by 44-54%, and knockdown of *LsLpR* in adult female lice reduced the number of offspring with 72% compared with control lice.

1. Introduction

In this thesis, genes involved in the metabolism and transport of lipids were investigated in *Lepeophtheirus salmonis* (*L. salmonis*) (Krøyer, 1837). Lipids are essentials for the growth, development and reproduction of animals. The research conducted during this study provides information about the essential roles of lipids in the reproduction of *L. salmonis*. This knowledge can be used in the future to control the infestation of this economically important ectoparasite in the aquaculture industry. In the introduction, the first part describes the parasite itself, host, life cycle and economical importance of this parasite in the aquaculture industry. The second part describe the lipid transport system in other organisms and how existing knowledge help us to understand the lipid metabolism and transport in this parasite.

1.1 Salmon lice infestation in aquaculture

The fish farming industry in Norway was started in 1960, and today Norway has the largest production of Atlantic salmon globally. The production of Atlantic salmon in Norway has grown continuously the last decades, from 630000 metric tons (2006) to 1233619 tons in 2016 [(https://www.ssb.no, https://www.statista.com/statistics/250262/top-atlantic-salmon-producers-from-aquaculture/)]. As the salmon farming industry is growing, the direct problems in terms of fish diseases as well as welfare and environmental issues have also increased. Among these problems, one of the major issues in aquaculture industry is the presence of sea lice. Sea lice is a major term which covers more than 500 species infecting different fish species, however, *Lepeophtheirus salmonis* (*L. salmonis*) and different species of Caligus are main ectoparasites of farmed as well as wild Atlantic salmon (Salmo salar) (1). In Norway, the infection on salmonids is dominated by *L. salmonis* while *Caligus elongatus* is found less frequently (2, 3). The presence of lice infections on farmed fish results in high economic losses for the aquaculture industry

in term of reduced growth, fillet quality, high mortality rate and treatment costs (medicinal and/or non-medicinal). In Norway only, the total cost to deal with lice in the year of 2014 was estimated at 351 million dollars (4). *L. salmonis* is commonly known as salmon louse due to specificity to infect salmonids. Due to the economic importance of *L. salmonis* as the major ectoparasite in salmonid farming industry of Norway, the thesis focused on this parasite.

The salmon louse cause not only huge economic losses in the aquaculture industry but is also considered a threat to wild salmonids (5-7). Spread of salmon lice to wild populations of salmonids is typically observed in the area of intense salmon farming (8, 9) with potential mortality of wild salmonids smolts (10, 11). However, the actual effect of lice originate from the farms to wild salmon population is hard to estimate (11). The parasitic stages of salmon louse live on the host and feed on skin, mucus and blood (12, 13). Due to grazing activity on the skin surface, the salmon louse damages the skin and cause haemorrhages typically on the head and back of their host (14, 15), which leads to loss of physical and microbial protective function of the host's skin, secondary infections, osmoregulatory stress and also responsible of high mortality if remain untreated (14-17). Salmon lice hosts viruses and it has been hypothesized that it act as vector of different viral diseases (18, 19).

Salmon lice infestations in the fish farming industry have mainly been controlled through medicinal compounds. These medicinal compounds include organophosphates, pyrethroids, avermectins, benzoyl urea and disinfectant (hydrogen peroxide). However, the effectiveness of most of these medicinal compounds has declined due to the development of resistance or reduced sensitivity in lice populations against these compounds (20-23). Resistance towards deltamethrin (Pyrethroid), azamethiphos (Organophosphate), emamectin benzoate (Avermectin) and hydrogen peroxide (disinfectant) has been reported along the Norwegian coast (24, 25). Moreover, medicinal compounds used to delouse the farmed salmon also

have a negative impact on other non-target species in the sea (26). Due to reduced sensitivity, issue of resistance and the negative environmental impact of these medicinal compounds, salmonid producers have also implemented alternative methods to control the lice infestation at the farms. These alternative methods are cleaner fish (27, 28), snorkel cages (29), warm and freshwater treatments (30-32) and anti-attachment diets. Cleaner fish are one of the most widely used methods to reduce lice abundance at the farm level but their welfare as well effectiveness have been questioned and can act as a vector for spreading other diseases (33-35). Snorkel cages are another preventive method to control lice where fish are kept deeper in the water to avoid infective lice larvae that are found mostly in the top 5 metres of the water column (36). However, this treatment method needs extra husbandry inputs. Warm and fresh water treatment has been developed to delouse the lice, however, these methods are not 100% effective (37) and it is possible that salmon lice may develop tolerance to these method (38). In conclusion, there is severe lack of efficient methods that also ensure good fish welfare. Medicinal compounds have been used at a large level to control the salmon lice; however, effectiveness of medicine itself is a challenging and also has negative effects of the environment. Emamectin benzoate was introduced in 1999 as the latest medicine to control sea lice and recently lufenuron (benzoyl phenyl-urea) introduced and approved in Chile but not in Norway. Therefore, it has become essential to look for new medicinal compounds or vaccine targets to control sea lice infestation in the aquaculture industry.

1.2 The salmon louse; host and geographical distribution

The salmon louse, *Lepeophtheirus salmonis* (*L. salmonis*) is a marine ectoparasitic copepod on salmonids from the genera *Salmo*, *Oncorhynchus* and *Salvelinus*. Two subspecies of *L. salmonis* are believed to be present in the Atlantic (*Lepeophtheirus salmonis salmonis*) and Pacific (*Lepeophtheirus salmonis onchorynchii*) oceans respectively (39). In the north Atlantic, *L. salmonis* has three hosts; Atlantic salmon

(*S. salar*) and sea trout (*Salmo trutta (Linnaeus*, 1758)) and Arctic charr *(Salvelinus alpinus (Linnaeus*, 1758)). In the Pacific Ocean, *L. salmonis* is commonly found on Oncorhynchus species such as rainbow trout, *Oncorhynchus mykiss* (*O. mykiss* (Walbaum, 1792)), pink salmon (*O. gorbuscha* (Walbaum, 1792)), chinook salmon (*Oncorhynchus tshawytscha)* sockeye Salmon (*O. nerka*) and chum salmon (*O. keta* (Walbaum, 1792)). In the Pacific Ocean *L. salmonis* has also been reported on non-salmonids species such as three-spine stickleback *(Gasterosteus aculeatus* Linnaeus, 1758) (40).

1.3 Life cycle, biology and interaction with host

The life cycle of the salmon louse consists of planktonic and parasitic stages and comprises a total of eight stages where each stage is separated by moult (41, 42). The planktonic phase consists of two nauplii (I/II) stages, and the free living copepodid stage, whereas the parasitic phase consists of parasitic copepodids, two chalimus (I/II), two preadults (I/II) and one adult stage **(Fig. 1)** (42, 43). Development of *L. salmonis* from eggs to the adults is influenced by temperature, and it takes approximately 32 days for males and 51 days for females at a water temperature of 10°C (44-46). The life cycle starts with the hatching of eggs from paired egg-strings carried by sexually mature adult females to produce free-swimming, non-parasitic nauplius I. Nauplii I larvae hatch into water columns, moult into nauplius II and further into infective copepodids. Both naupliar stages and the free-living copepodite are non-feeding, and their development and survival depend on the maternally deposited lipid and protein reserves within their yolk (47-50). They are passively transported with the water currents and can disperse over long distances, up to 100 km or more (51-53). Planktonic stages are positively phototactic and also have limited vertical swimming, enable to position them in the upper layers of water during the day and sink deep in the water at night, which may increase the probability of these larvae to interact with the host (54, 55). Planktonic stages exhibit positive

rheotaxis behaviour which enables them to avoid salinities below 30 ppt in case of nauplii, however, copepodids can be found in salinity between 16 to 20 ppt and also use the host and non-host semiochemicals in host selection (56-58). Once free living copepodids find a suitable host, they attach themselves to their host through second antennae, feed on mucus and skin of host and start parasitic life cycle (43, 59). The copepodids moults into the first of two immobile chalimus stages. Chalimus (I/II) attaches to their host via a frontal filament and obtain nutrition eating skin and mucus of the host (47, 60, 61). Chalimus II moult into the first pre-adult stage. Finally, the pre-adult II moults into adult stage. Pre-adult and adult stages are motile and continuously feed on host mucus, skin and underlying tissues and cause skin lesions (62, 63). Female lice are larger than males but develop at a slower rate (46). Once males become sexually mature, they locate and engage in a pre-copula with female louse and deposit spermatophores on the genital complex followed by guarding of the female lice to prevent polyandry (43, 64). But this mate searching, pre-copula and guarding does not fully prevent polyandry and evidence of genetic contributions by many males in single egg batches appears common (65). An adult female louse can inseminate several batches of eggs through already deposited sperms and can produce at least eleven pairs of egg-strings during a reproductive lifespan (66). Each egg-string contains several hundreds of eggs. Fertilisation takes place as the female louse release the egg and attaches the eggs externally to a hook. Embryos start to develop inside the egg-strings and as the embryos start to mature, the colour of egg-strings changes from light to dark. Once the larvae are fully developed, the egg-string membrane ruptures and the next generation of nauplii are released starting a new life cycle.
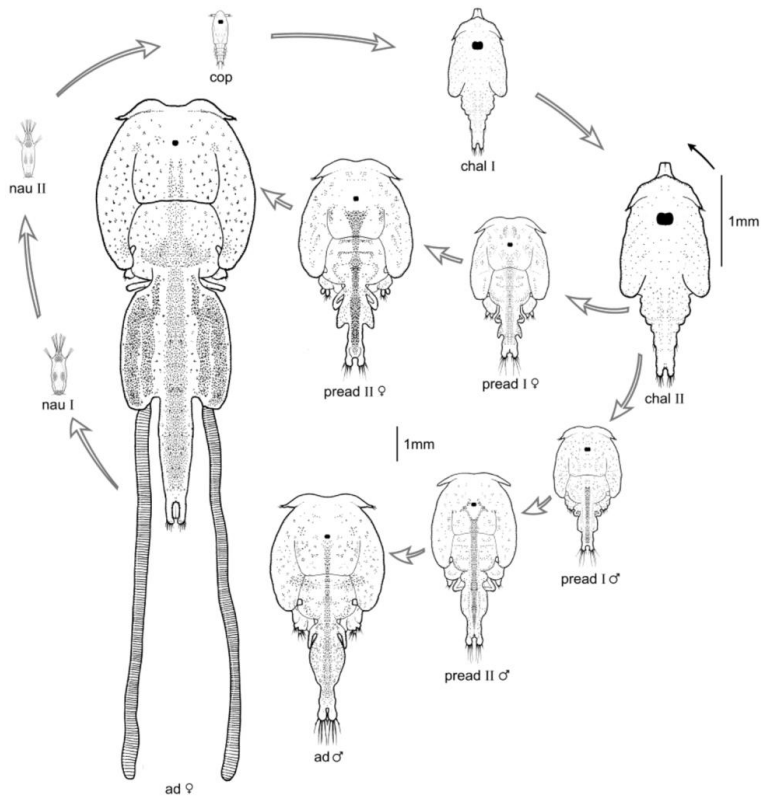
Fig. 1.Life cycle of salmon louse. The salmon louse life cycle has eight developmental stages. These stages consist of two planktonic naupliar stages (nau I and nau II), infectious copepodid (cop), two immobile chalimus stages (chal I and II), two mobile preadult stages (pread I and II) and finally adult stage (ad). The figure is taken from "SLRC *Lepeophtheirus salmonis* life cycle" by Sea Lice Research Centre which is licensed under a Creative Commons AttributionShareAlike 4.0 (International License). Scale bar = 1mm

1.4 Brief description of the lice anatomy

1.4.1 The cuticle and sub-cuticular tissue

A cuticle is a multifunctional rigid coat which covers the body of animals and makes a barrier between the animal and its external environment. Like other arthropods, salmon louse also contains cuticle which protects the animal from different pathogens, maintain body morphology, provide support to the appendages and internal tissues, serves as a site for the osmotic and respiratory exchanges and most likely also protect the animal from different pesticides (67). The cuticle is a rigid structure, and to grow, the animals must molt (ecdysis). Moulting is a complex process controlled by hormones where the new cuticle is produced underneath the old one with a large size so that animal can stretch and increase in length. Production of new cuticle takes place at the same time as the degradation of the old cuticle (68). Old cuticle detaches from the underlying epidermal layers, and a gap is generated, which is filled with moulting fluid consisting of different enzymes such as proteases, peptidases, chitinases and glucosidases (68, 69). This fluid degrades the old cuticle and release free amino acids. These amino acids are absorbed and reused in building proteins along with chitin for the synthesis of the new cuticle. The new cuticle is initially convoluted and can be expanded once the old cuticle has been shed. The new cuticle undergoes sclerotinization to harden the new cuticle.

The sub-cuticular tissue (Fig. 2) is a type of tissue which is distributed throughout the louse under the cuticle and consist of cells of variable shape, packed in an irregular pattern along with muscles and different glandular structures (70-72) and most recently renamed as sub-epidermal tissue (73). The tissue is believed to have a function similar to the liver or fat body and is the site of vitellogenin production (48, 71). Moreover, gene expression associated with fatty acid metabolism and lipid metabolism/transport has also been reported in this tissue (49, 74).
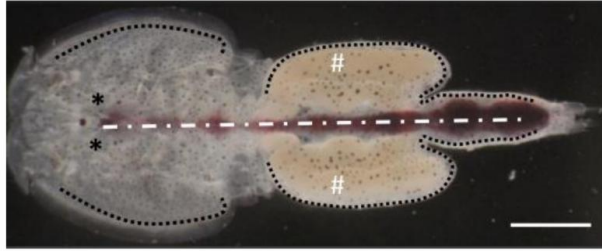
Fig. 2. Dorsal view of a sexually mature female louse. The black dotted line shows the area where sub-cuticular tissue is situated. A white straight dash-dotted line shows the position of the gut filled with blood. Asterisks (*) represent the positions of the ovaries and hashtags (#) represents the positions of the mature vitellogenic oocytes. Scale bar = 1mm

1.4.2 Gut

The gut (Fig. 2) of salmon louse is a tubular structure, running from the anterior part of the cephalothorax to the abdomen, which is composed of cuticle covered foregut, hindgut and long undifferentiated midgut (75, 76). Peristaltic movements occur continuously in the alimentary canal which moves the gut content forward and backwards. The midgut is the largest part of the intestine, and presence of three different types of cells has been identified in the epithelium of midgut (75). The type 1 cells contain large amounts of zymogen granules which suggest role of these cells in the enzyme production similar to hepatopancreatic F-cells of decapods (75, 77). The type II cells resemble to type I cells due to the presence of zymogen granules but different ultrastructure suggest that these cells perform absorptive function (75). Cells type III has some similarity with B-cells of decapods which suggest that these cells perform functions in the digestion and absorption.

1.4.3 Reproductive Organs

The ovaries of female lice are paired organs located on each side of the gut, anterior in the cephalothorax (Fig. 2). In adult female lice, ovaries continuously produce oocytes that are transported through the oviducts to the genital segment (78), where maturation of oocytes takes place. Adult female lice store large amounts of proteins (48, 71) and lipids (50) (Fig. 3) in the oocytes. Maternally produced lipids stored in oocytes during vitellogenesis and are used as a main source of energy during embryonic and larval development. A method to stain the lipids in louse was developed during this thesis work and results show the presence of both neutral (TAG) and phospholipids in the egg-strings and embryos (Fig. 3). Similarly, a previous study also shows that egg-string of adult female lice contain TAGs as the main neutral lipids whereas phosphatidylcholine and phosphatidylethanolamine as the major polar lipids (50). In salmon louse, the maturation of eggs inside eggstrings take around ten days at 10 °C, and it depends on the temperature (71). Like the ovaries, testes in the male are also paired organs and found on each side of the coalesced eyes in the cephalothorax region. Sperm produced in the testes are transported through the *vas deferens*, leading from the anterior part of the testis to the spermatophore sac found in the genital complex. The spermatophore sacs are located slightly posterior to the genital complex with an opening to the exterior through gonophores (78). A pair of cement glands can also be seen in the genital segment with the opening into the spermatophore sacs.
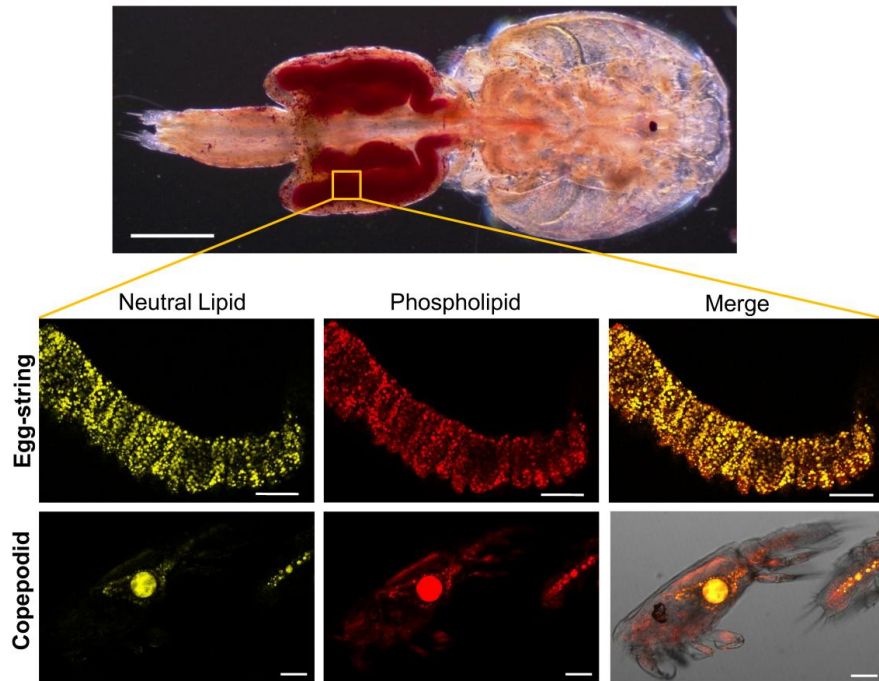
Fig. 3. Staining of lipids in salmon lice. (A) Adult female louse. Lipid reserves in egg-strings stained with Oil red O. (B) Egg-strings were removed from the genital segment and stained with Nile red staining for the detection of neutral lipids and phospholipids. (C) Localization of lipid droplets inside larvae stained with Nile Red staining. Scale bars A = 1mm, B = 250µm, C = 100µm

1.5 Lipid Transport

Lipids are the main source of metabolic energy. Lipids are also essential structural component of the cell membrane and other cellular compartments, important biological carrier for fat-soluble vitamins and play important roles in cell signaling. The three main types of lipids are triglycerides, phospholipids and sterols such as cholesterols. Triglycerides and cholesterols are hydrophobic in nature, whereas phospholipids are hydrophilic in one end. Animals obtain lipids through two different sources, diet or internal production by de novo synthesis pathway. In both cases,

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lipids must circulate from site of production or storage to site of utilization to ensure different cellular processes. The hydrophobic lipids like triglycerides and cholesterols cannot pass through the blood or extracellular fluids of animals and use special lipid transport vehicles like lipoproteins.

1.5.1 Lipoproteins and their compositions in different organisms

Lipoproteins are lipid-protein complexes which consist of a hydrophobic core of neutral lipids, surrounded by a single layer of phospholipids, unesterified cholesterol and a protein component, the apolipoproteins (Fig. 4). This non-covalent assembly of lipids and protein act as a vehicle for the transport of lipids between different tissues of animals.
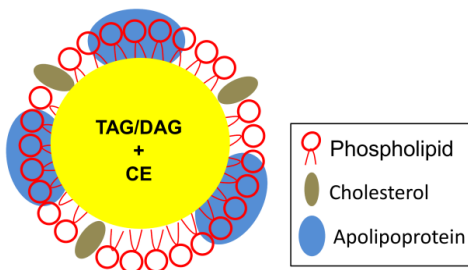
Fig. 4. Structure of a lipoprotein. Lipoproteins are composed of a layer of phospholipids with cholesterol and apolipoproteins and a core containing different classes of lipids. Abbreviation: Triglyceride (TAG), diglyceride (DAG), cholesterol ester (CE).

Lipoproteins can be divided into different classes (Fig. 5) depending on their size and density, lipid composition and the presence of apolipoproteins. For example in vertebrates, the transport of lipids is taken care of by chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) (79). Chylomicrons and VLDL are two major triglycerides (TAG) carrier lipoproteins found in vertebrates and supply TAGs

to different tissues of animals. Chylomicrons are produced in the intestine and transport dietary based TAGs throughout the animal body. The TAGs are released from the chylomicrons by the action of lipoprotein lipase (LPL) and convert chylomicrons into chylomicron remnants. VLDL is produced in the liver and transport endogenous TAGs from liver to different tissues. LDL is involved in the transport of cholesterol whereas; HDL plays an important function in the reverse cholesterol transport system. High amounts of cholesterol are removed from the peripheral tissues and transported back to liver by the action of HDLs (80).
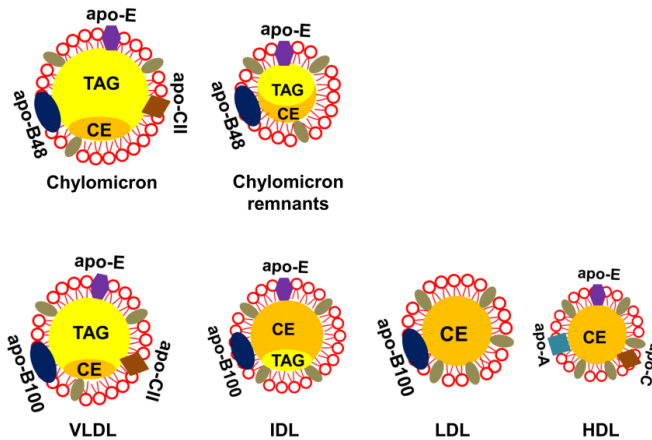
Fig. 5. Different classes of lipoproteins found in vertebrates. Lipoproteins differ in diameter, density, protein and lipid compositions. Chylomicrons, VLDL (very low density lipoprotein) and IDL (intermediate density lipoprotein) are enriched in triglyceride, while the LDL (low density lipoprotein) and HDL (high low density lipoprotein) are enriched in cholesterol. Density of lipoproteins increases as triglycerides (TAGs) percentage reduces and thus, diameter of lipoproteins is inversely proportional to the density. Among all lipoproteins, chylomicron has the lowest density and then density gradually increases from VLDL IDL, LDL to HDL.

The protein part of lipoproteins is made up of apolipoproteins. Apolipoproteins provide structural support to the lipoprotein, participates in lipoprotein biogenesis, and act as ligands for lipoprotein receptors and cofactors for enzymes involved in the

metabolism of lipoproteins. All lipoproteins of vertebrates contain apolipoprotein B (apoB) except HDLP which contain another apolipoprotein known as apoA-I (81, 82).

In insects, lipophorin is the main lipid transporting lipoprotein and distribute lipids between different tissues (83-86). In most insect species, diacylglycerol (DAG) is the major lipid class transport by lipophorins rather than TAG along with other lipids such as PL (phospholipids) and free fatty acids (87-91). In addition to the function as neutral lipids and PL carrier, lipophorin can also carry and distribute hydrocarbons and carotenoids from the site of synthesis to utilization (85). The protein component of insect lipophorin consist of apolipophorins II and I (apoLp-II/I). Both apoLp-II and I are produced from a common precursor through post-translational cleavage, where the molecular weight of apoLp-I is 240 kDa and apoLp-II being much smaller with a molecular weight of 75KDa (92).

In crustaceans, high density lipoprotein/β-glucan binding proteins (HDL-BGBPs) and large discoidal lipoproteins (dLPs) are two major lipoproteins involved in the transport of lipids (93-95). Results from mass spectroscopy and sequencing revealed that both dLp and the HDL-BGBP are products of two proteolytic cleavages of a single precursor protein (95). Most lipoproteins of crustaceans belong to high density lipoproteins (93, 96).

ApoB found in mammals, insects apoLp-II/I and dLPs of crustaceans all belong to the large lipid transfer protein (LLTP) superfamily. However, HDL-BGBP of crustacean does not belong to LLTP superfamily. In animals, LLTP protein superfamily has important functions in lipid transport, development, immunity, ageing and life span regulation (97-104). The other members of this protein family are vitellogenins and large subunit of microsomal triglyceride transfer protein (MTP). Vitellogenins are the most abundant yolk proteins found in oviparous animals, and are involved in the transport of nutrients to developing oocytes, including lipids. Most recent phylogenetic analysis of vitellogeneins in crustaceans show that they are closer to the

apoB of vertebrates and insects apolipophorin II/I and termed apocrustaceins (105). In salmon lice, two vitellogenins genes (*LsVit1* and *Lsvit2*) has been identified (71). MTP is a lipid transfer protein which is found in both vertebrates and invertebrates and essential for the assembly and secretion of apolipoproteins and vitellogenin (106-111).

1.5.2 Lipoprotein Receptors

Lipoproteins deliver lipids to the target cells through two different mechanisms: via endocytosis of complete lipoproteins by members of the low density lipoprotein (LDL) receptor (LDLR) family (Fig. 6) or through the action of lipoprotein lipases (112-115). This thesis will focus on the tissue uptake of lipids in lice through receptor-mediated endocytosis. During the endocytic process; cell surface receptor recognize lipoproteins and facilitate entry of lipoproteins into the cells where lipids are released by the process of hydrolysis and receptor recycles back to the cell surface for a new round of uptake (112-114). During the non-endocytic process, lipoproteins supply lipids to the cells by the action of lipoprotein lipases found on the surface of the cells.

Members of LDLR family (Fig. 6) play a major role in the metabolism of lipoproteins, receptor-mediated endocytosis, and other biological functions (116). In mammals, the LDL receptor binds to cholesterol-rich LDL to regulate cholesterol homeostasis, and mutations in this receptor lead to familial hypercholesterolemia (117-119). Other members of this superfamily include VLDL, Vg (vitellogenin) and Lp (lipophorin) receptors. VLDL receptor takes parts in VLDL-triglyceride metabolism and other functions such as cell proliferation, migration and differentiation (120). In oviparous animals, Vg and VLDL receptors have an important role in oocytes maturation because they transport Vg and VLDL into oocytes (121-123). The uptake of lipophorin in different tissues of insects, including developing oocytes is carried out by LpR (124-131). Recently, lipophorin receptors

have been identified in crustacean shrimp (*Pandalopsis japonica*) (132). LDLR members have five structural domains: A ligand binding domain (LBD), an epidermal growth factor (EGF) precursor domain, an *O*-linked sugar domain (OLSD), a transmembrane domain and a cytoplasmic domain (133). The LBD of LDLR family consists of ligand binding repeats and each repeat contains six cysteine residues. The LBD is essential for the ligand-receptor interaction (134). The second domain of LDLR family is called EGF precursor domain which is involved in the acid-dependent dissociation of ligands. The EGF precursor domain also contain three (A-C) EGF-precursor repeats and five F/YWXD tetra-peptide motifs important for the formation of β–propeller structure (135). The OLSD of LDLR family has unknown function and transmembrane domain anchors the receptor in the plasma membrane (113). The cytoplasmic domain is required for the clathrin-mediated internalization of receptor-ligand complex.
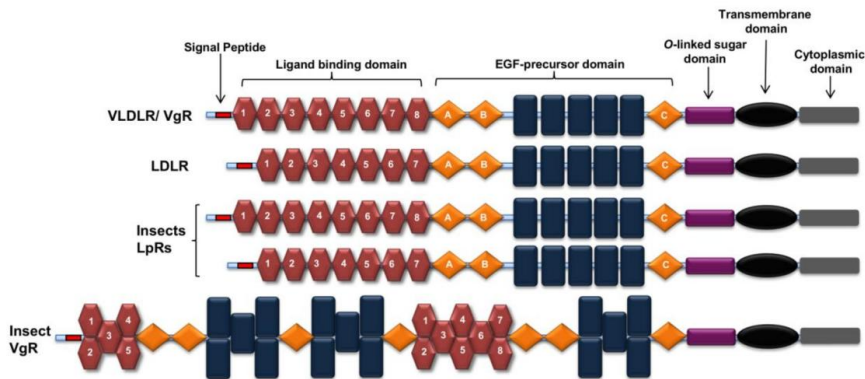
Fig. 6. Schematic comparison of members of low density lipoprotein receptor (LDLR) family in different organisms. All members consist of five identical structural domains. Vertebrates VLDLR/VgR contain eight ligand binding repeats in ligand binding domain (LBD). LDLR of vertebrates has seven repeats in LBD. Insects have two different LpRs based on the numbers of Ligand binding repeats. One type of LpR has seven whereas other has eights ligand binding repeats in LBD. Insect has longer VgR compared to vertebrates VgR due to the presence of two two LBD and EGF-precursor domains where first LBD contain six repeats and second LBD has eight ligand binding repeats.

The mechanism of lipid transport has been studied extensively in vertebrates and insects. In crustaceans, several lipoproteins has been found, however, it is not fully clear how they deliver lipids to different tissues of these animals. This is also the case in salmon louse where lipid transport system has not yet been studied in detail.

1.5.3 Brief description of lipid transport system in vertebrates

In vertebrates, different types of lipoproteins carry and distribute lipids through the animal body. For example, mammals have two TAG carry lipoproteins: apoB-48 containing chylomicrons produce in the intestine and deliver exogenous TAGs along with cholesterol, whereas apoB-100 containing VLDLs produce in the liver and transport endogenous TAGs and cholesterol (Fig. 7). Both chylomicrons and VLDLs pass through the circulation, hydrolyzed mainly by lipoprotein lipases found on the luminal surface of the endothelial cells and release fatty acids to peripheral tissues (Fig. 7) (136). Once lipids have been released, the chylomicrons are converted into chylomicron remnants whereas VLDL is converted into IDL and further to LDL. Chylomicron remnants and LDL particles become enriched in cholesteryl ester (CE) and are taken up by the liver or peripheral tissues through the LDL receptor (112, 113, 137-139). The LDLR interact with LDL through apoB while VLDL and IDL bind with LDLR via the apoE (140, 141). In this process of lipid delivery to the cells by LDLR, the degradation of apoB along with lipid component take place in the lysosomes while apoE containing remnants are recycled back (142, 143). Furthermore, during conversions of these lipoproteins, HDL act as donor or acceptor for exchangeable apolipoproteins and HDL also recycle the cholesterol back from the peripheral tissues to the liver through a procedure known as reverse cholesterol transport (144-147). Assembly and secretion of chylomicrons from the intestine and VLDL from the liver need large subunit of microsomal triglyceride transfer protein (MTP) (107, 148). MTP does not act as a vehicle for lipid transport and thus restrict to the intracellular compartment of the secretory pathway where it promotes the secretion of lipoproteins.
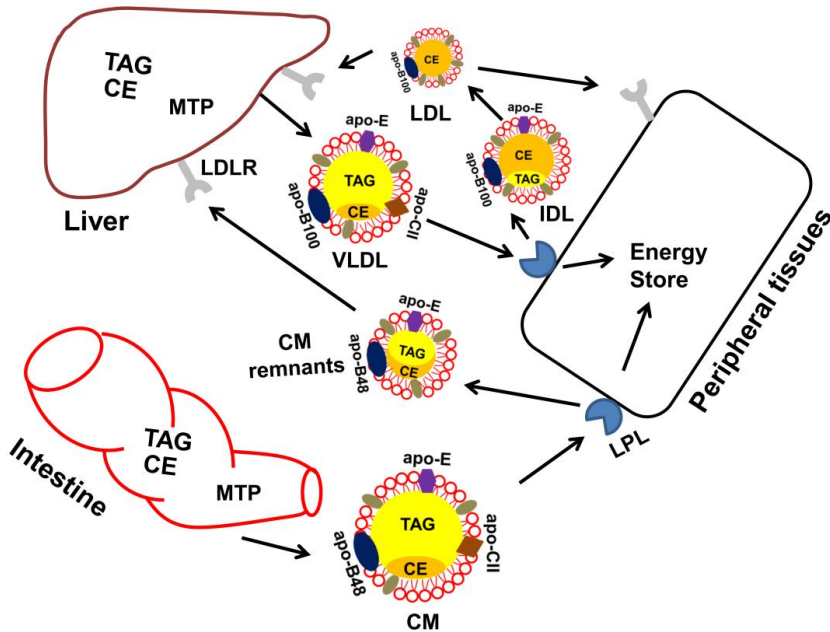
Fig. 7. Overview of the lipid transport system in the mammals. Lipids from the intestine and liver are transported in the extracellular fluids to the peripheral tissues through TAG-enriched lipoproteins. Assembly and secretion of these TAG-enriched lipoproteins from intestine and liver depend upon another protein known as MTP.

1.5.4 Brief description of lipid transport system in insect

Insects like other animals obtain lipids either through external dietary sources or synthesize de novo. The midgut of insects is the main site of lipid digestion (149-152). In insects, the main lipid carrying lipoproteins known as lipophorins produce and secrete from the fat body into the hemolymph to transport lipids from midgut (153, 154) to different tissues such as somatic tissues, ovaries and developing oocytes either for storage or utilization (Fig. 8) (84, 87, 90, 155, 156). Lipophorins can be of high or low densities depending on their lipid and protein compositions (86, 157).

High-density lipophorin (HDLp) is composed of two apolipoproteins: apolipoprotein I (apoLp-I) and apolipoprotein II (apoLp-II) with a lipid content of 30-50% (86, 92, 158). Low-density lipophorin (LDLp) has higher lipid content (up to 62%) with several molecules of a third apolipoprotein, apoLp-III in addition to apoLp-II/I (155, 159, 160). The production of LDLp occurs when large amounts of lipids need to be mobilized (86, 155).

Lipophorin shuttles lipids different tissues with or without the use of receptor mediated mechanisms and also without being accumulated or degraded inside the cells (115, 131, 161, 162). However, accumulation of lipophorin has been observed inside yolk of some insect species (163, 164). In the fat body cells of insects, lipophorin (HDLp) supply lipids through receptor mediated endocytosis (153, 162, 165, 166). On the other hand, the supply of lipids to muscle cells through lipophorin takes place without the lipophorin receptor. During insect flight, muscle cells need large amounts of lipids and LDLp supply a large number of stored lipids from the fat body to muscle cells. For high demands of lipids to muscle cells, adipokinetic hormone (AKH) releases large numbers of DAGs from the fat body cells (167). The released DAGs are then taken up by the extracellular HDLps and convert into LDLp (168). LDLp reaches to the muscle cells, release fatty acids by the action of lipoprotein lipase and converts into HDLp (115, 169-171). The process of lipid uptake from the gut or fat body to lipophorin is not well documented, but existence and activity of another lipoprotein, lipid transfer particle (LTP) may explain this lipidation process (172, 173).
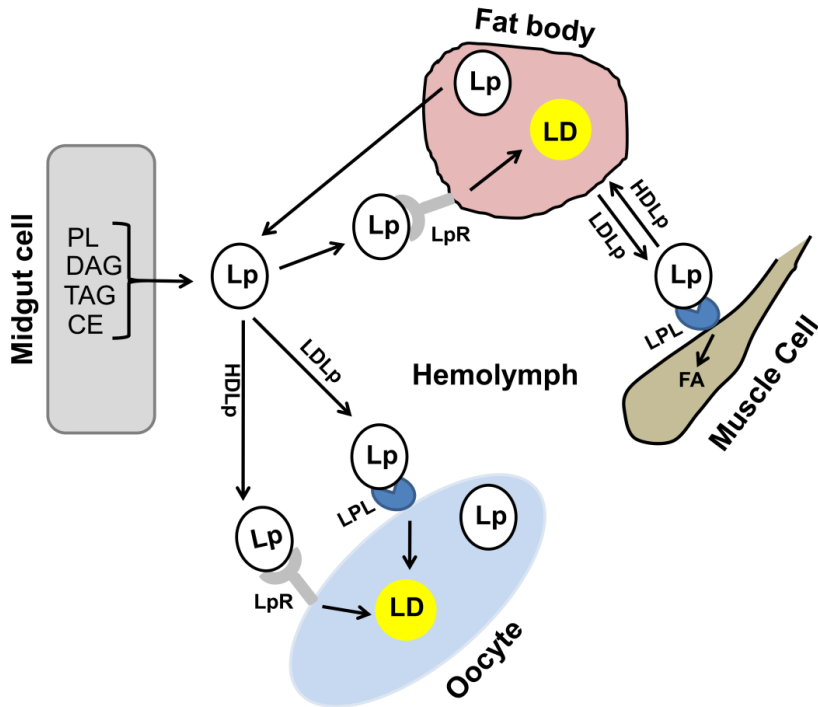
Fig. 8. The lipid transport system in an insect. Lipophorin obtains lipids from the midgut, circulate them through the hemolymph and so that they reach to different organs for the supply of lipids.

During oogenesis, females deposit large amounts of lipids to the yolk as a main source of energy during embryo and larval development. The capacity of the oocytes to synthesize fatty acids is limited, and therefore obtains most of their lipids from maternal gut or fat body cells (86, 174, 175). In insects, vitellogenin facilitates about 8-15% of total supply of maternal lipids to oocytes (86). Thus major supply of maternal lipids to oocytes is facilitated by lipophorin either in the form of HDLp or LDLp depend upon the insect species (86, 175, 176). HDLp supply lipids to developing oocytes through receptor mediated endocytosis and specific receptor of HDLp has been found in several insect species (124, 125, 177-179). Although HDLp has effective role in the supply of lipids to oocytes, but contribution in total supply of oocytes lipids is lesser than LDLp, however, LDLP is not taken up by oocytes

through receptor mediated endocytosis mechanism (175). Lipoprotein lipases (LPL) hydrolyze neutral lipids of both HDLp and LDLp, but LPL has a high affinity towards LDLp, and therefore LDLp is consider more effective than HDLp in the delivery of lipids to oocytes (175, 180). LDLp is not produced in all insect species, and therefore changes in the density of HDLp have been observed. For example, in *A. aegypti* high demand of oocytes lipids is fulfilled through the increased levels of HDLp (179, 181, 182). HDLp which is taken up by oocyte through receptor mediated endocytosis, release lipids inside oocytes and recycle back for another round (179, 183).

2.   Aims of the study

The salmon louse *Lepeophtheirus salmonis* is a marine ectoparasitic copepod of salmonid fish that causes significant economic losses in the salmon farming industry and represent a threat to wild populations of salmonids. Control of salmon lice at the farm level has historically mainly relied on a few available chemical compounds. Recently reduced sensitivity towards most of these chemical compounds has caused serious problems in management of this parasite. Therefore, there is a need to accumulate more information about the biology of this parasite to identify new methods and principles for the control of lice. Lipids are important for the growth and reproduction of lice. Lipids are crucial components of cell membranes, essential energy source and play an important role during cell signalling. Female lice also accumulate large amounts of lipids in oocytes where it is utilized for embryogenesis and larval development. However, the transport of lice lipids to various tissues, particularly oocytes, has not been studied in detail. Lack of accumulation of lipids in oocytes may cause arrest in the process of embryogenesis which ultimately leads to a broken lifecycle. Therefore, identification of key components of lipid metabolism and study transport of lipids in lice especially to oocytes, may provide insight that can be used in future development of means to control this parasite in the aquaculture industry.

Aims of the thesis are:

- ❖ To characterize the function of microsomal triglyceride transfer protein (MTP), as an essential protein for the assembly of lipoprotein and lipid metabolism

- ❖ To identify the apolipoproteins in the salmon louse and characterise function in egg-development through knock-down studies.

❖ To characterise the lipoprotein binding receptor (LsLpR) transcript and protein expression patterns and investigate its role through RNAi in the reproduction of female salmon louse.

3. Abstract of the Papers

3.1 Microsomal triglyceride transfer protein (MTP) in the salmon louse

Microsomal triglyceride transfer protein (MTP) is an endoplasmic reticulum resident protein which plays an important role in lipid metabolism and transport. MTP has been investigated mostly in mammals and in some egg-laying animals, however no studies reported on crustaceans. The aim of this study was to investigate the importance of *L. salmonis* MTP (*LsMTP*) in the reproduction and lipid metabolism of adult female lice.

During reproduction, adult female salmon louse (*Lepeophtheirus salmonis*) produces a large number of eggs with high lipid contents. Lipids deposit into oocytes is used as an source of energy and lipids for embryos and larvae. Like in other oviparous organisms (86, 184, 185), female lice are likely to transport these lipids through the hemolymph using lipoproteins. Current studies suggest that assembly and secretions of lipoproteins are under the influence of intracellular protein known as MTP (186). In salmon lice, we identified three *L. salmonis* MTP (*LsMTP*) transcript variants with a capacity to encode two different protein isoforms. Homology modelling of LsMTP predicted three β-sheets (N, C, and A) and a central helical domain as found in MTPs from other species (107, 187). In adult female lice, differential transcription of LsMTPs was found in the sub-cuticular tissues, the intestine, the ovary, and in the maturing eggs. The function of MTP in the reproduction and lipid metabolism of female lice was studied through RNA interference (RNAi). RNAi-mediated knockdown of LsMTP in lice shows that females produced offsprings with significantly less neutral lipids in their yolk and with only 10-30% survival of copepodids. These results suggest that LsMTP has an important role in the

reproduction and lipid metabolism of adult female *L. salmonis* and can be used as a target to control this important, challenging parasite in the aquaculture industry.

3.2 Characterization of lipophorin receptor at the molecular level in the female salmon lice

The aim of this study was to characterize the lipoprotein binding receptor (LsLpR) in the salmon louse at the molecular level and investigate its role through RNAi in the reproduction of female salmon louse.

In oviparous animals, the sexually mature female transport maternal lipids to developing eggs, where lipoprotein receptor found on the surface of oocytes play a vital role in the uptake of these lipids (129, 131). In female salmon lice, most probably similar mechanism exists; however, it has not been reported before. In this study, a full-length lipoprotein receptor, named as *L. salmonis* lipophorin receptor (LsLpR) was identified from the genome. RACE, cDNA sequencing and RT-PCR analysis predicted the gene extends over 16 exons with transcript length of 4007 nucleotides and predicted ORF of 952 amino acids. A protein structural analysis showed five functional domains, very similar to LpRs from insects and decapods (132, 133, 188). Phylogenetic analysis grouped the LsLpR with LpRs from decapods and insects. Expression analysis at various stages of lice confirmed that the highest expression of *LsLpR* transcripts were found in the copepodids and adult female lice. In adult females, both *LsLpR* transcript and protein were found in the ovary and vitellogenic oocytes. In larvae, *LsLpR* transcripts were found in the neuronal somata of the brain and the intestine. Lipid staining, Oil Red O confirmed the storage of neutral lipids in vitellogenic oocytes and ovaries of adult females and the yolk of larvae. The RNA interference (RNAi) was performed to confirm the function of LsLpR in reproduction and lipid metabolism of L. Salmonis. In larvae knockdown of

LsLpR, transcript levels were decreased by approximately 50% while silencing of LsLpR in female lice produced 72% less off spring when compared to control groups.

3.3 RNAi-mediated silencing of apolipoproteins and their role in the reproduction of female lice

In salmon lice, the role of apolipoproteins in the lipid transport and during reproduction has never been described before. The aim of this study was to identify apolipoproteins and uncover their function in the supply of lipids to oocytes during their development inside the female genital segment.

Apolipoprotein associated lipoproteins facilitate the transport of lipids in animals (79). In oviparous organisms such as insects, the role of apolipoprotein in the transport of lipids to reproductive organs has been described in detail (90, 109, 156). In salmon lice, the female likely uses similar apolipoproteins as found in other oviparous animals for the supply of lipids, particularly to oocytes. In the present study, two apolipoprotein encoding genes (*LsLp1* and *LsLp2*) were identified from the salmon lice genome and their roles in the reproduction of female lice were studied. RT-qPCR analysis at various stages of lice confirmed the expression of *LsLp1* and *LsLp2* throughout all tested stages*,* particularly high expression levels in adult stages*.* In-situ hybridization and RT-PCR confirmed the transcription of *LsLp1* and *LsLp2* in sub-cuticular tissue and intestine of adult female lice. Silencing of *LsLp1* and *LsLp2* in female lice using RNA interference resulted in reduced expression of both transcripts. Knockdown of *LsLp1* in female lice produced significantly less offspring as compared to control lice, whereas knockdown of *LsLp2* in female lice caused no reduction in the number of offspring. These results suggest that *LsLp1* has an important role in female reproduction.

4. General Discussion

In animals, lipids (triglycerides, phospholipids and cholesterol) function as an energy reserve, provide structural support to cell membranes and serve as a precursor for hormones. Lipoproteins are lipid carrying vehicles that mediate transport of dietary and endogenous lipids between different tissues in animals. Lipoproteins are made up of different classes of lipids and protein components known as apolipoproteins. Apolipoproteins belong to the LLTP superfamily and includes apolipophorin of insects, vitellogenins of vertebrates and invertebrates, apolipocrustacein and mammalian apolipoprotein B (apoB) (86, 189-193). Apolipoproteins are not only involved in the development and reproduction of animals (98, 99, 194), but also performs other functions related to immunity, ageing and regulation of lifespan (100, 103, 195, 196). Another member of this LLTP superfamily is known as MTP and according to sequence and structural similarities; its role in the biogenesis of lipoproteins has been described in several vertebrates and invertebrates species (109, 111, 197-199).

Genes involve in the biogenesis of lipoproteins such as MTP (**Paper I**), apolipoproteins (**Paper III**) and ligand to apolipoprotein, lipophorin receptor (**Paper II**) have been well studied in different organisms. However, the information in crustaceans is limited, particularly their importance related to reproduction and embryogenesis. Here, these genes were characterized at the molecular level and their importance in the reproduction of female salmon lice was assessed through RNAi knock down studies.

4.1 Microsonal triglyceride transfer protein (MTP) in the salmon lice

A member of LLTP superfamily, MTP (**Paper I**) involved in lipid metabolism by performing its role in the biosynthesis of lipoproteins was identified in the genome of salmon lice.

4.1.1 Sequence and structural analysis of MTP

Three transcript variants of *MTP* were identified in salmon lice and all contain variable sizes of 5' UTRs (**Fig. 1, Paper 1**). Two of the three transcripts encoded identical proteins of same lengths (819 amino acids) while the third transcript encodes a slightly larger protein (827 amino acids) harbouring a different N-terminal signal peptide. Splice variants of MTP have been reported in other organisms like mice and human (200-203). Mice contain two isoforms, although with similar lipid transfer activity (200, 201). A recent study in human confirmed the presence of two splice variants known as MTP-B and MTP-C (203). In *MTP*, the process of alternate splicing plays important function to regulate cellular levels of *MTP* through the introduction of distinct promoter regions and unique 5' UTRs. Moreover, these promoter regions and unique 5' UTRs contain elements that change the translational efficiency and thus enable the cell to optimize the activity of MTP. The amino acid sequence of salmon louse MTP has relatively little similarity (approximately 22% identity) to MTP orthologues in other species. Similarly, also other organisms have large variation in MTP at the sequence level. For example MTP of invertebrates and vertebrates have less than 25% primary sequence identity, however, the secondary and tertiary structures of the MTP orthologues apparently remain conserved throughout evolution (187). Analysis of predicted secondary and tertiary structures shows that MTP of the salmon louse has similar domain composition (N-terminal β-sheet, central helical domain, C and A β-sheets) to what have been predicted in MTPs from other species (**Fig. 2 and Supplementary Fig. 1, Paper I**).

4.1.2 Role of MTP in the biosynthesis of lipoproteins

MTP orthologues have been found in many organisms; particularly in vertebrates (109, 111, 199, 204-207) and their role in the assembly and secretion of lipoproteins have been acknowledged. In mammals, apoB containing lipoproteins are produced in the intestine (chylomicrons) and liver (VLDL) where MTP control synthesis and secretion of these lipoproteins.

In oviparous animals, the role of MTP has been confirmed in the production of vitellogenins and lipophorins. In *Xenopus laevis,* MTP is involved in the biogenesis of vitellogenin (197). In *Drosophila*, MTP is essential for the lipidation of the fat body originating lipoprotein called lipophorin (109) which transport phospholipids as a major lipid class rather than triglycerides (199, 208). The lipidation of lipophorin in *Drosophila* occurs in two steps. Initially, lipophorin is produced and secreted from the fat body as a phospholipid-rich particle and its lipidation is dependent of MTP whereas, in the second step phospholipid-rich lipophorin are recruited to the gut and further loaded with sterols and diacylglycerols in the presence of another lipid carrying particle known as large lipid transfer particle (109). Defecation suppressor of Clk (DSC-4) is a homolog of MTP found in the intestine of nematode, *Caenorhabditis elegans (C. elegans)* (111). In worms, the intestine is not only a digestive organ but also act as a secretory organ for yolk proteins, vitellogenins. In *C. elegans*, mutations and RNAi of *dsc-4* suppress the germline delay and egg-laying but no change has been observed in the rate of postembryonic development.

In salmon lice, MTP transcripts were found in sub-cuticular tissue, intestine, oocytes and ovaries (**Fig. 3, Paper I**). Sub-cuticular tissue of salmon lice is functionally similar to the liver (74) and previous studies showed that production of yolk precursors such as vitellogenins and yolk associated protein (LsYAP) occur in this

tissue (48, 71). Recently we have shown that two apolipoproteins (**Fig.3, Paper 3**) in salmon lice are also transcribed in the sub-cuticular tissue and in the intestine. Production of MTP in the apolipoprotein producing tissues has been described previously in mammals and oviparous animals. In salmon lice presence of apolipoproteins and MTP transcripts in sub-cuticular tissue and intestine may suggest that apolipoproteins based lipid transport system exist in these tissues and produces lipoproteins for delivery of lipids to various tissues in the lice.

4.1.3 Knockdown of *MTP* reduce egg production and larval survival

Salmon louse obtains all nutrients by feeding on its host. In salmon louse, the main site of lipid storage has been observed in the developing eggs of female **(Fig. 3B)** and yolk of larvae **(Fig. 3C).** Previous studies in salmon lice showed that the main lipids in eggs are triacylglycerol (TAG) and cholesterol followed by polar lipids such as phosphatidylcholine and phosphatidylethanolamine (209). Lipids inside the developing eggs of lice are deposited during the process of vitellogenesis. In salmon lice, delivery of lipids from intestine to developing oocytes is not documented, but like other animals it is believed that lipoproteins are involved in the transport of lipids from intestine to other tissues including eggs of lice. In intestine of mammals, lipidation and secretion of chylomicrons depend upon MTP.

Several studies confirm the role of MTP in the lipoprotein biogenesis; additionally, a few studies have demonstrated its essential function in the transport of lipids to developing embryos. For example, homozygous knockout of the *MTP* gene in mice is lethal to the embryo (210). In MTP-knockout mice, embryonic lethality is caused by the lack of lipoproteins in the yolk sac (210) because of loss of lipids supply to developing embryos. In *C. elegans,* disruption of *dsc-4* through RNAi or mutation reduces germline delay and egg-laying (111). Here in salmon lice, MTP transcript was knocked down in both preadult and adult female lice and effects were studied on

the import of lipids to the developing oocytes and further in embryogenesis and larval development. Knockdown of *MTP* in preadult female resulted in egg-strings of shorter length (**Paper 1, supplementary Fig. 2**) and 90% less hatched embryos as surviving embryos compared to female from control group. Results of this experiment confirmed that MTP does play a critical role in embryogenesis egg production, most probably by supplying lipids, the main energy source during embryogenesis and larval development. The second RNAi experiment, which was carried out in newly molted adult female lice confirmed that knock down of *MTP* results in the production of larvae with drastically lower levels of lipids (**Paper 1, Fig. 6**) compared to animals in the control group. Here, knockdown studies of MTP confirm its importance in the female salmon lice reproduction and lipid metabolism, however, the role of MTP in the biogenesis of lipoproteins was not studied. For this purpose, further information on types of lipoproteins, lipid composition and associated apolipoproteins in the salmon lice is needed.

4.2 Apolipoproteins of salmon lice

Apolipoproteins bind to lipids to form lipoproteins and are involved in the transport of lipids through circulation to various tissues of animals. In salmon lice aplolipoproteins were identified and their role in the reproduction of female lice was studied.

4.2.1 Sequence and domain organization of apolipoproteins

The domain analysis (**Fig. 8A Paper III**) of apolipoproteins confirms the presence of N-terminal LpD_N domain (SM00638), DUF1943 (SM001169), DUF1081 (pfam06448). In addition to these described domains, many members of LLTP superfamily does contain a single Von Willebrand domain (vWD) which is found near the C terminus. Here in salmon lice two cDNA sequences (**Fig. 9B**) encoding apolipoprotein 1 (*LsLp1*) and apolipoprotein 2 (*LsLp2*) were identified based on

sequence and domains structural similarities. The genomic organization revealed that *LsLp1* gene has 11 exons and span nearly 14 kbp, whereas *LsLp2* gene is composed of 7 exons and span nearly 12 kbp (**Fig. 9B**). Further analysis revealed that both *LsLp1* and *LsLp2* reside on the same super contig of the *L. salmonis* genome with a distance between them of 16 kb. The amino acid sequences of both salmon lice apolipoproteins have 56 to 61% similarities with apolipoproteins from other species. Domain organization revealed that LsLp1 contain all domains except the C-terminal vW domain whereas LsLp2 does contain only vW domain.
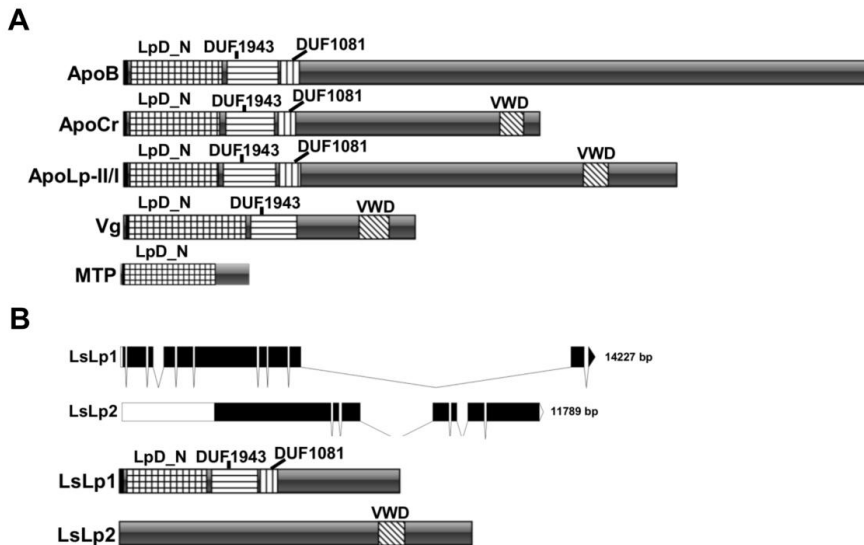
Fig. 9. Members of LLTP superfamily and organization of two apolipoproteins of L. salmonis. Domain organization of apolipoproteins, vitellogenin and MTP. (B) Exon-intron organization of *LsLp1* and *LsLp2*. *LsLp1* consists of 11 exons, whereas, *LsLp2* has seven exons and six introns. Boxes represent the exons and protein-coding region of each gene is shown as black. The white box in the predicted *LsLp2* transcript represents the 5'-UTR. The domains from the *L. salmonis* apolipoproteins were predicted by the SMART algorithm and CD database, and further compared with other members of LLTP superfamily.

The presence of single vW domain in the LsLp2 and three domains (LpD_N domain, DUF1943 and DUF1081) in LsLp1 was suggesting perhaps both proteins are translated from the same single gene. Multiple RACE experiments in combination with PCR attempts were carried out to combine both *LsLp1* and *LsLp2* transcripts. However, initial RACE and PCR experiments did not provide any clue which supports that LsLp1 and LsLp2 encoded by one gene. Moreover, expression levels of both *LsLp1* and *LsLp2* were evaluated in different developmental stages of lice by using qRT-PCR analysis. Results showed that *LsLp1* has relatively higher expression in all the developmental stages of lice as compared to *LsLp2* (**Fig. 2, Paper III**). However, localization of both *LsLp1* and *LsLp2* transcripts were found in sub-cuticular tissue and intestine (**Fig. 3, Paper III**) of female lice. The functions of both transcripts were evaluated through knock down of both cDNA in RNAi experiments and results showed that *LsLp1* knocked down animals have short egg-strings and produce lower numbers of copepodids as compared to control animals. While knock downed of *LsLp2* in female louse fail to provide any abnormal phenotype such as short egg-strings or reduce number of offspring compared with control female lice. On the base of results collected from expression and functional studies concluded that *LsLp1* and *LsLp2* are two independent transcripts.

4.2.2 Expression of apolipoproteins in sub-cuticular tissue and intestine of salmon lice

Lipoproteins distribute lipids from the site of synthesis or storage to different tissues of animals. As mentioned previously, lipoproteins are complexes of different classes of lipids and proteins called apolipoproteins. Apolipoproteins not only direct these lipoproteins, but also provide essential structural support, which means that in the absence of these apoproteins, the lipidation of these particles does not take place. Relevant examples like mammalian liver/ intestine originated apoB (211) and insects fat body based apoLp-II/apoLp-I are involved in the secretion and transport of lipoproteins between different tissues (85, 212, 213). In crustaceans, two major lipoproteins, high density lipoprotein/β-glucan binding proteins (HDL-BGBPs) and

large discoidal lipoproteins (dLPs) have been found. The expression of HDL-BGBPs has been found in hepatopancreas, intestine and muscles (214, 215). In case of dLP, tissue specific expression has not been studied yet, however it is suggested that dLP would has the same expression sites since dLP is derived from the same precursor together with HDL-BGBP (93). In salmon lice, the expression of both *LsLp1* and *LsLp2* was found in sub-cuticular tissue and intestine of adult female **(Paper III, Figure 3A-3C)**. Antibodies against LsLp1 and LsLp2 were not available and therefore it is unclear if proteins are also expressed in the same tissues of female lice. However, mRNA expression studies suggested that *LsLp1* and *LsLp2* both produced in intestine and sub-cuticular tissues and perhaps secrete into the hemolymph for the supply of different classes of lipids to various tissues of adult female lice.

4.2.3 Knock down of *LsLp1* reduces reproductive capacity of female lice

Some functional studies in insects have suggested that lipophorin have a role in the lipid metabolism and embryo development. In the tsetse fly, knockdown of apoII/I causes low levels of lipids in the hemolymph, delay in the development of oocytes and extended larval gestation (216). In *Drosophila*, the blockage of *Lp* (lipid transporter) reduces levels of lipids in the hemolymph whereas, levels of lipids increases in the midgut (109). In *Anopheles*, silencing of the apolipophorin precursor known as retinoid and fatty-acid binding glycoprotein (RFABG) showed its involvement in egg development and survival of motile zygotes (ookinetes) (217). Several lipoproteins have been found in crustaceans. However, unlike insects and vertebrates, functional studies of lipoproteins in crustaceans have not been available in detail. Similarly in salmon lice, functional studies of lipoproteins have not been documented before. Here we identified two apolipoprotein homologs in salmon lice and described their role in the reproduction of the female lice through RNAi. Two genes, *LsLp1* and *LsLp2* were identified as apolipoproteins. RNAi based knockdown confirmed that females injected with *LsLp1* dsRNA alone or in combination with

LsLp2, produce short egg-strings compared to control groups (**Paper 3, Figure 7**). Similarly, numbers of copepodids produced from eggstrings of female injected with *LsLp1* dsRNA were also significantly reduced (**Paper 3, Figure 7**). These results suggest that adult female lice need *LsLp1* for egg production. Furthermore during RNAi experiments it has also been observed that complete stop in the development of eggs or embryos were not achieved. Therefore, it is important to conduct more functional studies in female lice to check the protein levels of LsLp1during it's silencing. It is possible that during the period of *LsLp1* knock downed, the levels of mRNA reduced but protein levels were not reduce to that level where complete stop in the development of eggs or embryos can be achieved. Moreover in salmon lice, it is possible that other lipoproteins may also be involved in the transport of lipids along with LsLp1. In insects, both lipophorin and vitellogenin supply maternal lipids to the insect eggs (86). In female salmon lice, viellogenins and vitellogenins like proteins (71) have been found and it is possibility that they may be involved in the supply of maternal lipids to oocytes for successful embryogenesis and larval development.

4.3 Lipoprotein receptor (LpR) in salmon lice

Lipoprotein receptors (**Fig. 6**) are well characterized evolutionarily ancient cell surface proteins involved in the recognition and internalization of lipoproteins for the supply of lipids to different tissues of animals. In mammals, the LDL receptor is required for cholesterol-rich LDL endocytosis (112-114). In egg-laying vertebrates such as chicken; VLDL/Vg receptor is involved in the uptake of VLDL/Vg during oocytes development and thus recognized as essential for female reproduction. (218). In insects, lipophorin is the main lipid transfer particle in the hemolymp, and its internalization is facilitated by LpR through receptor-mediated endocytosis (125-131, 219).

4.3.1 Sequence and structural analysis of LpR

In salmon lice, a single transcript of *LpR* was identified based on sequence and structural identities (**Fig. 1, Paper II**). The LpR of salmon lice (LsLpR) shared 46% identity with LpR of shrimp, 48–53% identity with LpR of insects, and slightly lower levels of similarity with VLDLRs of oviparous vertebrates and LDLRs of mammals. A typical LpR consists of five functional domains, ligand binding domain, epidermal growth factor precursor homologous domain, O-linked sugar domain, a transmembrane domain and a cytoplasmic domain. In salmon lice, domain organization analysis confirmed the presence of all five functional domains (**Fig. 1, Paper II**). Evolutionary relationship analysis with other lipoprotein receptors showed that *LpR* of salmon lice is closely related to decapods and insect *LpR*s (**Fig. 2, Paper II**). These results suggest that LpR of salmon lice has the same function in the lipid metabolism as found in other organisms and therefore considered as a critical component in the lice during reproduction and involved in the accumulation of lipids inside oocytes during their development inside the genital segment.

4.3.2 Expression and functional analysis of *LsLpR* in larvae and female lice

LpR has been found in different species of insects and its function in the uptake and accumulation of lipids in oocytes inside ovaries (86, 130, 131) is well established. Other than ovaries, LpR has also been found in different somatic tissues of adults and in larvae of insects (124, 128, 131, 219-221). In salmon lice, one gene similar to *LpR* of insects and *LDLR* of vertebrates was found. Gene expression analysis in different stages of salmon lice showed highest *LsLpR* transcript levels in larvae and in the adult female relative to chalimus I (**Paper 2, Figure 3**). Furthermore, *in situ* hybridization confirmed the presence of *LpR* transcripts in the ovary and vitellogenized eggs of adult female, whereas in larvae, the *LpR* was found in the neuronal somata of brain and in the intestine (**Paper 2, Figure 5A-5E**). At protein level, the existence of

LsLpR was detected in the ovaries and maturing oocytes of female lice (**Paper 2, Figure 5F and 5G**). The expression of *LsLpR* in ovaries and vitellogenized eggs of adult female and in somatic tissues of larvae of salmon louse suggest its important function in the recognition and internalization of lipid carrying particles like lipoproteins.

To evaluate the function of this receptor in salmon lice, RNAi experiments were conducted in larvae (**Paper 2, Figure 6**) and in female lice (**Paper 2, Figure 7**). RNAi experiments in larvae showed no visible defects in development and in the swimming performance of *LsLpR* dsRNA treated animals. Moreover, *LsLpR* dsRNA treated larvae consumed same levels of yolk lipids as found in larvae of control groups (**Paper 2, Figure 6B-D**). Larvae of salmon lice are lecithotrophic (43) and depend upon maternal yolk sac reserves as a nutrition during their development until they find suitable host. The presence of *LsLpR* in intestine of larvae suggested that receptor provides binding site for lipoprotein which obtain lipids from the yolk and circulate them to different tissue of larvae for development. In larvae of salmon lice, it was speculated that RNAi of *LsLpR* may stop the development of larvae due to complete or partial loss of lipids mobilization from the yolk to different tissues. However, this were not the results from RNAi experiments in larvae, maybe because the degree of knock downed achieved for *LsLpR* were not sufficient to reduce or disrupt the lipid transport from the yolk to other tissues of larvae. Secondly, even though the mRNA levels were reduced, it is possible that protein levels were still at about the same levels as were before the RNAi experiment. Similar results were found in the knock down of tsetse fly *LpR*, where receptor was significantly knocked down without any changes in the lipid levels of hemolymph.

In female lice, RNAi experiments were conducted in preadult II stage and experiments were terminated when female lice had become adult and produced second pair or egg-strings. In all RNAi experiments, no significant changes in the transcript levels of *LsLpR* were observed (**Paper 2, Figure 7A**). Moreover, in all

experiments, females produced normal egg-strings and normal development of all offspring to copepodids were also found **(Paper 2, Table 2).** But, in one out of three RNAi experiment the number of copepodids that hatched from the egg-strings of *LsLpR* dsRNA treated female lice was lower than the control group lice. In insects, similar RNAi results were found in *S. ricini* (221)*. Here female pupae of *S. ricini* were injected with *LpR* dsRNA, but did not result in considerable changes in the level of mRNA compared to control animals and ovary development and egg production were found to be normal. In another *LpR* RNAi experiment conducted in *B. germanica*, the levels of Lp were reduced in the ovary. However, no changes in ovarian development or fertility were observed (129). Moreover, the effects of *LpR* RNAi in the fat body of *B. germanica* began to disappear after three days, and levels of *LpR* mRNA and Lp contents began to increase which suggest that *LpR* silencing occurred for a short period only.

Another RNAi experiment was conducted in salmon lice where nauplii were treated with *LsLpR* dsRNA. When nauplii molted into copepodids, some copepodids were collected to check the knock down efficiency of *LsLpR* and remaining copepodids were used to infect the fish and follow them until they reached into adult females (approximately 60 days after infection). The RNAi experiment was terminated once females from control group produced second pair of egg-strings. All females from both *LsLpR* knock downed and control groups were collected and further check the efficiency of RNAi. Results showed that silencing of *LsLpR* was about 60% in copepodids and no significant change in the knock down of *LsLpR* was observed in adult females **(Paper II, Figure 7C)**. Further RNAi experiments in adult females **(Paper II, Figure 7B)** showed that maximum knock down of *LsLpR* was 30% at day 15. Perhaps this knock down efficiency of *LsLpR* was not enough to show abnormal phenotypes in adult female salmon lice. Secondly, protein levels of lipophorin receptor were not tested after all RNAi experiments.

5.   Conclusions and future works

In this present work, lipid transport and metabolism were studied in adult female louse to determine the importance of lipids for development and survival of female louse itself, for egg production and for embryogensis and development of larvae.

Paper I addresses the active role of MTP in reproduction and lipid metabolism in adult female lice. MTP promotes biosynthesis of lipoproteins essential for the transport of apolar lipids to various organs of animals. In salmon lice, MTP was identified for first time and its functions during reproduction of female were studied by using RNAi. Results show that *MTP*-knocked down females produce embryos with fewer lipids in their yolk and 70-90% embryos coudnot developed into copepodids. Results of RNAi experiments in female lice suggested that MTP is essential during reproduction which may play an important role in the lipoprotein-based supply of maternal lipids to developing eggs. MTP is compulsory factor for the production of lipoproteins as studied in different animals. In salmon lice, role of MTP in the biosynthesis of lipoproteins was not investigated. Mostly MTP expressed in tissues where lipoprotein biosynthesis occurred as observed in different animals. In salmon lice presence of *MTP* transcripts in the intestine and sub-cuticular tissues indicate its active involvement in the production of lipoproteins in these tissues. To address its function in the biosynthesis of lipoproteins, further functional studies are needed. Functional studies like silencing of MTP in salmon lice can be used to observe the lipids and lipoproteins levels in the hemolymph and in MTP-expressing tissues to figure out its involement in the biosynthesis of lipoproteins.

In paper III, two apolipoprteins (*LsLp1* and *LsLp2*) of salmon lice were identified and functional studies were carried out in female lice. Since apolipoprteins are essential part of lipoproteins and these lipoproteins circuate lipids throughout body of animals. In adult female lice, the major lipid contents were observed in eggs and therefore it was assumed that knock down of *LsLp1* and *LsLp2* may disrupt complete or partial

supply of maternal lipids to eggs inside egg-strings. Functional studied through RNAi showed that *LsLp1* dsRNA treated females produced short-eggstrings with less numbers of offsprings as compare to females from control groups. These results indicate that *LsLp1* is involved in the development of eggs inside eggstrings. During these functional studies, transcript levels of *LsLp1* or *LsLp2* were estimated but protein levels were not measured. Therefore further functional studies in female lice are suggested to follow the levels of these apolipoproteins in hemolymph and expressing tissues such as subcuticular tissues and intestine. Moreover, levels of lipids should also be measured in hemolymph and in different tissues of female salmon lice during knock down of *LsLp1* or *LsLp2*. More results from functional studies could explain the contribution of these lipoproteins in the supply of lipids to different tissues particularly to eggs of female lice. Moreover, role of vitellogenins and vitellogenin like proteins need to be addressed in the supply of maternal lipids to eggs of female lice. These results could explain the contribution of vitellogenins and vitellogenin like proteins for the accumulation of lipids inside eggs of female lice along with lipoproteins such as LsLp1and LsLp2.

In oviparous animals, eggs obtain most of their lipids through receptor-mediated endocytosis of lipoproteins. In insects, lipophorin receptor is essential for uptake and accumulation of lipids by eggs and other tissues. In paper II, lipophorin receptor (LpR) was identified and its role in the lipid metabolism and reproduction of female lice was characterized for the first time at the molecular level. Presence of *LpR* transcripts and proteins in ovaries and eggs indicate that the receptor may be able to mediate the endocytic uptake of salmon lice lipoproteins. RNAi-mediating knock down of the lipophorin receptor was conducted in female lice. However, effects of RNAi were not sufficient to conclude if receptor is involved in the uptake of lipoproteins. During knock down of *LpR*, it is possible that levels of lipids and lipoproteins increased in hemolymph of salmon lice. Therefore, it is suggested that further functional studied need to be carried out along with quantification of lipid

contents in hemolymph and in other tissues of lice. LpR affinity towards lipoproteins was not investigated and further ligand-binding assays are suggested in cell culture to evaluate the function of this receptor in the salmon lice.

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Microsomal triglyceride transfer protein in the ectoparasitic crustacean salmon louse (*Lepeophtheirus salmonis*)⒮

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Abstract   The salmon louse, *Lepeophtheirus salmonis*, is an endemic ectoparasite on salmonid fish that is challenging for the salmon farming industry and wild fish. Salmon lice produce high numbers of offspring, necessitating sequestration of large amounts of lipids into growing oocytes as a major energy source for larvae, most probably mediated by lipoproteins. The microsomal triglyceride transfer protein (MTP) is essential for the assembly of lipoproteins. Salmon lice have three *L. salmonis* MTP (*LsMTP*) transcript variants encoding two different protein isoforms, which are predicted to contain three β-sheets (N, C, and A) and a central helical domain, similar to MTPs from other species. In adult females, the LsMTPs are differently transcribed in the subcuticular tissues, the intestine, the ovary, and in the mature eggs. RNA interference-mediated knockdown of *LsMTP* in mature females gave offspring with significantly fewer neutral lipids in their yolk and only 10–30% survival. The present study suggests the importance of LsMTP in reproduction and lipid metabolism in adult female *L. salmonis*, a possible metabolic bottleneck that could be exploited for the development of new anti-parasitic treatment methods.—Khan, M. T., S. Dalvin, F. Nilsen, and R. Male. **Microsomal triglyceride transfer protein in the ectoparasitic crustacean salmon louse (*Lepeophtheirus salmonis*). *J. Lipid Res.* 2017. 58: 1613–1623.**

Supplementary key words   lipid transport proteins • lipid transport • gene expression • lipid and lipoprotein metabolism • lipoproteins • RNA interference • Nile Red • Oil Red O • sea lice

The microsomal triglyceride transfer protein (MTP) was first reported as an endoplasmic reticulum resident protein that catalyzes the transfer of neutral lipids between membranes (1). Later it was found that MTP is also essential for the synthesis and secretion of lipoproteins containing apoB (2). MTP belongs to the large lipid transfer protein superfamily and it functions as a transporter of lipids in the assembly of nascent lipoprotein particles within the endoplasmic reticulum (3). This protein family also contains other members with a central role in animal reproduction and lipid circulation, such as vitellogenins, vertebrate's apoB, and insect apolipophorin (apoLp)-II/I (4, 5).

MTP is a heterodimeric protein complex composed of two distinct subunits, a large subunit of, typically, 99 kDa containing a lipid transfer activity and a multifunctional 58 kDa protein disulfide isomerase (PDI) (6, 7). The MTP large subunit, for simplicity named only MTP, is composed of three structural motifs: the N-terminal β-barrel (N-sheet), the central α-helix domain and C-terminal β-sheet (C-sheet), and three functional domains (lipid transfer, membrane-associating, and apoB binding) (8, 9). The N-sheet β-barrel is involved in the recognition of the N terminus of apoB, the central α-helix interacts with both apoB and PDI, and the C-sheet β-sheet has lipid binding as well as transfer properties (8).

In mammals, MTP is essential for the assembly and secretion of apoB-containing lipoprotein, chylomicrons in the intestine, and VLDLs in the liver (10), and thereby facilitates delivery of triglyceride and cholesterol to the peripheral tissues. In humans, homozygous mutations in the *MTP* gene abolish secretion of apoB-lipoproteins and reduce the lipid level in plasma, resulting in abetalipoproteinemia (11). Studies in the mice show that homozygous knockout of the *MTP* gene is lethal to the embryo (12). This phenotype is ascribed to the lack of lipoprotein synthesis and massive accumulation of lipid droplets in the cells of yolk sac endoderm (12), indicating that the yolk sac has lost its ability to produce lipoproteins and deliver lipids to the developing embryo.

Abbreviations: apoLp, apolipophorin; DIG, digoxigenin; dsRNA, double-stranded RNA; ef1α, elongation factor 1α; Lpp, lipophorin; LsMTP, microsomal triglyceride transfer protein from *Lepeophtheirus salmonis*; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; RACE, rapid amplification of cDNA ends; RMSD, root mean square deviation; RNAi, RNA interference; UTR, untranslated region.

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⒮ The online version of this article (available at http://www.jlr.org) contains a supplement.

In oviparous organisms, such as frog (13), *Drosophila* (14, 15), and worms (16), MTP has a similar function in the secretion of lipoproteins as found in mammals. The secretion of frog vitellogenin A-1 is MTP dependent (13), as the secretion is enhanced only when coexpressed with MTP. In *Drosophila melanogaster*, the transport of lipids between organs is carried out by a single apoB-family lipoprotein, lipophorin (Lpp) (14). The lipidation of Lpp occurs in two continuous steps with the help of two distinct lipid transfer proteins, MTP and large lipid transfer particle. Initially, Lpp is released from the fat body as a phospholipid-rich particle through the MTP-dependent mechanism and reaches the gut, where it is loaded with sterols and diacylglycerols via large lipid transfer particle. A homolog of the large subunit of MTP, named defecation suppressor of Clk (DSC-4), was found in the intestine of *Caenorhabditis elegans* (16).

The salmon louse (*Lepeophtheirus salmonis*) is a marine ectoparasitic copepod that infests salmonids in the Northern Hemisphere. The salmon louse feeds on the blood, mucus, and skin of hosts and represents a major health and fish welfare issue that causes large economic losses in the Atlantic salmon (*Salmo salar*) farming industry (17) and also poses a considerable threat to wild salmonids (18). The lifecycle of the salmon louse consists, in total, of eight stages, each separated by a molt (19). The first are two stages of free-living nauplius larva followed by one infective copepodid stage. This is followed by two chalimus stages (where the parasites are firmly attached to the host), two preadult stages (with clear morphological sex difference), and finally the adult stage. Before host attachment, larvae are lecithotrophic, dependent on energy from maternally deposited lipid and protein reserves within the yolk (20, 21). The sexually mature adult female continuously produces eggs carried in two egg-strings. Under laboratory conditions, female salmon lice can survive for at least 455 days and produce more than 11 pairs of egg-strings (22). During egg production, the female louse incorporates massive amounts of yolk proteins (21, 23) and lipids into the growing oocytes. The predominant lipids in the eggs are neutral lipids, triacylglycerol, and cholesterol, followed by polar lipids, such as phosphatidylcholine and phosphatidylethanolamine, but fatty acid composition varies with the composition of the food received by the host salmon (20). The mechanism of lipid accumulation in the growing oocytes has not been described in salmon lice. However, lipids are absorbed in the intestine and are likely to be transported with the hemolymph via lipoproteins, for example, to the oocytes. A highly efficient lipid uptake and transport can be predicted to secure the high production of eggs in salmon lice and dispersal of louse larvae in the environment.

In the present study, we identified a gene encoding MTP from *L. salmonis* (*LsMTP*). We hypothesize that LsMTP may be involved in the lipoprotein-based supply of lipids from the intestine of a female salmon louse to growing oocytes. To this end, *LsMTP* was characterized and three transcript variants were identified. Silencing of the *LsMTP* gene using RNA interference (RNAi) affected production of eggs and

reduced the viability of the developing larvae due to less neutral lipids in their yolk. Our results suggest that LsMTP has a crucial role in the reproduction of female salmon lice.

MATERIALS AND METHODS

Sampling of salmon lice

A laboratory strain of salmon lice, *L. salmonis* (22), was kept on Atlantic salmon (*Salmo salar*) in tanks with a continuous supply of seawater (temperature 10°C and salinity 34.5 ppt). Fish were fed a commercial diet daily. Nauplii I/II and copepodids were obtained from hatching egg-strings in hatching incubators supplied with the same seawater. Chalimus, preadult, and adult stages of lice were sampled from fish. Prior to sampling, fish were anesthetized with a mixture of benzocaine (60 mg/l) and metomidate (5 mg/l) in seawater. All the experiments and maintenance of salmon were carried out according to the Norwegian animal welfare legislation.

For stage-specific quantitative (Q)-PCR, five biological replicates were collected from each stage. The following life stages and number of animals were collected for each replicate. Nauplius I (n = 100), nauplius II (n = 100), planktonic copepodid (n = 100), chalimus I (n = 10), chalimus II (n = 10), preadult I male and female (n = 1), preadult II male and female (n = 1), adult male (n = 1), young adult female and adult female (n = 1). For the starvation experiment, adult female lice were collected from fish and kept in seawater for 1–4 days. All the samples were stored in RNAlater™ (Ambion) and kept overnight at 4°C prior to storing at −20°C for further use.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRI-reagent (Sigma-Aldrich) according to the manufacturer's instructions. The concentration and purity of isolated RNA was confirmed using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The isolated total RNA samples were treated with amplification grade DNaseI (Invitrogen) as per manufacturer's instructions. For Q-PCR, DNase-treated total RNA (250 ng) was used for cDNA synthesis with Affinity Script QPCR cDNA synthesis kit (Stratagene) and diluted 10 times with nuclease-free water prior to storage at −20°C. For PCR, 1 µg total RNA was reverse transcribed using a qScript cDNA SuperMix (Quanta Bioscience).

Genome analysis, PCR, cloning, and sequencing of *LsMTP* gene

LsMTP-coding sequence was identified in the Ensembl database (http://r9ywwtvj.ensemblgenomes.org/Lepeophtheirus_salmonis/Info/Index) and the salmon louse genome database (accession: EMLSAT00000001530) (LiceBase, https://licebase.org/) with homology to known human (NCBI: X91148.1) and *Drosophila* MTP (FlyBase: FBgn0266369). The GenBank accession numbers of the three MTP sequences reported here are: LsMTP-A, MF063064; LsMTP-B, MF063065; and LsMTP-C, MF063066. PCR was carried out using GoTaq Flexi DNA polymerase (Promega) as per the manufacturer's protocol. The 5′ and 3′ rapid amplification of cDNA ends (RACE) was conducted with SMARTer RACE cDNA amplification kit (Clontech) as instructed in the users' manual. The 5′ and 3′ RACE-Ready cDNAs were synthesized from the total RNA of adult females and used for RACE-PCR. Gene-specific primers for 5′ and 3′ RACE are listed in supplemental Table S1. PCR products were cloned into pCR™ 4-TOPO® vector using the TOPO TA cloning kit for sequencing (Life Technologies) followed by transformation into *Escherichia coli* TOP10 cells.

Clones were verified by PCR with M13 forward and reverse primers (supplemental Table S1). PCR products of positive clones were cleaned with ExoSAP-it (Affymetrix) and sequenced using BigDye Terminator v3.1 reagent (Applied Biosystems) at the sequencing facility of the University of Bergen.

In situ hybridization

To confirm the in situ hybridization specificity, two different single stranded digoxigenin (DIG)-labeled RNA probes of 476 bp and 604 bp lengths corresponding to different regions of LsMTP transcripts (Fig. 1) were synthesized separately from cDNA using the DIG RNA labeling kit (Roche). Primers used for the synthesis of sense and antisense RNA probes are listed in supplemental Table S1. The concentration and labeling efficiency of probes was assessed by spectrometry (Nanodrop ND-1000) and with a spot test on nylon membrane, respectively. In situ hybridization was carried out in paraffin-embedded sections of adult female lice, as previously described by Kvamme, Frost, and Nilsen (24) and Dalvin, Nilsen, and Skern-Mauritzen (25) with some modifications. Tissue sections were deparaffinized with Histoclear (National Diagnostic) instead of xylene and proteinase K treatment was done for 13 min. Hybridization of probes (500 ng/100 µl) was carried out at 65°C for 16–20 h. Sections were incubated with anti-DIG-alkaline phosphatase Fab fragments (Roche) and visualized using nitroblue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche). The localization of LsMTP transcripts was detected with antisense probes and sense probes were used as negative controls.

Real-time Q-PCR

Q-PCR was performed on Applied Biosystem 7500 real-time PCR system using PowerUp SYBR Green Master Mix (Applied Biosystem) as per the manufacturer's recommendations. The Primers used in Q-PCR are listed in supplemental Table S1. The salmon louse elongation factor 1α (ef1α) was used as a reference (26). Two-fold serial dilutions (six dilutions) of cDNA were used to create a standard curve for efficiency calculation. As the efficiency of the assay ranged from 95% to 100%, all the assays were carried out simultaneously for LsMTP and ef1α using the same cDNA and master mix along with two negative controls, a non-template control and a no reverse transcriptase control. All the samples were run in duplicate, and Ct (cycle threshold) values were averaged. The final results were analyzed using the $2^{-\Delta\Delta CT}$ method (27). The Q-PCR analysis was performed on lice recovered from two RNAi experiments. Primers used in Q-PCR for the detection of downregulation in the RNAi experiments were designed outside the double-stranded RNA (dsRNA) fragments. For each RNAi experiment, five representative adult females from the control group and LsMTP dsRNA-treated group were analyzed. Animals from the control group were used as a calibrator to calculate relative expression. Relative expression levels of three variants of LsMTP in different developmental stages of salmon lice were also determined by Q-PCR using copepodids as a calibrator. For the starvation experiment, animals (n = 5) were collected on days 0, 1, 2, and 4, and after 2 days of refeeding on the host fish. Relative expression of LsMTP was calculated using day 4 for calibration.

RNAi

dsRNA was prepared according to the Megascript RNAi kit (Ambion). Two different fragments targeting different regions of LsMTP mRNA (Fig. 1) were amplified by PCR from primers with T7 promoter sequence, previously used for synthesis of in situ hybridization probes (supplemental Table S1). A fragment of 850 bp from cod trypsin (CPY185) was used as a control

(23). Respective PCR products were used as templates for the synthesis of sense and antisense RNAs by in vitro transcription using T7 polymerase. For synthesis of dsRNA sense and antisense, RNAs were pooled and incubated at 75°C for 5 min followed by slow cooling to room temperature. The purified dsRNA concentrations were measured with Nanodrop ND 1000 Spectrophotometer, and a final concentration of 600 ng/µl was used for injections.

Two RNAi experiments were conducted separately in female lice. The first knockdown of LsMTP was carried out with dsRNA fragment 1 (LsMTP Fr 1) in newly molted preadult II females. In the second experiment, LsMTP silencing was done with dsRNA fragment 2 (LsMTP Fr 2) in young adult female lice. Both RNAi experiments were performed as described by Dalvin et al. (23). In each experiment, female lice were injected for LsMTP dsRNA and control dsRNA separately. After injection of dsRNA, lice were kept in seawater for 3 h and put back (n = 30–32) on three fish for every dsRNA fragment with equal numbers of dsRNA-treated female and untreated male lice. Both RNAi experiments were terminated when control dsRNA-injected female lice produced second pairs of egg-strings. Female lice were examined for gross morphology and imaged along with egg-strings for further egg-string measurement. Afterwards, egg-strings from females of both experiments were removed gently with forceps, placed into individual hatching incubators and closely examined every day. The offspring from the first RNAi experiment were evaluated visually and counted at 9 days post hatching when control animals had developed to copepodids. Nauplii from the second RNAi experiment were collected, visually evaluated, and counted between 6 and 8 h post hatching and closely followed through molting to nauplii II and further to copepodids. Neutral lipid content was detected and quantified in nauplii I using lipid stains (see below).

Lipid analysis

Seven independent replicates of groups of 25 nauplii hatched from control and LsMTP dsRNA-injected female egg-strings from the second RNAi experiment were used for qualitative and semi-quantitative analysis of neutral lipids.

Oil Red O staining. Nauplii I were collected from hatching incubators, washed three times with cold PBS, and fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 2 h. Oil Red O stain was performed using the methods described (28) with some modifications. Fixed nauplii were washed three times with cold PBS, resuspended in 60% isopropanol for 10 min, and stained with Oil Red O stain (Sigma-Aldrich) for 30 min. After staining, nauplii were washed in cold PBS, rinsed with 60% isopropanol, mounted, and photographed with a Leica Model MZ6 stereo microscope.

Nile Red staining. Nile Red stain was used to detect neutral lipids in unfixed nauplii I according to (29) with the following modifications. Nauplii I were washed three times with cold PBS, stained with 1 ug/ml of Nile Red (Sigma-Aldrich) in PBS for 30 min, and imaged directly with a Leica TCS SP5 confocal microscope. Neutral lipids were visualized by excitation at 543 nm and fluorescence detection at 635 nm.

Semi-quantification of total nauplii I neutral lipids. The semi-quantification of total neutral lipids of nauplii I was carried out using Oil Red O stain. After fixation and staining with Oil Red O, the excess stain was washed away with 60% isopropanol. Oil Red O stain was extracted from nauplii using 200 µl of 100% isopropanol and absorbance was measured at 500 nm in duplicate.

Background signal was subtracted using 100% isopropanol as a background control.

Bioinformatics analysis

The Staden package (30) was used for DNA sequence assembly, editing, and analysis. Multiple sequence alignment was done in BioEdit version 7.2.5 (31) using ClustalW. Accession numbers of MTP protein sequences from other species included in the multiple alignment were as follows: *Homo sapiens* (NCBI: NP_000244.2), *Salmo salar* (NCBI: XP_014050992.1), *Danio rerio* (NCBI: NP_998135.1), *D. melanogaster* (NCBI: NP_610075.2), *Zootermopsis nevadensis* (NCBI: KDR21635.1), *Daphnia magna* (NCBI: JAN30039.1), *Scylla olivacea* (Uniport: A0A0P4WDH4), and *Xenopus tropicalis* (NCBI: XP_002934813.1). Signal peptides were predicted using Phobius (http://phobius.sbc.su.se/) and SignalP server (http://www.cbs.dtu.dk/services/SignalP/). Conserved domain (LpD-N) in the protein sequences was analyzed in Conserved Domain Database (32). Secondary structures of proteins were predicted using JPred4 (33) or PSSpred (http://zhanglab.ccmb.med.umich.edu/PSSpred/) and three-dimensional structures of proteins were resolved using Phyre2 online server (34). All predicted structures of proteins were refined using Modrefiner (35) and visualized using Pymol software.

RESULTS

LsMTP gene encodes three transcript variants

One DNA sequence encoding *LsMTP* was identified in the salmon louse genome. To obtain the full-length transcript sequence, 5′ and 3′ RACE were carried out using primers specific to the *LsMTP* sequence, which revealed three transcript variants named *LsMTP-A*, *LsMTP-B*, and *LsMTP-C* (**Fig. 1A**). Variant *LsMTP-A* contained alternative exon 1, which was part of the 5′ untranslated region (UTR) sequence in variant *LsMTP-B*, but not present in variant *LsMTP-C* (Fig. 1A, B). Predicted open reading frames of *LsMTP-B* and *LsMTP-C* variants, both started from the alternative start codon located in the intronic region of variant *LsMTP-A* (Fig. 1A, B). The details of the all LsMTP variants are summarized in Fig. 1D.

Sequence and structural analysis of *LsMTP* variants

The *LsMTP-B* and *LsMTP-C* transcripts encoded an identical protein of 819 amino acids, while the LsMTP-A was 827 amino acids and contained a different N-terminal signal peptide (Fig. 1C). The LsMTP-A and LsMTP-B/C isoforms contained a conserved region, named the lipoprotein N-terminal domain (SMART accession SM00638), found in lipid transport proteins, including vitellogenins, apoLp, and apoB. A BLASTP search in the UniProtKB/Swiss-Prot revealed LsMTP as most closely related to an uncharacterized protein from the crab *Scylla olivacea* (25% identity) and MTP of *Daphnia magna* (23% identity). A similar identity of LsMTP was also found with other functionally known MTPs like *Homo sapiens* (21.8%), *Mus musculus* (21.1%), *Danio rerio* (21.8%), *Gallus gallus* (21.0%), and *D. melanogaster* (21.7%). Alignment to MTP of its host, the Atlantic salmon (NCBI: XP_014050992.1) showed 22% identity to LsMTP.

Secondary and tertiary structures of the mature LsMTP (without signal peptide) were modeled along with other MTP orthologs (human, *Drosophila*, frog, and worm) using a homology modeling approach. The modeling template was the X-ray crystal structure of the silver lamprey (*Ichthyomyzon unicuspis*) lipovitellin, the mature form of vitellogenin (PDB ID: 1LSH) (36), also used for domain database annotation and in similar modeling studies (37, 38). All the modeled structures (**Fig. 2**, supplemental Fig. S1) displayed similar domain composition made up of an N-terminal β-barrel (N-sheet), a central helical domain, and two β-sheets (C-sheet and A-sheet) toward the C terminal, in agreement with the lipovitellin template. Moreover, structural similarity between the MTPs was calculated by performing structural alignments in PyMol with salmon louse MTP structure as a reference. The average distance between the atoms of the superimposed proteins, calculated as root mean square deviation (RMSD), was found to be 2.52, 3.07, 3.07, and 10.2 Å for *H. sapiens*, *D. melanogaster*, *X. tropicalis*, and *C. briggsae*, respectively.

In the N-sheet, the salmon louse MTP model predicted 13 antiparallel β-strands (Fig. 2A, B), similar to the available lipovitellin structure where 11 of the 13 β-strands formed a barrel-like conformation (9). The central helical domain of the salmon louse MTP model consisted of 17 α-helices arranged in an inner and outer layer (Fig. 2A, B), as in the lipovitellin structure. The C-sheet domain in the lipovitellin X-ray structure contained two β-sheets (C and A), which were also found in the sea louse MTP model. The two β-sheets formed a hydrophobic pocket (Fig. 2A), which included a number of hydrophobic residues (37). Sequence alignment of the conserved N-sheet, the central helical domain, and the C-sheet of MTP from salmon lice, crustaceans, vertebrates, and insects showed that the two cysteines known to form a disulfide bond in the N-sheet domain of lamprey lipovitellin (9) were conserved (C156-C182 in lipovitellin, C101-C120 in LsMTP) (Fig. 2B, C). This disulfide linkage in the N-sheet domain was essential to stabilize the barrel-like conformation formed by the β-strands. Walsh et al. (39) found an amino acid (D169) in the N-sheet domain, which was important to the formation of an internal salt bridge with amino acids K187 and K189. Missense mutation (D169V) destroyed this salt bridge, which led to loss of PDI binding as well as lipid transfer activity. In the N-sheet domain of LsMTP, amino acid D96 formed an internal salt bridge with amino acids R115 and K117 (Fig. 2B).

The central helical structure in lipovitellin was stabilized by a disulfide linkage (C451-C486 in lamprey lipovitellin, C391-C396 in LsMTP) (Fig. 2A). The residues in the salt bridge (R547-E574 in lamprey lipovitellin, R473-E502 in LsMTP) in the helical domain of lipovitellin were also conserved in the LsMTPs (Fig. 2D). In lipovitellin, this salt bridge was important to tie together helices 14 and 16 and increased the stability of the local fold. Further, the MTP-specific sequence (Fig. 2E), which was not present in apoB, apoLp, vitellogenin, phospholipid transfer proteins, and other lipid transfer proteins (40), was also conserved in the salmon louse MTP. Two helixes (helix-A and helix-B) in
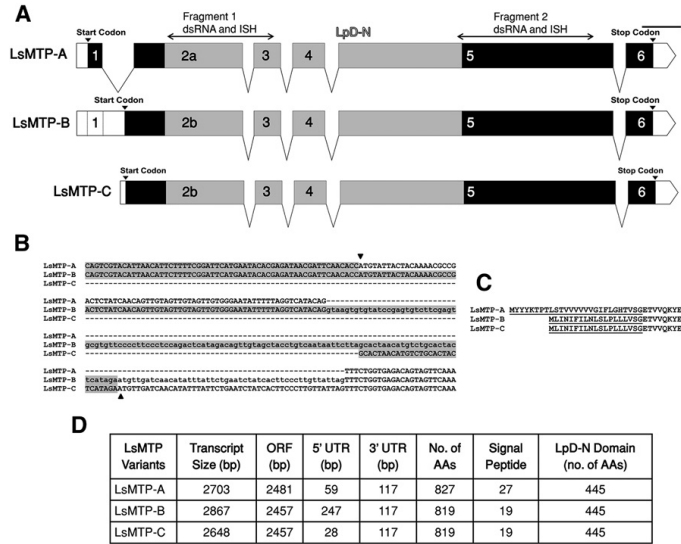
Fig. 1. The organization of the *LsMTP* gene. A: Genetic structure of the three variants of *LsMTP*. *LsMTP-A* consisted of six exons with an initiator codon in exon 1. The 5′ UTR is represented with a white box. *LsMTP-B* was generated due to intron retention, with exon 1 as part of the 5′ UTR. *LsMTP-C* arose due to exon 1 skipping. Lipoprotein N-terminal domain (LpD-N) is shaded with gray. The positions of the fragments used for RNAi and in situ hybridization RNA probes (ISH) are also shown. Scale bar = 200 bp. B: Multiple nucleotide alignments of the 5′ UTR sequences of three *LsMTP* variants. The 5′ UTR nucleotide sequences of three variants are highlighted in gray. The arrowheads indicate the start codon (ATG). Lowercase letters represent the intron sequence. Gaps are displayed as dashed lines. C: N-terminal amino acid sequence alignment of three LsMTP variants. The predicted signal peptides for three variants are underlined. D: Overview table. The table lists the size of the variants, open reading frame (ORF), 5′ and 3′ UTRs, signal peptide, and LpD-N domain size in number of amino acids (AAs).

the A-sheet of human MTP were known to be involved in lipid transfer activity (37). The amino acid, leucine-734, in helix-A was important in lipid transfer and conserved in vertebrates. However, in helix A (A-sheet) of LsMTP, isoleucine (I653) was found at this position (Fig. 2E).

Tissue distribution of *LsMTP* transcripts in the adult female lice

In order to localize the site of expression of *LsMTP* in the adult female louse, in situ hybridization was performed on sections. Two independent probes targeting different parts of the transcript detecting all the three variants of *LsMTP* were utilized. The two probes revealed the same localization pattern of *LsMTP* transcripts in the female (**Fig. 3B**–E). *LsMTP* transcripts were detected in the sub-cuticular tissue (Fig. 3B), intestine (Fig. 3C), ovaries (Fig. 3D), and vitellogenic oocytes in the genital segment (Fig. 3E). No positive signal was detected in slides treated with sense probes (negative control).

Differential expression of *LsMTP* transcript variants in different tissues by RT-PCR

RT-PCR with variant-specific primers (supplemental Table S1) showed a differential expression of *LsMTP* variants among the examined tissues. *LsMTP-A* was mainly expressed in the sub-cuticular tissue and ovaries (Fig. 3F). *LsMTP-B* was detected in the ovaries, with relatively low

abundance in the sub-cuticular tissue, while *LsMTP-C* was present in sub-cuticular tissue, intestine, ovaries, and vitellogenic oocytes (Fig. 3F).

Expression level of *LsMTP* variants in different developmental stages

Analysis of mRNA levels using Q-PCR showed that all three splice variants of *LsMTP* were expressed at all developmental stages of salmon lice (**Fig. 4**). Expression levels of variants *LsMTP-A* and *LsMTP-B* were highest in adult male and female stages as compared with other stages (Fig. 4). However, relative expression of *LsMTP-A* was reduced in the adult female when compared with the young adult female (newly molted females) and in the adult male, the expression of *LsMTP-B* was relatively higher than *LsMTP-A*. Moreover, the expression of *LsMTP-C* was relatively stable, with the highest expression levels found in nauplius I (Fig. 4).

Knockdown of *LsMTP* gene inhibits egg production and reduces larva survival

Two experiments with RNAi knockdown of *LsMTP* were performed, one in newly molted preadult II females (n = 30) and a second in young adult females, using dsRNA targeting cod trypsin in the negative control groups (**Table 1**). Both RNAi experiments were terminated when the adult females from the control groups produced the
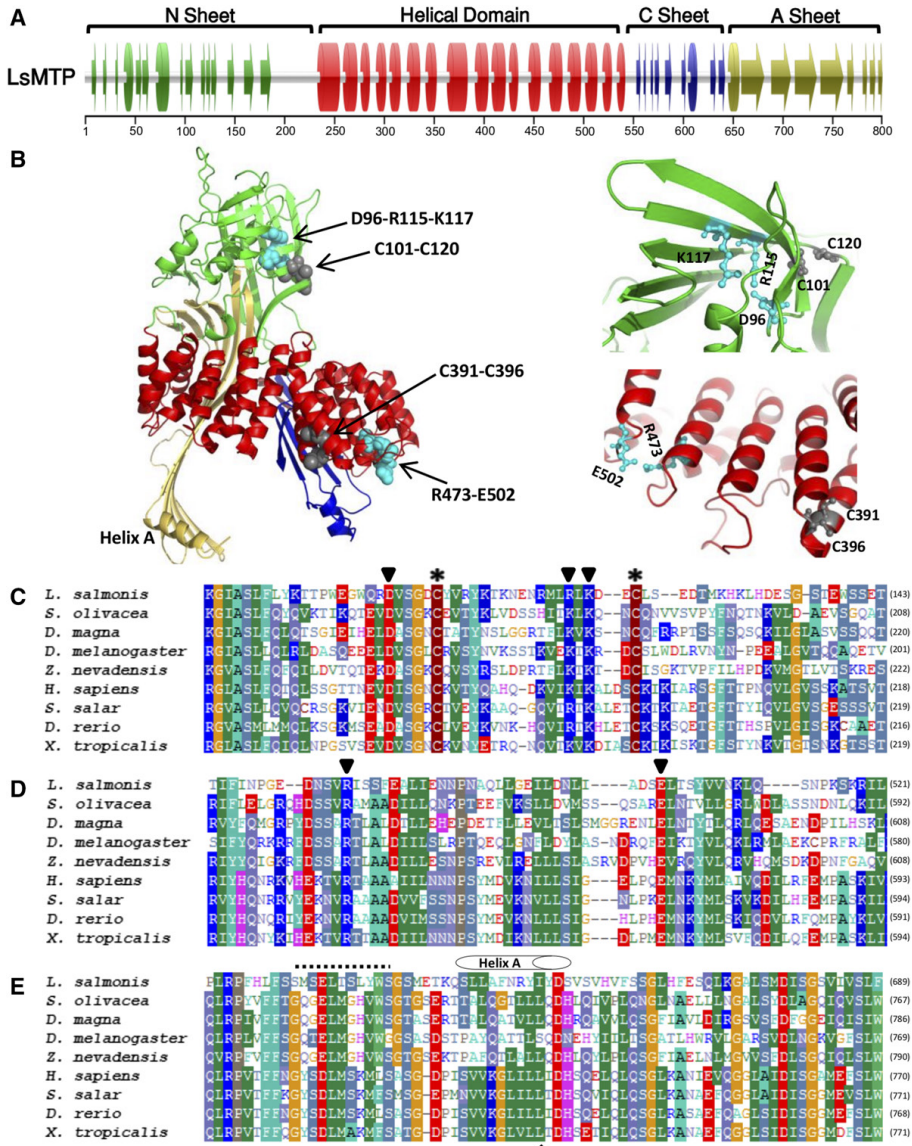
Fig. 2. Structural analysis of LsMTP. A: Predicted secondary structures of LsMTP. LsMTP model consists of four functional domains: N-terminal β-sheet, central helical domain, C β-sheets, and A β-sheets. The ruler for amino-acid numbering is shown below. B: The tertiary structure of LsMTP was modeled using PHYRE protein structure prediction program. The left panel represents the full view of the ribbon structure of LsMTP protein, with cysteine residues (gray spheres) and residues of the salt bridges (cyan spheres). The right panel represents the zoom view of the interior of the N-terminal β-sheet (upper) and central helical domain (lower). Disulfide linkages have been formed between C101-C120 and C156-C182, whereas salt bridges have been formed between D96-R115-K117 and R473-E502. C, D: Multiple alignments of the conserved N-sheet and central helical domain of LsMTP with other MTPs. The conserved cysteine residues are shown with asterisks and residues of the salt bridges are highlighted with inverted triangles. E: Multiple alignment of the MTP-specific sequence. This region was present in salmon louse MTP and contained helix A. The black arrow below the sequence indicates the amino acid position (L734 in human MTP) important for the lipid transfer activity (37). Isoleucine (I653) was found at this position in salmon lice. The dotted line shows the helix as predicted by Jpred4 and PSSpred, which has not been described before in other MTPs.
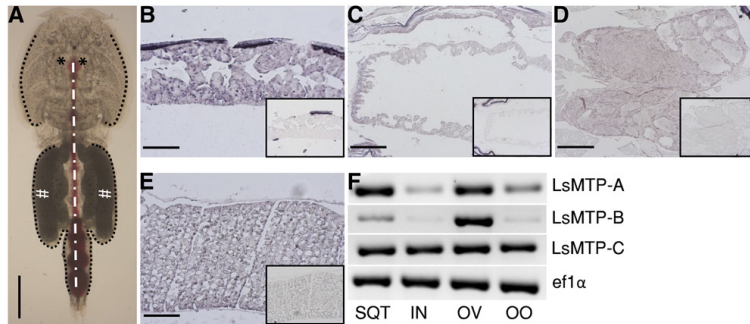
Fig. 3. In situ hybridization and RT-PCR analysis of *LsMTP* mRNAs in various tissues of an adult female salmon louse. A: Dorsal view of an adult female without egg-strings. The black dotted line indicates the area where sub-cuticular tissue is situated; a white straight dash-dot line represents the gut filled with blood. Asterisks (*) and hashtags (#) represent the positions of the ovaries and mature vitellogenic oocytes, respectively. B–E: Cross-sections of sub-cuticular tissue (B), intestine (C), ovaries (D), and vitellogenic oocytes (E) hybridized with antisense probes or sense probes (small inserts) as negative controls. F: RT-PCR analyses from cDNA templates of different tissues of adult female lice using *LsMTP* variant-specific primers. RT-PCR analysis of *ef1α* was carried out to determine the quantitative variations of *LsMTP* transcripts among samples. SQT, sub-cuticular tissue; IN, intestine; OV, ovaries; OO, oocytes. Scale bars = 1 mm (A), 200 μm (B, E), 100 μm (C, D).

second pair of egg-strings to be certain that maturing eggs received dsRNA treatment. In the first experiment, RNAi knockdown of *LsMTP* in newly molted preadult II females (n = 30), the downregulation of *LsMTP* was highly significant and the levels of *LsMTP* were reduced by 95% compared with control animals (**Fig. 5**). The knockdown of *LsMTP* had no lethal effect on the adult lice. However, females injected with *LsMTP* dsRNA produced shorter and curly egg-strings compared with control females, which produced normal straight egg-strings, (supplemental Fig. S2). A very strong reduction (90%) in the number of surviving copepodids produced from the females treated with *LsMTP* dsRNA was observed, as compared with control groups (Table 1). The few larvae that hatched went through

molting, apparently as normal, and developed into copepodids. This low number of surviving larvae could be caused by reduced lipid deposition in the developing oocytes, while still sufficient for some larvae to survive. To address this possibility and to further confirm the specificity of RNAi-mediated gene silencing, a second RNAi was performed in newly molted young adult females (n = 32) that should give higher initial MTP transcript level, and therefore somewhat higher survival rate of the larvae. Downregulation of *LsMTP* gene expression was approximately 90% compared with control (Fig. 5), with the remaining absolute levels 2-fold higher than in the first RNAi experiment. All animals in the control group produced normal egg-strings, while about 50% (11 females) of the
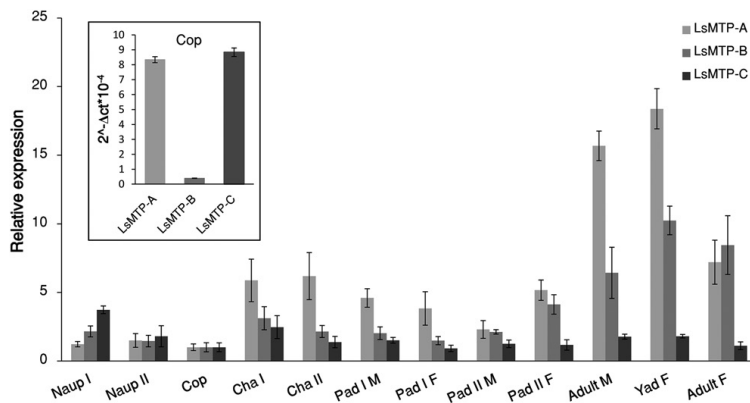

Fig. 4. Expression of the transcript levels of three different variants of *LsMTP* in various developmental stages of the salmon louse relative to transcript level in the copepodids. The insert shows the expression of the three variants in the copepodids. Naup I, nauplii I; Naup II, nauplii II; Cop, planktonic copepodids; Cha I, chalimus I; Cha II, chalimus II; Pad I M, preadult I male; Pad I F, preadult I female; Pad II M, preadult II male; Pad II F, preadult II female; YAD, young adult female. Error bars represent the SD (n = 5 for each stage).

	Injected Female Lice	Recovered Female Lice	Number of Females that Produced Egg-Strings	Length of Egg-Strings (mm)	Number of Hatched Nauplii	Number of Hatched Copepodids
Experiment 1						
Control	30	12	12	18.8 ± 1.6 (n = 9)	Not counted	322 ± 109 (n = 9)
RNAi (fragment 1)	30	10	7	7.4 ± 3.6 (n = 6)	Not counted	33 ± 16 (n = 5)
Experiment 2						
Control	30	23	23	18.4 ± 1.8 (n = 20)	348 ± 78 (n = 7)	314 ± 67 (n = 7)
RNAi (fragment 2)	32	22	11	15.5 ± 3.8 (n = 7)	262 ± 97 (n = 6)	86 ± 61 (n = 6)

RNAi-treated females still produced egg-strings. These egg-strings were short and curly when compared with egg-strings of control females (supplemental Fig. S3). Furthermore, a significant number (~72%) of hatched nauplii from LsMTP-treated females did not develop to copepodids when compared with the control group (Table 1).

To explore the level of lipids in larvae from controls and RNAi knockdown animals, nauplii hatched from the egg-strings were stained to detect neutral lipids with Nile Red and Oil Red O. Similar results were obtained with the two different stains. Nauplii produced from females injected with LsMTP dsRNA had no or fewer lipid droplets in their yolk as compared with nauplii produced from females injected with control dsRNA (Fig. 6A–F). Total neutral lipids were measured using Oil Red O in hatched nauplii of LsMTP and control RNAi-treated females. As expected, a significant reduction (83%) of total neutral lipids was seen in nauplii of LsMTP RNAi-treated females (Fig. 6G).

Starvation reduces the LsMTP mRNA level in adult female lice

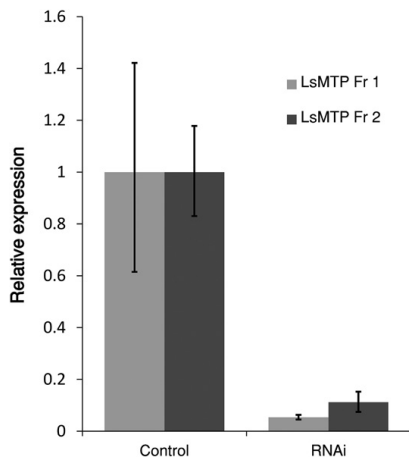Adult female lice obtain important nutrients, such as lipids, from the fish host's blood and skin. To determine whether LsMTP mRNA expression was directly correlated with food uptake, adult female lice were starved by removal from the host, and then refed on the fish host. Samples for Q-PCR analysis were collected at 0 (control), 1, 2, and 4 days, and 2 days of refeeding. The expression of LsMTP was reduced 70–85% following starvation and increased upon refeeding (Fig. 7).

DISCUSSION

MTP has been studied in many organisms, particularly in vertebrates, due to its important role in lipid metabolism. Three transcript variants of LsMTP, referred to as LsMTP-A, LsMTP-B, and LsMTP-C (Fig. 1), were identified. LsMTP-A mRNA is predicted to encode an 827 amino acid protein, whereas LsMTP-B and LsMTP-C encode an 819 amino acid long protein (LsMTP-B/C). Two isoforms of MTP (MTP-A and MTP-B) have been identified in mice due to alternative first exons, and both isoforms are effective in lipid transfer activity (41, 42). Recently, two splice variants (MTP-B and MTP-C) have been found in humans (43), and it has been concluded that alternative splicing and the presence of distinct promoter regions play a key role in the regulation of cellular MTP levels. Additionally, specific 5′ UTRs containing elements that alter translation enable the cell to optimize MTP activity. The predicted protein isoforms of L. salmonis have only 21% to 23% sequence identity to MTPs of other species. Similarly, MTP proteins from other invertebrates (insects and nematodes) have been reported to have less than 25% sequence identity compared with human and zebrafish (15, 40). Therefore, a considerable identity difference is present between the MTPs of invertebrates and vertebrates.

Further structural analysis predicted that LsMTP protein contains β-sheets (N, C, and A) and a central α-helix C-terminal domain (Fig. 2). Similar structural domains have also been found in MTP of H. sapiens, as well as in other MTP orthologs such as D. rerio, D. melanogaster, C. elegans (40), and M. amblycephala (44). The RMSD was found to be 2.52, 3.07, 3.07, and 10.2 Å for H. sapiens, D. melanogaster, X. tropicalis, and C. briggsae, respectively. The high RMSD found for C. briggsae could be connected with lower numbers of β-strands in the N-terminal domain (supplemental Fig. S1). Of the other protein sequences tested, the results indicate a significant overall structural similarity.

LsMTP transcripts were found in the sub-cuticular tissue (tissue with a functional resemblance to insect fat body and vertebrate liver) and intestine. In the salmon louse, the sub-cuticular tissue is the site where two vitellogenins
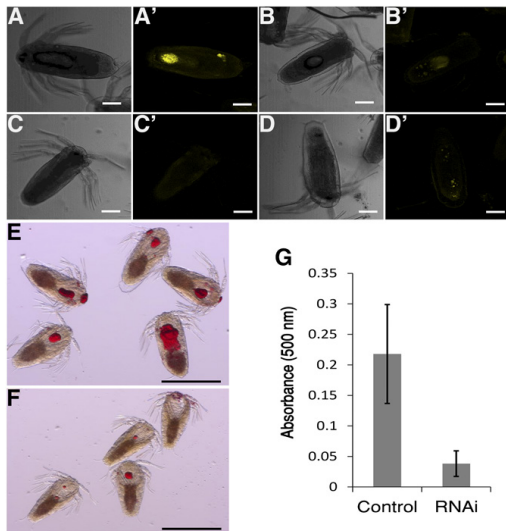

Fig. 5. Inhibition of LsMTP transcript by RNAi in adult female salmon lice. The expression level of LsMTP was quantified by Q-PCR in adult females injected with dsRNA in preadult (Fr 1) and young adult (Fr 2) females against control. The results represent the mean ± SEM of five biological replicates from each treatment group. Significant downregulation of LsMTP was found as compared with control (t-test, P < 0.05).

Fig. 6. LsMTP transports maternal neutral lipids to developing embryos. Bright field (A–D) and confocal fluorescence (A'–D') nauplii stained with Nile Red to visualize the lipid droplets. Accumulation of maternal neutral lipids was reduced in the nauplii of females injected with *LsMTP* dsRNA (C'–D') as compared with nauplii produced from females injected with control dsRNA (A'–B'). Nauplii hatched from females injected with control dsRNA (E) and *LsMTP* dsRNA (F) were stained with a nonfluorescent dye (Oil Red O). Nauplii hatched from females injected with *LsMTP* dsRNA accumulate fewer neutral lipids (F), as compared with nauplii hatched from females treated with control dsRNA (E). G: Semi-quantification of neutral lipids with Oil Red O stain in the nauplii of females treated with control and *LsMTP* dsRNAs. Neutral lipids were reduced significantly (83%) in hatched nauplii of *LsMTP* dsRNA-treated females. Results are represented as the mean ± SD of nauplii (n = 25) hatched from seven independent replicates of control and *LsMTP* dsRNA-injected females. Scale bars = 100 µm (A/A'–D/D'), 500 µm (E, F).

(21) and yolk-associated protein (23) are produced. In *C. elegans*, *MTP* transcripts were found in the intestine, which is reported to function as a secretory organ of vitellogenins (16). Similarly, in *D. melanogaster*, *MTP* transcripts were found in the fat body (an organ analog to vertebrate adipose tissue and liver), and MTP proteins are reported to promote the production of Lpp and large lipid transfer particles (14). *MTP* transcripts were also found in vertebrates in tissues expressing apoB, such as liver, intestine, retina (45), kidney (46), myocardium (47), placenta, and yolk sac (48–50). Here, MTP functions as an essential chaperone for the assembly and secretion of apoB-lipoproteins. *LsMTP* transcripts were also found in the ovaries and oocytes of the salmon louse. In vertebrates, the *MTP* is found in the ovaries and testis, where *apoB* is not expressed (51). The function of MTP in these lipid-affluent tissues is unknown. However, it is likely that MTP may play an important role in lipid trafficking and/or storage (52, 53). Further studies are needed to demonstrate the tissue-specific function of LsMTP in the salmon louse.
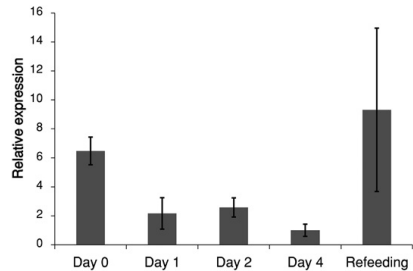

Fig. 7. Starvation reduces *LsMTP* transcript levels in adult female lice. Significant reduction of the expression level of *LsMTP* was found in starved animals, as compared with control animals (day 0) (*t*-test: $P < 0.05$). Refeeding (2 days) increased the expression of LsMTP as compared with starved and control (day 0) animals. Error bars represent the SD for each time point (n = 5).

Expression levels of the three *LsMTP* transcript variants were also analyzed in some key adult female louse tissues (Fig. 3F). The observed differences between expression of the three *LsMTP* variants in different tissues of adult female lice are similar to observations in vertebrates, such as mice and humans (41–43). For example, mice have two isoforms of *MTP*. The *MTP-A* isoform is expressed predominantly in the liver, intestine, and heart, whereas *MTP-B* is mainly found in adipose tissue. Similarly, in humans, splice variant *MTP-B* is found in various tissues, whereas *MTP-C* is expressed mainly in brain and testis.

In copepodids, the *LsMTP-A* and *LsMTP-C* transcripts are clearly the most abundant forms (Fig. 4, insert). Assessing the *LsMTP* transcript levels throughout the *L. salmonis* life cycle, relative to copepodids, showed that *LsMTP-A* and *LsMTP-B* forms varied with highest levels in adult lice of both sexes, whereas *LsMTP-C* was relatively stably transcribed. The salmon louse larval stages are nonfeeding and survive on maternally provided energy, whereas the parasitic stages have access to a stable and abundant amount of energy. The steep increase in the *LsMTP-A* variant (and to some extent the *LsMTP-B* variant) in adults probably reflects increased demand of lipids for gamete production, particularly in females, because these forms are expressed in the sub-cuticular tissue and ovary (Fig. 3F). Our data indicate that, in the intestine, *LsMTP-C* is the most abundant form (Fig. 3F) and have a stable expression levels in the various developmental stages (Fig. 4). These results indicate that *LsMTP-C* is involved in lipoprotein maturation and secretion from intestinal cells and that increased intestinal capacity is a result of growth (i.e., increased number of enterocytes).

To investigate the effect of starvation, adult females were removed from their hosts. A significant reduction in expression of *LsMTP* was seen over time and refeeding increased the expression, as compared with starved and control (day 0) animals. This indicates that expression of *LsMTP* directly depends on the availability of food in the intestine and/or lipids in the blood-feed, or is affected indirectly due to downregulation of other lipid-carrying lipoproteins. The effect of feeding on *MTP* mRNA expression has been studied in *D. rerio* and a significant pretranslational

increase in *MTP* expression was seen in the anterior intestine (54). In mammals, in contrast, *MTP* mRNA expression was not significantly changed in the intestine and liver due to fasting, but a moderate pretranslational increase was noted because of high fat and cholesterol diets (55–58).

LsMTP is expected to play an important role in the transport of yolk lipids from the intestine to growing oocytes through the secretion of lipoproteins. The results of RNAi in young adult females clearly showed that the larvae of *LsMTP* knockdown female lice had a significantly lower reserve of maternally deposited lipids in their yolk, as confirmed by our qualitative and quantitative lipid analysis (Fig. 6A–G). Furthermore, a significant reduction in the number of copepodids was also noted (Table 1). To our knowledge, no information about the function of MTP is available in other crustaceans. However, knockout of the *MTP* gene in homozygous mice was embryonic lethal, due to the failure of the yolk sac to deliver lipids to the developing embryos (12). Similarly, disruption of *dsc-4*, a homolog of *MTP* in *C. elegans*, by RNAi or mutation, suppresses the germline delay and egg-laying (16). Moreover, MTP has also been shown to be important for the secretion of Lpp and large lipid transfer particles in the hemolymph of *D. melanogaster* and *MTP* mutant larvae; neutral lipid accumulates in the gut due to loss of lipoproteins (14). MTP is also important for yolk lipid utilization and absorption of dietary neutral lipids in larvae of *D. rerio* (59). The significant reduction in egg production and mortality of copepodids suggests that LsMTP has an important function in the reproduction and lipid metabolism of salmon lice. The specific mechanism for the reduction of lipids in the embryos of the *LsMTP* knockdown female is not known. However, it can be suggested that LsMTP is essential for the secretion of lipoproteins, similar to mammalian lipoproteins, and serves as a vessel for the transport of lipids from the intestine to oocytes or other tissues of the salmon louse.

In summary, we identified *MTP* in the salmon louse, and structural analysis revealed that LsMTP has similar functional domains found in MTP homologs from other species. From results of expression analysis together with functional studies, it can be concluded that LsMTP has an important role in the lipid metabolism and reproduction of salmon lice. Results from our study further demonstrated that LsMTP could be used as a target for the control of reproduction in the female lice. However, further investigations are required to characterize associated lipoproteins and mechanisms of lipid loading and secretions in salmon lice. ⬛

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II

Molecular characterization of the lipophorin receptor in the crustacean ectoparasite *Lepeophtheirus salmonis*

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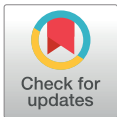
Abstract

The Salmon louse (*Lepeophtheirus salmonis*) is a marine ectoparasite of salmonid fish in the Northern Hemisphere and considered as a major challenge in aquaculture and a threat to wild populations of salmonids. Adult female lice produce a large number of lipid-rich eggs, however, the mechanism of maternal lipid transport into developing eggs during salmon louse reproduction has not been described. In the present study, a full-length *L. salmonis* lipophorin receptor (*LsLpR*) consisting of 16 exons was obtained by RACE and RT-PCR. The predicted ORF was 952 amino acids and structural analysis showed five functional domains that are similar to LpR of insects and decapods. Phylogenetic analysis placed the LsLpR together with LpRs from decapods and insects. Expression analysis revealed that the relative abundance of *LsLpR* transcripts was highest in the larvae and adult female lice. In adult females, the *LsLpR* transcripts and protein were found in the ovary and vitellogenic oocytes whereas, in larvae, the *LsLpR* transcripts were found in the neuronal somata of the brain and the intestine. Oil Red O stain results revealed that storage of neutral lipids was found in vitellogenic oocytes and ovaries of adult females, and in the yolk of larvae. More-over, RNA interference (RNAi) was conducted to demonstrate the function of *LsLpR* in reproduction and lipid metabolism in *L. salmonis*. In larvae, the transcription of *LsLpR* was decreased by 44–54% while in an experiment *LsLpR* knockdown female lice produced 72% less offspring than control lice.

Introduction

The salmon louse (*Lepeophtheirus salmonis*) is a marine ectoparasitic copepod that infests salmonids in Norway, Scotland, Ireland and Canada. It feeds on blood, mucus and skin of hosts in sea water, which leads to major health and welfare issues of fish and results in a major economic losses in the Atlantic salmon (*Salmo Salar*) farming industry [1]. The salmon louse has also been considered to be a threat to wild salmonids [2]. The life cycle of the salmon louse comprises of eight developmental stages, each stage separated by a moult [3]. The free-living

stages consist of two nauplius stages, and an infectious copepodid stage. After the settlement of copepodids to host fish, there are two immobile chalimus stages where the louse is anchored to the host through frontal filaments, followed by three mobile stages: two pre-adult stages and one adult stage. Eggs hatch into free-living nauplius I larvae, the first of three larval stages of *L. salmonis* that are lecithotrophic. These larvae stages rely on stored nutrients imported to the eggs during vitellogenesis and the free-living copepodids must settle to a fish host before they run out of energy [4].

Once the adult female louse becomes sexually mature, a continuous production of eggs is initiated in the ovaries. The oocytes migrate from the ovaries to the genital segment where they grow and mature forming two genital complexes with vitellogenic oocytes. The eggs are fertilized and deposited as a pair of egg-strings which the female carry externally until all eggs are hatched. Like other oviparous animals, salmon lice store large amounts of yolk proteins [5, 6] and lipids [7] in the developing oocytes to secure energy for embryogenesis and early larval development. In general, yolk lipids provide energy, building blocks for the developing cell membranes, and precursors for prostaglandin and steroid hormones. The major neutral lipid found in eggs and larvae (nauplius II) of *L. salmonis* is triacylglycerol (TAG), whereas the major polar lipids are phosphatidylcholine and phosphatidylethanolamine [7]. Despite the existing knowledge of lipid classes in oocytes and larvae of *L. salmonis*, mechanism of lipid distribution and uptake in developing oocytes is scarce. Hence, improved understanding of mechanism for lipid uptake will enhance the knowledge regarding oocytes maturation and can potentially be used in anti-parasitic strategies.

In animals, lipids are transported in the aqueous environment of the circulatory system in lipid-protein complexes named lipoproteins. A lipoprotein particle consists of a hydrophobic core of neutral lipids surrounded by a single layer of phospholipid molecules, unesterified cholesterol and apolipoproteins. Mammals have two different TAG-rich lipoproteins involved in lipid transport: chylomicrons from the intestine and very low-density lipoproteins (VLDL) from the liver, delivering neutral lipids to target tissues through lipoprotein lipase-mediated lipolysis. After lipolysis, chylomicrons convert into chylomicron remnants and VLDLs change into intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDLs). These remnants particles become enriched in cholesteryl ester (CE) and supply cholesterol to the liver or peripheral tissues through receptor-mediated endocytosis. In contrast to mammals, the major lipoprotein in the hemolymph of insects is lipophorin (Lp) [8–10] which functions as a reusable shuttle for the delivery of lipids to various tissues including oocytes [11–16]. In some insects, Lp is accumulated inside the developing oocytes and becomes itself part of the yolk [17]. Two forms of Lp are found in insects, high-density lipophorin (HDLp) and low-density lipophorin (LDLp) which has 30–50% and up to 62% lipid contents respectively [18, 19]. The HDLp contains one molecule of apoLp I and one molecule of apoLp II. However, when large amounts of lipids are mobilised during insect flight, extra copies of apoLp III are associated with HDLp and formation of LDLp occurs which contains much more lipids than HDLp [10, 12, 20]. Other than Lp, a small contribution of vitellogenin (Vg) has also been suggested in the transport of lipids to growing oocytes of insects [15, 18].

The LDL receptor (LDLR) is a member of the LDLR superfamily. In mammals, LDLR binds cholesterol-rich LDL and internalizes it through receptor-mediated endocytosis. During endocytosis, the receptor releases lipoprotein into the lumen of the endosome and the receptor is recycled back to the surface of the cell available to new rounds of endocytic uptake [21–23]. The role of LDLR is to maintain the cholesterol homoeostasis and mutations in this receptor lead to familial hypercholesterolemia [24, 25]. Another member of the LDLR superfamily, termed VLDL/Vg receptor (VLDLR/VgR) plays a major role in reproduction of chicken as it mediates the uptake of VLDL and Vg in the developing oocytes [26]. In arthropods, the LDLR

family member lipophorin receptor (LpR) binds and transport lipophorin to the developing oocytes through receptor-mediated endocytosis. The LpR gene was first characterized at the molecular and functional level in the locust, *Locusta migratoria* [27] and later cloned and characterized in several insect species [28–35]. Recently, three lipophorin receptors (LpR1, LpR2A and LpR2B) from shrimp (*Pandalopsis japonica*) have been characterised [36]. Similar to other members of LDLR family, LpR contains five functional domains: A ligand binding domain, an epidermal growth factor (EGF) precursor homologous domain, an O-linked sugar domain, a transmembrane domain and a cytoplasmic domain. In insects, the LpR has been reported to play an important function in lipid metabolism as well as in the reproduction. The expression of LpRs takes place predominantly in the reproductive organs and is responsible for lipid accumulation in growing oocytes. Studies of mutants have shown that LpR2 of *D. melanogaster* has an important role in the transport of lipids to growing oocytes [34]. Similarly, RNAi experiment showed that LpR is involved in the uptake of Lp in *B. germanica* [32].

In this study, a gene encoding a lipophorin receptor (LsLpR) containing the conserved domain structure was identified in salmon louse. To our knowledge, this is the first report on the characterization of a member of LDLR superfamily in *L. salmonis*. The receptor was found to be expressed in all developmental stages, but predominantly in larval and adult female lice. The receptor mRNA and protein were found exclusively in the ovaries and oocytes of the adult females. In larvae, the transcripts were found in several tissues. Furthermore, RNAi experiments were conducted in larvae and female lice confirming this function.

Materials and methods

Sampling of salmon lice

A laboratory strain of salmon lice, *Lepeophtheirus salmonis* [37] was maintained on Atlantic salmon (*Salmo salar*) in tanks, supplied with a continuous flow of seawater at 10˚C and 34.5 ppt salinity. Fish were hand fed daily with commercial dry pellets. Nauplii I/II and free-living copepodids were obtained from egg-strings, hatched in flow-through incubators with the same supply of seawater. Chalimi, pre-adult and adult stages of lice were sampled from fish. Before sampling, fish were anaesthetized with a mixture of benzocaine (60mg/l) and methomidate (5mg/l) in seawater. All the experiments were performed according to the Norwegian animal welfare legislations and approved by Norwegian Food Safety Authority (Mattilsynet).

Five biological replicates were collected from each developmental stage of the salmon lice for stage-specific RT-qPCR. The following life stages and pooled number of animals were harvested for each replicate: Nauplius I (n = 100), nauplius II (n = 100), planktonic copepodid (n = 100), chalimus I (n = 10), chalimus II (n = 10), preadult I male and female (n = 1), preadult II male and female (n = 1), adult male (n = 1) and adult female (n = 1). All the samples were collected in RNAlater[TM] (Ambion) and kept overnight at 4˚C before long time storage at -20˚C.

Isolation of RNA and cDNA synthesis

Total RNA was isolated using TRI reagent (Sigma-Aldrich) as per manufacturer's instructions. The concentration and purity of isolated RNA was confirmed using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). Following RNA isolation, 1 µg of total RNA was treated with amplification grade DNaseI (Invitrogen) as per manufacturer's instructions. For RT-qPCR, 300 ng of total DNase-treated RNA was used for the synthesis of cDNA with Affinity Script QPCR cDNA Synthesis Kit (Stratagene) and diluted 10 times with nuclease free

water prior to storage at -20°C. For RT-PCR, 1µg of total RNA was reverse transcribed using a qScript cDNA SuperMix (Quanta Bioscience).

Genome analysis, PCR, cloning and sequencing of LsLpR

The LpR sequences from *Bombyx mori* (GenBank: AB211594) and *Blattella germanica* (GenBank: AM403063) were used to identify candidate LpR genes in the salmon louse genome database (Licebase https://licebase.org/). Two genes (stable IDs: EMLSAG00000008639 and EMLSAG00000009473) were predicted to encode LpRs according to the lowest e-value criteria. However, SMARTer RACE (rapid amplification of cDNA ends) demonstrated that these two predicted genes were part of the same gene. The 5′ and 3′ RACE was conducted with SMARTer RACE cDNA Amplification Kit (Clontech) as instructed in the users' manual. Total RNA isolated from an adult female was used to synthesize the 5′ and 3′ RACE-Ready cDNAs using gene-specific primers (Table 1). PCR products were cloned into pCR™ 4-TOPO® vector using the TOPO TA Cloning kit for sequencing (Life Technologies) followed by transformation into *Escherichia coli* TOP10 cells. PCR products of positive clones were cleaned with ExoSAP-it (Affymetrix) and used as templates for sequencing using M13 forward and reverse primers. All the sequences were assembled, and the single transcript was reconfirmed by RT-PCR. The complete mRNA sequence of LsLpR has been deposited in GenBank (MF435899).

Table 1. Primers used during this study.

Name	Sequence (5'-3')	Analysis
LpR48_5RACE	CTCCACAATCATCCTCTTGATCACAAACCCAAC	RACE
LpR_3RACE-3	GCAAGGCATCAGAAGAAGGCAATGGATCTCG	RACE
LpR-F	TCCATCTCTTCTGTTTGCACAT	PCR
LpR-R	ACAACGATAGATCGCCATGA	PCR
LpR-F2	GCGTGTCTCAAGGGTCACAT	PCR
LpR-R2	CACGTCTGATCACATCCTCCA	PCR
M13_f	GTAAAACGACGGCCAG	TOPO cloning
M13_r	CAGGAAACAGCTATGAC	TOPO cloning
LpRORF-F	ATGATACGTTTCTCAACATA	PCR
LpRORF-R	CGAATTGATGACCTCCTCTGA	PCR
LpRp-F T7	TAATACGACTCACTATAGGGGCACCCATTGATGAAGGTAA	dsRNA, Fragment 1
LpRp2-R T7	TAATACGACTCACTATAGGGGATGACCATTGGGACTTGCT	
LpRp-F	GCACCCATTGATGAAGGTAA	
LpRp2-R	GATGACCATTGGGACTTGCT	
LpRp-FT7	TAATACGACTCACTATAGGGGAAACTGGGCGGATGAGTCA	dsRNA, Fragment 2, In situ
LpRp-RT7	TAATACGACTCACTATAGGGGTTCCCGTATCTGTCCAATA	
LpRp-F	GAAACTGGGCGGATGAGTCA	
LpRp-R	GTTCCCGTATCTGTCCAATAGA	
LpRp-F3 T7	GAAATTAATACGACTCACTATAGGGTAACGAGACTGCCGGATTCA	dsRNA Fragment 3
LpRp-R3 T7	GAAATTAATACGACTCACTATAGGGACAGCATGATCTCTTGGTTCAC	
LpRp-F3	TAACGAGACTGCCGGATTCA	
LpRp-R3	ACAGCATGATCTCTTGGTTCAC	
LPR_SY_F4	TCTCATTTCCACCATCATCG	RT-qPCR
LPR_SY_R4	GCCAACGCAATGTTTCACTA	RT-qPCR

RACE, Rapid Amplification of cDNA Ends: TOPO, DNA topoisomerase: PCR, Polymerase Chain Reaction: RT-qPCR, Quantitative reverse transcription PCR: Insitu, Insitu hybridization: dsRNA, double-stranded RNA.

https://doi.org/10.1371/journal.pone.0195783.t001

Phylogenetic analysis

Protein sequences of lipoprotein receptors were obtained from GenBank protein database. These included the vertebrate VLDLRs (Very low density lipoprotein) of *Canis lupus familiaris* (NP_001273907), *Oryctolagus cuniculus* (BAA01874), *Rattus norvegicus* (NP_037287), *Mus musculus* (AAH13622), *Bos taurus* (NP_776914), *Macaca mulatta* (AAR83314), *Pan troglodytes* (XP_520460), *Homo sapiens* (NP_003374); the vertebrate VgRs (Vitellogenin receptors) of *Oncorhynchus mykiss* (CAD10640), *Morone americana* (AAO92396), *Oreochromis aureus* (AAO27569); the vertebrate LDLRs (Low density lipoprotein receptors) of *Mus musculus* (CAA45759), *Homo sapiens* (AAA56833), *Bos taurus* (NP_001160002), *Sus scrofa* (AHF51842), *Chiloscyllium plagiosum* (AAB42184), *Rattus norvegicus* (NP_786938); three lipoprotein receptors (LpRs) of shrimp *Pandalopsis japonica*: LpR1(AHL26189), LpR2A (AHL26190) and LpR2B (AHL26191), and insect LpRs of *Aedes aegypti* (AAQ16410), *Drosophila melanogaster* (NP_733119), *Rhyparobia maderae* (BAE00010), *Locusta migratoria* (CAA03855), *Blattella germanica* (CAL47126), *Bombyx mori* (BAE71406), *Galleria mellonella* (ABF20542); the crustacean VgRs of *Marsupenaeus japonicas* (BAH57291), *Penaeus semisulcatus* (AAL79675), Penaeus monodon (ABW79798), Macrobrachium rosenbergii (ADK55596), *Palaemon carinicauda* (AHB12420), *Pandalopsis japonica* (AHL26192); the insects VgRs of *Drosophila melanogaster* (AAB60217), *Anopheles gambiae* (EAA06264), *Aedes aegypti* (AAK15810), *Solenopsis invicta* (AAP92450), *Periplaneta Americana* (BAC02725), *Rhyparobia maderae* (BAE93218), *Blattella germanica* (CAJ19121). Multiple sequence alignment was performed in BioEdit version 7.2.5 [38] using the clustalW. All the gaps and divergent regions were removed. The aligned protein sequences were exported to Mesquite Version 3.2 [39] and nexus format file was generated. The best-fit model for the protein evolution was obtained from ProtTest V. 3.2 [40] based on the Bayesian Information Criterion (BIC). Phylogenetic analysis was performed with MrBayes v. 3.2 [41] using model (WAG+I+G). To root the tree, the RME2 sequence of *Caenorhabditis elegans* (AAD56241) was used as an outgroup. Two independent Monte Carlo Markov (MCM) chains were executed and sampled every 100 generations for a total of 1000000 generations to approximate the posterior probabilities. The quality of output data was assessed in Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/) and trees were obtained using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

In situ hybridization

Single stranded Digoxigenin (DIG) labelled RNA probe of 571 nt was synthesized from cDNA using the DIG RNA labelling kit (Roche). Primers used for the synthesis of sense and antisense RNA probes are listed in Table 1. The concentration and quality of the probes were determined by spot test and spectrometry (Nanodrop ND-1000). *In situ* hybridization was performed in paraffin embedded sections of adult female lice and copepodids as previously described by Dalvin *et al.* [42] and Eichner *et al.* [43] with some modifications. Histoclear (National Diagnostic) was used to deparaffinize the sections and proteinase K treatment was carried out for 18 minutes. Sections were hybridized with DIG-labeled RNA probes (1500 ng/100µl) at 65˚C for 20 hr. Afterward, sections were incubated with anti-DIG-alkaline phosphatase Fab fragments (Roche) and visualized using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche). Sense probe was used as a negative control. Pictures were obtained with an Axio Scope.A1 microscope (Zeiss).

Immunofluorescence

Immunofluorescence was performed on paraffin-embedded sections of adult female lice. Tissue sections were deparaffinized and rehydrated in a series of graded alcohols. Tissues were

blocked with 5% goat serum and 0.1% BSA for 30 minutes. After blocking, sections were incubated with 1:200 dilution of a polyclonal antibody of *Blattella germanica* LpR [32] (a generous gift from Maria-Dolors Piulachs, Institut de Biologia Evolutiva, IBE, Barcelona, Spain). The primary antibodies were detected using goat-anti-rabbit Alexa fluor 488 conjugated secondary antibodies (1: 100, Invitrogen) for 1 hr at room temperature. Sections were washed and mounted with ProLong Antifade mounting media containing DAPI (Life Technologies). Pictures were obtained using a Leica fluorescence microscope.

Hematoxylin and erythrosine staining

Paraffin-embedded sections of copepodids were stained with hematoxylin and erythrosine according to the procedure as described by Eichner et al. [43]. Briefly, sections were incubated at 65°C for 30 min, dewaxed in histoclear followed by rehydration in a series of graded alcohols. Afterwards, slides were put into distilled water and stained with hematoxylin (Shandon Instant Hematoxylin, Thermo Scientific) for 2.5 min and with 1% erythrosine (Certistain, Merck) for 1.5 min. After staining, slides were washed several times in distilled water and mounted in Histomount (Invitrogen).

Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was carried out on Applied Biosystem 7500 Real-Time PCR system using PowerUp SYBR Green Master Mix (Applied Biosystem) according to the manufacturer's instructions. Primers used in RT-qPCR are listed in Table 1. A standard curve was generated using a two-fold serial dilution (six dilutions) of cDNA to estimate the RT-qPCR assay efficiency. RT-qPCR was performed under the following conditions: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. At the end of the amplification cycles, a melting curve analysis was performed at 60–95°C. As the efficiency of the assay ranged from 95% to 100%, all the assays were carried out simultaneously for *LsLpR* and *ef1α* using the same cDNA and master mix along with two negative controls, a non-template control (NTC) and a reverse transcriptase negative control (-RT). The salmon louse Elongation factor 1 alpha (*ef1α*) was used as a reference gene [44]. All samples were run in duplicate under the following conditions, and Ct (cycle threshold) values were averaged. The expression levels of *LsLpR* was normalized to the expression level of *ef1α*, and the final results were analyzed using $2^{-\Delta\Delta CT}$ method [45]. Relative expression of *LsLpR* in all RNAi experiments was calculated using the control group as a calibrator. Relative expression levels of *LsLpR* were determined in various developmental stages of salmon louse using chalimus I as a calibrator.

Production of double-stranded RNA (dsRNA)

Double-stranded RNA (dsRNA) was prepared using Megascript RNAi kit (Ambion). Three different fragments targeting different regions of LsLpR mRNA were amplified by PCR with primers including T7 bacteriophage promoter sequence. The lengths of the dsRNA fragments were, fragment 1; 804 bps (corresponding to nt 1690 to nt 2494 in *LsLpR* mRNA GenBank accession no MF435899), fragment 2; 571 bps (nt 1235 to nt 1804) and fragment 3; 489 bps (nt 2652 to nt 3140). A fragment of 850 bp from cod trypsin (*CPY185*) was used as a control [46]. PCR products were used as templates for the synthesis of sense and antisense RNAs by in vitro transcription using T7 polymerase. Equal volumes of sense and antisense RNAs were pooled, incubated at 75°C for five min and slowly cooled to room temperature. Finally, dsRNAs were purified; concentrations were measured with Nanodrop ND 1000 Spectrophotometer and stored at -20°C until further use.

RNA interference (RNAi) in nauplii

To knock-down the *LsLpR* in nauplius I larvae, three RNAi experiments were performed separately and each experiment was repeated five times. The first and second RNAi experiments were performed using dsRNA fragment 1 and fragment 2 respectively. The third RNAi experiment was conducted using a combination of dsRNA fragment 1, and 3. Primers used to produce dsRNA used in each experiment are shown in Table 1. All RNAi experiments were performed according to procedure as described in [47]. For all experiments control group was included and animals were treated with dsRNA complementary to *CYP185*. Briefly, egg-strings were gently removed from adult female lice and transferred to flow-through wells. After hatching from the egg-strings, approximately 50 nauplius I larvae were collected for each experimental group in 150 µl of seawater and transferred into Eppendorf tube cap. Nauplii I larvae were incubated overnight (17h) with 1.5 µg of dsRNA. When nauplius I larvae had molted into the nauplius II stage, all animals were transferred into incubators with flow-through sea water supply. *LsLpR* dsRNA-treated animals were inspected daily to detect any abnormal phenotype and the experiment was terminated when the animals reached the copepodid stage 7 days post-hatching (dph). Animals were sampled into RNAlater™ (Ambion) for RT-qPCR analysis.

Knock-down of LsLpR in pre-adult and adult female lice

The *LsLpR* gene transcript knock-down experiments were done in pre-adult II female lice using previously described three non-overlapping dsRNA fragments. In each experiment, female lice were injected with dsRNA as described in [46] and kept in sea water for 4 hrs. Afterwards, equal numbers (n = 10–13) of dsRNA treated female and untreated male lice were put back on a single fish and a total of three fish were used in each experiment. Each RNAi experiment was terminated when control dsRNA injected female lice had become adults and had produced the second pair of egg-strings. Female lice with or without egg-strings were photographed and examined for changes in gross morphology. Subsequently, the egg-strings were gently removed with forceps, placed into individual hatching incubators and monitored daily to record hatching and developmental progress. Larvae were counted at 9 dph when all control animals were fully developed to copepodids. All lice were sampled and collected in RNAlater (Ambion) for RT-qPCR analysis.

In a single experiment, adult female lice (n = 30) were injected with a combination of *LsLpR* dsRNA fragment 1 and 3. Five injected female lice plus equal amount of untreated male lice were put back per single fish and a total of six fish were used. Same numbers of lice and fish were used for the control group. Lice were recovered after 5, 10 and 15 days post-injection for RT-qPCR analysis.

Infections of Atlantic salmon with LsLpR knock down copepodids

For infection trials, RNAi was carried out on nauplii I larvae as described above. Five biological parallels each contained approximately 50 nauplii I larvae were treated either with a combination of *LsLpR* dsRNA fragment 1 and 3 or control dsRNA. After that, all the samples were transferred into incubators with flow-through sea water supply. When nauplii molted into copepodids, around 20 copepodids were collected from each parallel for RT-qPCR analysis and remaining copepodids were used for infection of two fish. Each fish in a single fish tank was infected with 60 copepodids according to protocol as described in [43]. The same procedure was followed for the control group. The experiment was terminated when adult female lice of control group produced second pair of egg-strings. All female lice with or without egg-strings were inspected for any gross morphology changes and photographs were taken under

microscope. Egg-strings were removed from lice and put into hatching incubators, while female lice were collected for RT-qPCR analysis. Copepodids hatched from these eggs were counted at 9–10 dph.

Oil Red O staining

Adult female lice were collected directly from the host. Nauplii and copepodids were collected from hatching incubators. Unfertilized eggs from the genital segment and ovaries were dissected from the adult female lice. All the samples were washed three times with cold PBS and fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4). Female lice were fixed overnight while larvae, ovaries and unfertilized eggs were fixed for 2 hrs. Oil Red O stain was performed according to the previously described method [48] with some modifications in the length of time when adult lice were stained. After fixation, all samples were washed three times with ice-cold PBS and resuspended in 60% isopropanol for 10–30 minutes. Larvae and tissue samples were stained with Oil Red O (Sigma-Aldrich) for 0.5 hr while adult lice were stained for 2 hrs. After staining, samples were washed in ice-cold PBS and rinsed with 60% isopropanol. Pictures were obtained with a Leica Model MZ6 stereomicroscope directly or after mounting. For semi-quantification of total neutral lipids, stain was extracted from RNAi copepodids using 200 μl of 100% isopropanol and absorbance were measured at 500 nm in duplicates. Background signal was subtracted using the 100% isopropanol as a background control.

Protein modelling and bioinformatics analysis

Three-dimensional structure of extra-cellular domains (ligand binding domain from repeat R3-R8 and EGF-precursor domain) of LsLpR protein was modelled using Phyre2 online server [49]. Modelled protein structure was refined using Modrefiner [50]. Calcium ions binding sites were predicted using Raptor X Binding online server (http://raptorx.uchicago.edu/BindingSite/) or COACH for protein-ligand binding site prediction (http://zhanglab.ccmb.med.umich.edu/COACH/) [51]. Various domains of LsLpR protein was predicted using SMART (http://smart.embl-heidelberg.de/) [52]. Signal peptide was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [53]. Molecular weight and the theoretical isoelectric point of protein was predicted on expasy (http://web.expasy.org/compute_pi/).

Results

Sequence analysis of LsLpR

A full-length cDNA encoding LsLpR was isolated from adult females of *L. salmonis*. The full-length transcript was 4007 nucleotides, containing an open reading frame (ORF) of 2859 bp, a 5′-untranslated region (UTR) of 162 bp and a 3'UTR of 986 bp. The ORF of *LsLpR* encodes a putative protein consisting of 952 amino acids, with the signal peptide at position 1–23, the predicted molecular weight (Mw) of 107.04 kDa and the theoretical isoelectric point (pI) of 4.81. The exons-introns analysis revealed that LsLpR gene is composed of 16 exons spanning 115.1 kbp (Fig 1A). The second intron was the largest, spanning about 44.2 kbp.

A BLAST search (http://www.uniprot.org/blast/) against the UniProtKB/Swiss-Prot revealed that LpR of *L. salmonis* shared 46% identity (70.8% similarity) with the Lipoprotein receptor 1 from the crustacean *Pandalopsis japonica* and ~48–53% identity (~74–77% similarity) with insect LpRs such as *Locusta migratoria* (migratory locust), *Aedes aegypti* (yellow fever mosquito), *Galleria mellonella* (wax moth), *Bombyx mori* (silk moth), *Blattella germanica* (German cockroach) and *Drosophila melanogaster* (fruit fly). Besides insect LpRs, the *L. salmonis* LpR shared ~38–40% identity (~66–68% similarity) with VLDLRs of oviparous vertebrates
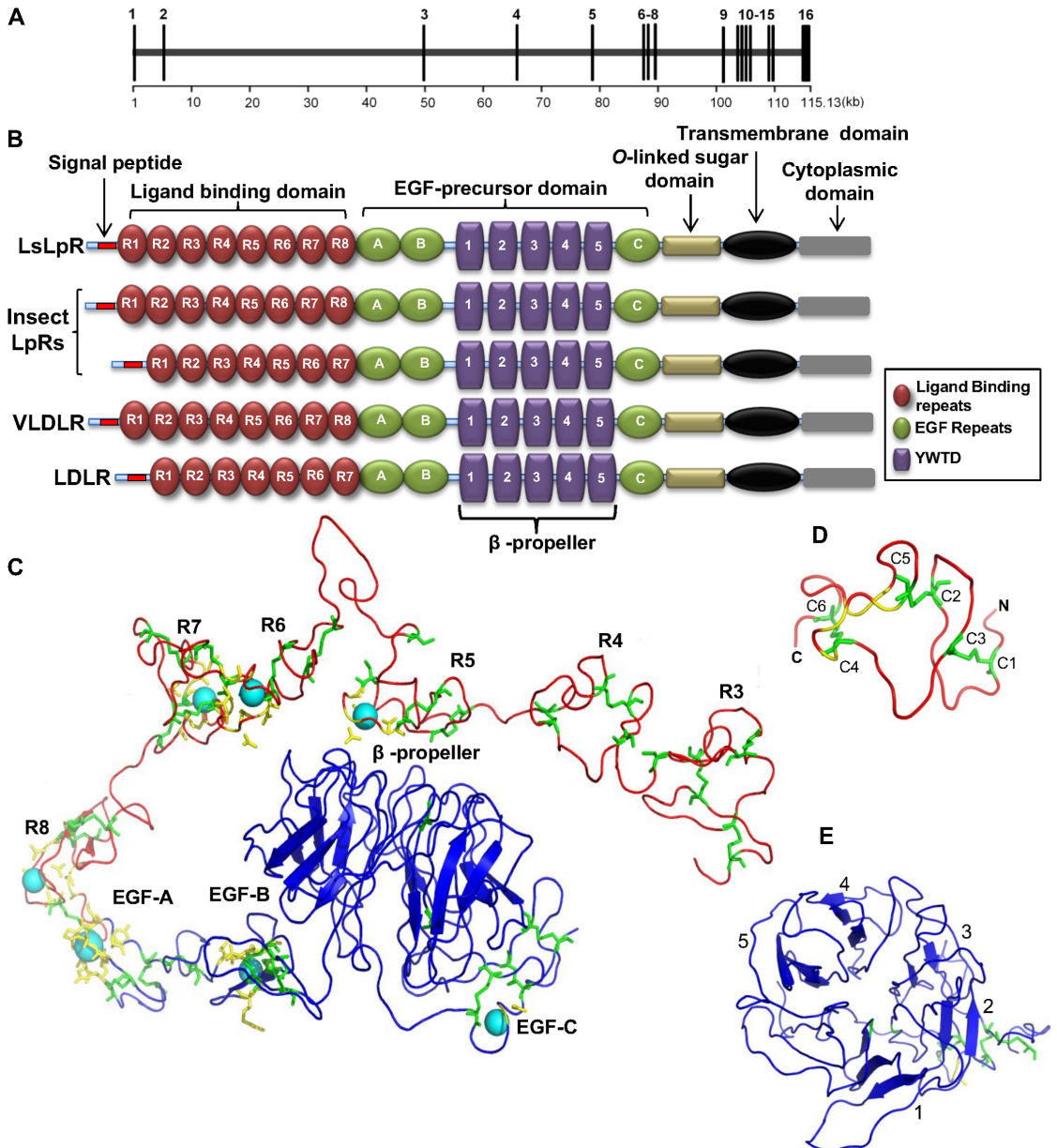
Fig 1. Exon-intron organization and structural analysis of LsLpR. (A) LsLpR gene is composed of 16 exons separated by 15 introns and spanning a genomic region of 115.13 kbp. (B) Domains organization of LsLpR with other members of LDLR family. (C) Modelled structure of extracellular domains of LsLpR using PHYRE protein structure prediction program. Cysteine residues are coloured green, yellow residues provide pocket for calcium ion and bound calcium ions are shown as cyan spheres. (D) Single repeat from ligand binding domain shows the three disulphide bonds (C1-C3, C2-C5 and C4-C6). (E) Top view of β–propeller domain with five F/ YWXD motifs.

such as *Salmo salar*, *Danio rerio* (zebrafish), *Gallus gallus* (chicken), *Anas platyrhynchos* (duck) and *Xenopus tropicalis* (frog) and ~36% identity (~67% similarity) with LDLRs of mammals including *Homo sapiens* (human), *Sus scrofa* (wild boar), *Bos taurus* (cattle), *Mus musculus* (mouse) and *Rattus norvegicus* (rat).

Structural analysis of LsLpR

To analyze the structural and functional domains of LsLpR which are common to members of LDLR superfamily, SMART annotation and multiple protein sequence alignment was carried out. The ligand binding domain (LBD) of LsLpR contained eight cysteine-rich repeats (Fig 1B and S1 Fig). Each cysteine repeat contained six cysteines as shown in modelled extracellular region of LsLpR (green residues in Fig 1C) based on X-ray crystal structure of human LDLR (PDB ID: 1N7D) used as a template [54]. These six cysteines in each repeat formed three pairs of disulphide bonds (C1-C3, C2-C5 and C4-C6) (Fig 1D) which was essential for ligand-receptor interaction [55]. Furthermore, in each repeat a Ca^{2+} binding site was predicted as shown in R5-R8 (Fig 1C and S1 Table) which was considered essential for disulphide formation and correct folding of LpR [56, 57]. Next to the LBD followed the epidermal growth factor (EGF) domain which was important for acid-dependent dissociation of ligands. The EGF domain was composed of three EGF-precursor repeats, and each repeat contained six cysteine residues that made up three pairs of disulphide bonds and a Ca^{2+} binding site (Fig 1C). The EGF domain also contained five F/YWXD tetra-peptide motifs (S2 Fig) required for the formation of a β–propeller structure (Fig 1E) [58]. The predicted *O*-linked sugar domain of LsLpR was composed of a short amino acids sequence consisting of 69 amino acids with phosphorylation sites. The predicted transmembrane domain (TMD) of LsLpR (Fig 1B) contained 23 amino acids helix (AGFMAGVAIGIGAGVILLLFLVL) which was greatly enriched in hydrophobic residues as seen in other LpRs and in other members of LDLR family. TMD-helix acts as membrane anchor [22, 23]. The TMD was followed by the cytoplasmic domain. The cytoplasmic domain of LsLpR carried one copy of NPXY motif (S3 Fig) that is needed for the clathrin-mediated internalization of receptor-ligand complex, and well conserved in LpRs and members of LDLRs family belonging to other species [59]. Presence of several phosphorylation sites in the cytoplasmic domain of LpRs suggested that they are involved in the signal transduction [59]. However, so far there has been no experimental data in insects which support the signal transduction function of LpR [60].

Phylogenetic analysis

Phylogenetic analysis to reveal evolutionary relationships between LsLpR and lipoprotein receptors from other species is shown in Fig 2. The analysis showed that LsLpR was grouped together with LpRs from decapods and insects. The analysis also revealed that vertebrate lipoprotein receptors (VLDLRs, LDLRs and VgRs) were closely related to each other and closest to decapod/insect LpRs than to VgRs of decapods and insects.

Expression of LsLpR and distribution of lipids

RT-qPCR analysis was conducted to measure the expression level of *LsLpR* in the different developmental stages of the salmon louse. Expression of *LsLpR* was detected in all the tested developmental stages, with the lowest expression detected in chalimus and pre-adult stages (Fig 3). In larval stages, the lowest expression of *LsLpR* was seen in nauplii I, gradually increased in nauplii II and reached the highest observed level in copepodids (Fig 3). In the mobile stages, the highest *LsLpR* transcript level was detected in the adult female (Fig 3).
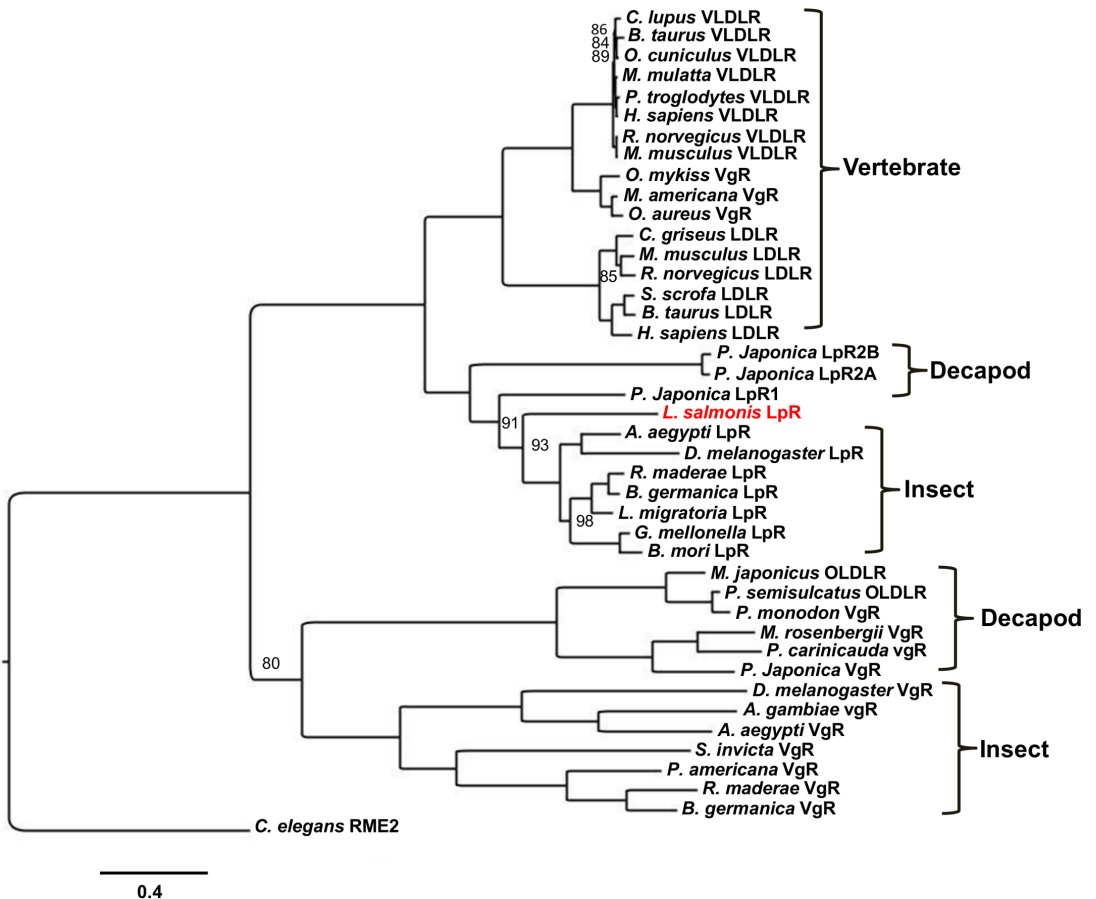
Fig 2. Phylogenetic tree of selected lipoprotein receptors from vertebrates and invertebrates. The tree was generated using Bayesian methods. LpR of L. *salmonis* (LsLpR) is shown in red. The yolk receptor (RME2) of the nematode (*C. elegans*) was used as an out-group. The nodes are labelled with posterior probabilities and for clarity only values < 100 are shown. The scale bar represents 0.4 amino acid substitutions per site.

https://doi.org/10.1371/journal.pone.0195783.g002

Neutral lipids were detected in adults and larvae of salmon louse by Oil Red O stain (Fig 4). In adult stages (Fig 4A and 4B), storage of lipids was detected in adult females (Fig 4B), mainly in unfertilized eggs and ovaries (Fig 4I and 4II). In larval stages (Fig 4C–4E), maternally derived lipids were found in the yolk (Fig 4C and 4D), which were utilized by the larvae before their settlement to new host fish (Fig 4E).

Distribution of LsLpR transcripts in adult female lice and copepodids

In situ hybridization was performed to examine the distribution of *LsLpR* transcripts. In copepodids, the highest expression of *LsLpR* transcripts was found in the neuronal somata of the brain and the intestine (Fig 5A). In adult female lice (Fig 5C), *LsLpR* transcripts were detected in the lumen of the coiled tubules of the ovaries (Fig 5D) and the outer membranes of the
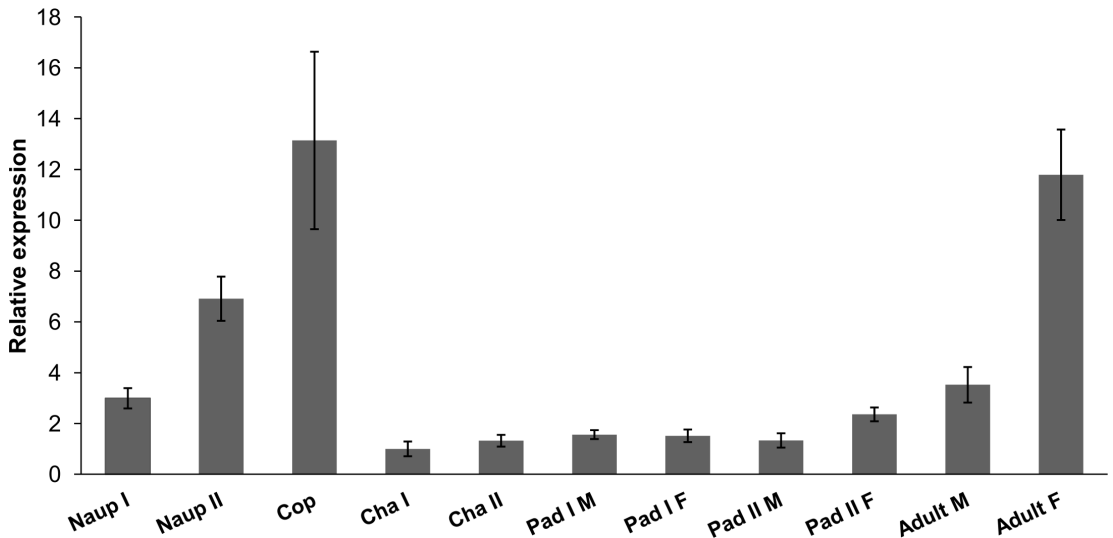
Fig 3. Expression analysis of the *LsLpR* in various developmental stages of the salmon louse. Expression levels of *LsLpR* in chalimus I was set as 1. Error bars represent the standard deviation (n = 5 samples for each stage). Abbreviations: Naup I, Nauplii I: Naup II, Nauplii II: Cop, free-living copepodids: Cha I, Chalimus I: Cha II, Chalimus II: Pad I M, Preadult I male: Pad I F, Preadult I female: Pad II M, preadult II male: Pad II F, Preadult II female.
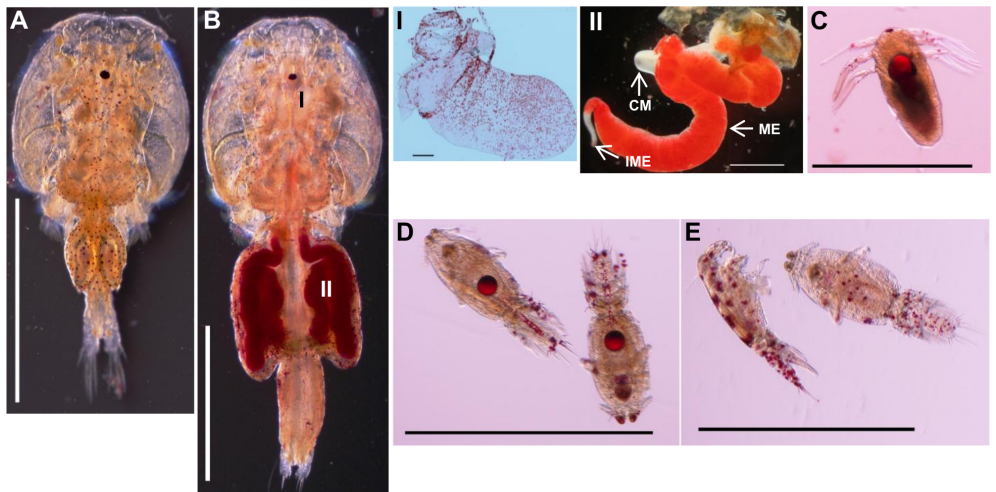
Fig 4. Staining of neutral lipids in salmon lice. Detection of neutral lipids by Oil Red O stain. Adult male (A) and adult female (B). Storage of lipids was detected mainly in mature eggs (II) but also in the ovary (I), of adult female lice. Maternally deposited lipids were found as droplets in the yolk of hatching nauplii (C). A reduction in lipid reserves was noted in copepodids of 7 dph (D) compared to newly hatched nauplii and no lipid droplets were found in copepodids (E) after 10 days of their hatching. Scale bars = (A, B, BII, C-E) 1 mm, (BI) 200 μm. Abbreviations: CM, cement gland; ME, mature eggs; IME, immature eggs.

vitellogenic oocytes ([Fig 5E](#)). Furthermore, semi quantitative RT-PCR was performed using cDNA of the selected tissues of the adult female lice confirming the results from the *in situ* hybridization ([S4 Fig](#)).
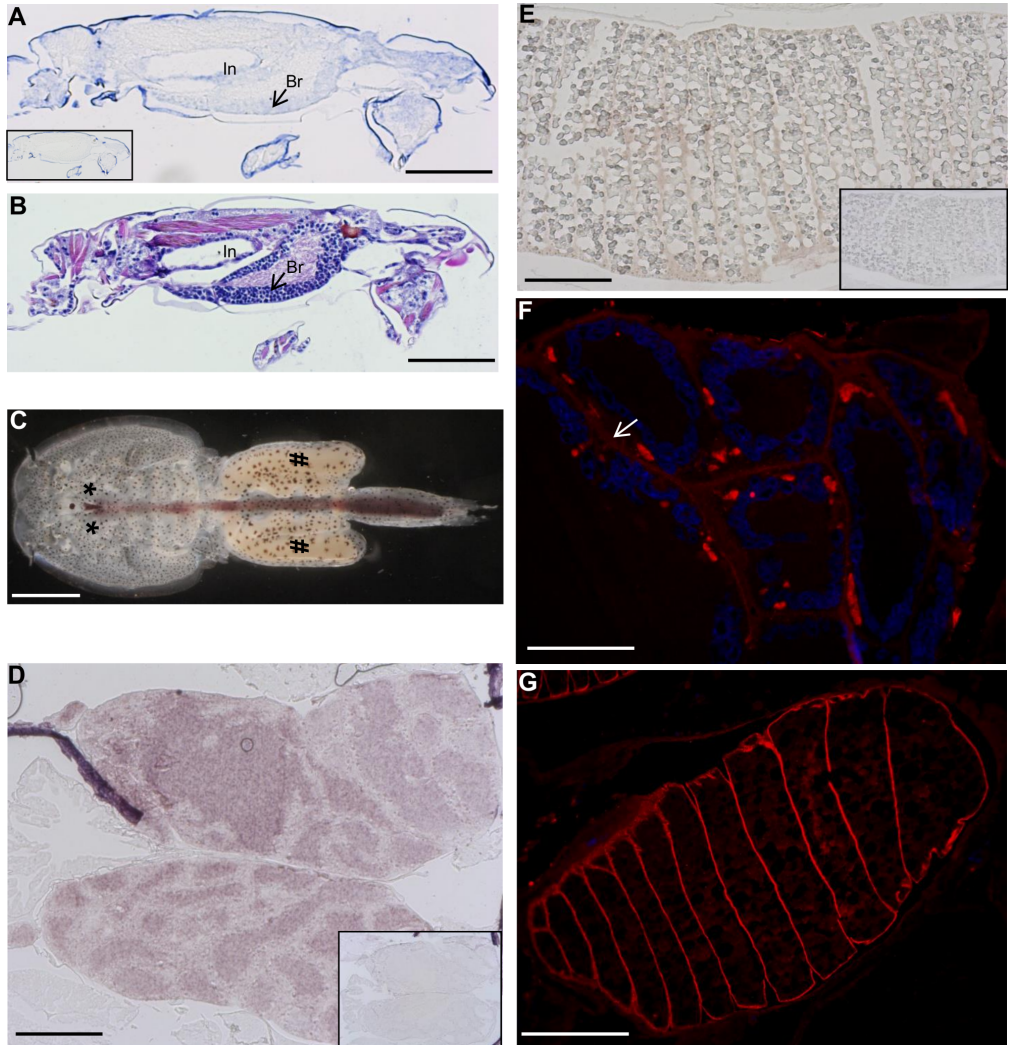

Fig 5. Localization of LsLpR mRNA and protein in the salmon lice. (A), (D) and (E) in situ hybridization. (A) Localization of the *LsLpR* transcripts in the intestine (In) and neuronal somata of the brain (Br) of copepodid. (B) Parallel slide of the copepodid stained with hematoxylin and erythrosine. (C) Dorsal view of an adult female without egg-strings. The asterisks (*) and hashtags (#) indicate the positions of the ovaries in the cephalothorax and mature vitellogenic oocytes in the genital segment of adult female louse respectively. (D) Localization of the *LsLpR* mRNA in the lumen of the ovarian tubules. (E) Localization of the *LsLpR* mRNA in the vitellogenic oocytes in the genital segment. No stain was seen in slides (small inserts) hybridized with sense RNA probe. (F) and (G) immunofluorescence with anti LpR. (F) Distribution of LsLpR protein was found in elongated structures, at the inner side of the tubular membrane (white arrow) together with the nuclei of the oocytes (nuclei were stained blue with DAPI). (G) Distribution of the LsLpR protein in the outer membrane of the vitellogenic oocytes. Scale bars indicate (A-B, E) 200 μm, (C) 1 mm, (D and G) 100 μm, (F) 50 μm.

Distribution of LsLpR protein in adult female lice

The presence of LsLpR protein was detected in sections of adult female lice using antibodies raised against LpR of *Blattella germanica* (see Materials and Methods). In ovaries, LsLpR protein was localized in elongated structures, found at the inner side of the tubular membrane together with the oocytic nuclei (Fig 5F). LsLpR was also seen in the outer membrane of the vitellogenic oocytes (Fig 5G), where the *LsLpR* was transcribed (Fig 5E). Moreover, no fluorescence was detected in the control slides treated with secondary antibody only.

Knockdown of LsLpR in nauplii by RNA interference (RNAi)

RNAi was induced in nauplius I to access the functional role of *LsLpR* in the larval stages. Three dsRNA fragments (see materials and methods) were produced and utilized in separate RNAi experiments. In the first and second RNAi experiments dsRNA fragment 1 and dsRNA fragment 2 were utilized and transcription of *LsLpR* was decreased by 54% and 44% as compared to control groups respectively (Fig 6A). The third RNAi experiment was conducted using a combination of dsRNA fragment 1, and 3 and *LsLpR* expression was decreased by 50% as compared to control animals (Fig 6A).

However, no gross phenotype or change in survival between control and *LsLpR* dsRNA treated groups was observed. No major difference was found in the lipid staining in the yolk of copepodids developed from nauplii treated with dsRNAs against *LsLpR* and control (Fig 6B–6D).

Knockdown of LsLpR in Pre-adult II and adult female lice by RNAi

Three separate RNAi experiments were conducted in pre-adult II female lice and analysed when adult females from control groups had produced the second pair of egg-strings. Eggs from all experimental groups were followed through hatching and development to copepodids. Each experiment was performed with a single dsRNA fragment, or with a combination of two dsRNA fragments (Table 1). The level of *LsLpR* transcripts was measured by RT-qPCR in adult female lice. No significant reduction in mRNA expression levels was observed in lice injected with single dsRNA fragment or a combination of two dsRNA fragments at the time of termination (Fig 7A). Moreover, no significant effect on morphology and survival rate was noted between females injected with *LsLpR* or control dsRNA, but the number of hatched copepodids per adult female was significantly lower (reduced by 72% ($p < 0.05$, *t* test)) in the *LsLpR*-injected group of experiment 2 compared to the control group (Table 2).

To see if duration of dsRNA treatment influenced knock down efficiency, adult female lice were injected with LsLpR dsRNA (fragments) and the level of *LsLpR* transcripts was measured at days 5, 10 and 15 (Fig 7B). RT-qPCR results showed that RNAi of *LsLpR* gene could not be detected before day 10. At day 15 transcript levels were reduce by 30% compared to control ($p < 0.05$, *t* test).

Infection trial and LsLpR knock down

LsLpR knock down (fragments 1 + 3) in nauplii I and level of transcripts were measured by RT-PCR in copepodids (7 dph). In copepodids transcription was decreased by 60% compared to the control group (Fig 7C). Afterwards, Atlantic salmon were infected with the copepodids from the knock down experiment in single fish tanks and maintained on the fish until the lice had developed into adults. Adult female lice were collected and expression of *LsLpR* was measured by RT-PCR. No significant reduction in transcript levels was observed in the adult female lice when compared to control group (Fig 7C). The number of lice recovered from
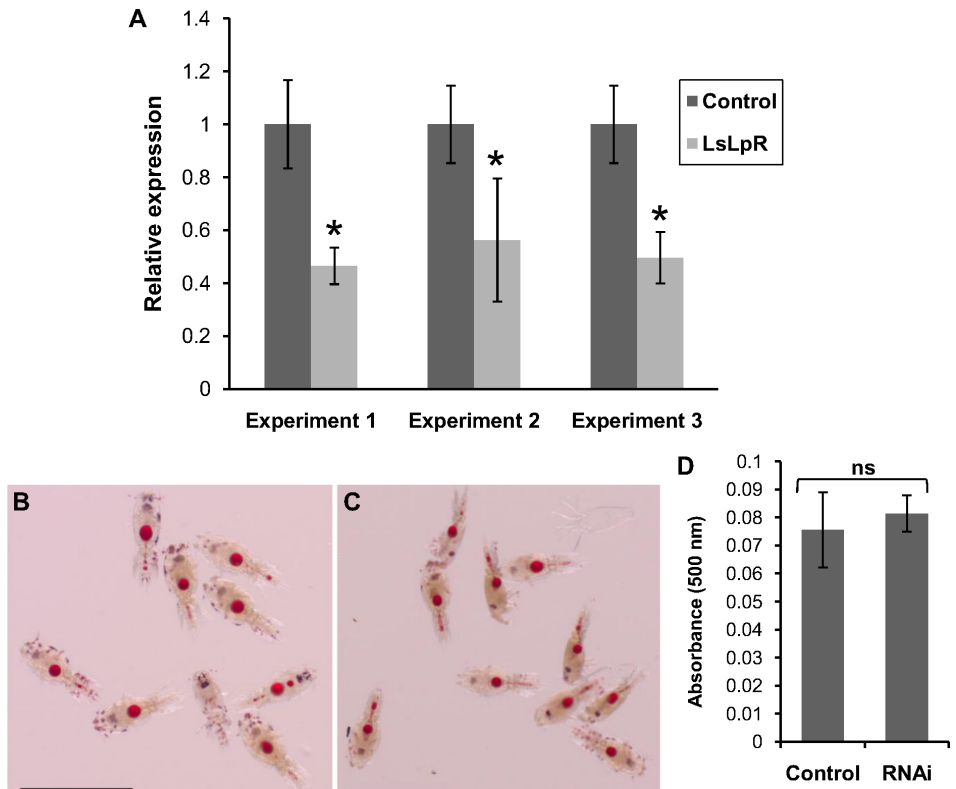
Fig 6. Effect of RNAi on *LsLpR* transcript and lipid levels in copepodids. (A) Relative Expression of *LsLpR* in the copepodids (7 dph) after knock downed in nauplius larva. Error bars show standard deviation. Asterisk represents significant difference (independent-samples T-test, p < 0.05) in mRNA levels of *LsLpR* between the control group (n = 5) and the knock-down group (n = 5). (B-D) Detection of neutral lipids by Oil Red O stain. Lipid contents in the copepodids hatched from *LsLpR* (fragments 1 + 3) (B) and control dsRNAs treated nauplii (C). Semi-quantification of total neutral lipids with Oil Red O stain in copepodids (n = 5, each replicate contains 25 animals) developed from nauplii treated with control and *LsLpR* dsRNAs (fragments 1 + 3) (D). No significant difference (independent-samples T-test, p > 0.05) was found between control group and *LsLpR* dsRNA-treated group. Scale bars = (B-C) 1 mm.

https://doi.org/10.1371/journal.pone.0195783.g006

LsLpR dsRNA treated group was 30% less than the number of lice recovered from control dsRNA treated group (Table 3). However, no gross abnormal phenotype difference was observed between control and *LsLpR* dsRNA treated groups. Female lice of both groups produced normal egg-strings and equal number of hatched copepodids were found from both groups (Table 3).

Discussion

In this study, a molecular characterization of the *LpR* from the salmon louse was carried out for the first time. A single copy gene encoding *LsLpR* was identified in the salmon lice genome. Exon-intron organization revealed that LsLpR gene is composed of 16 exons separated by 15 introns. The organization of exons-introns in silkworm, *B. mori* for LpR gene has previously been described [31]. The silkworm LpR1 (*Bm*LpR1) was composed of 16 exons interrupted by
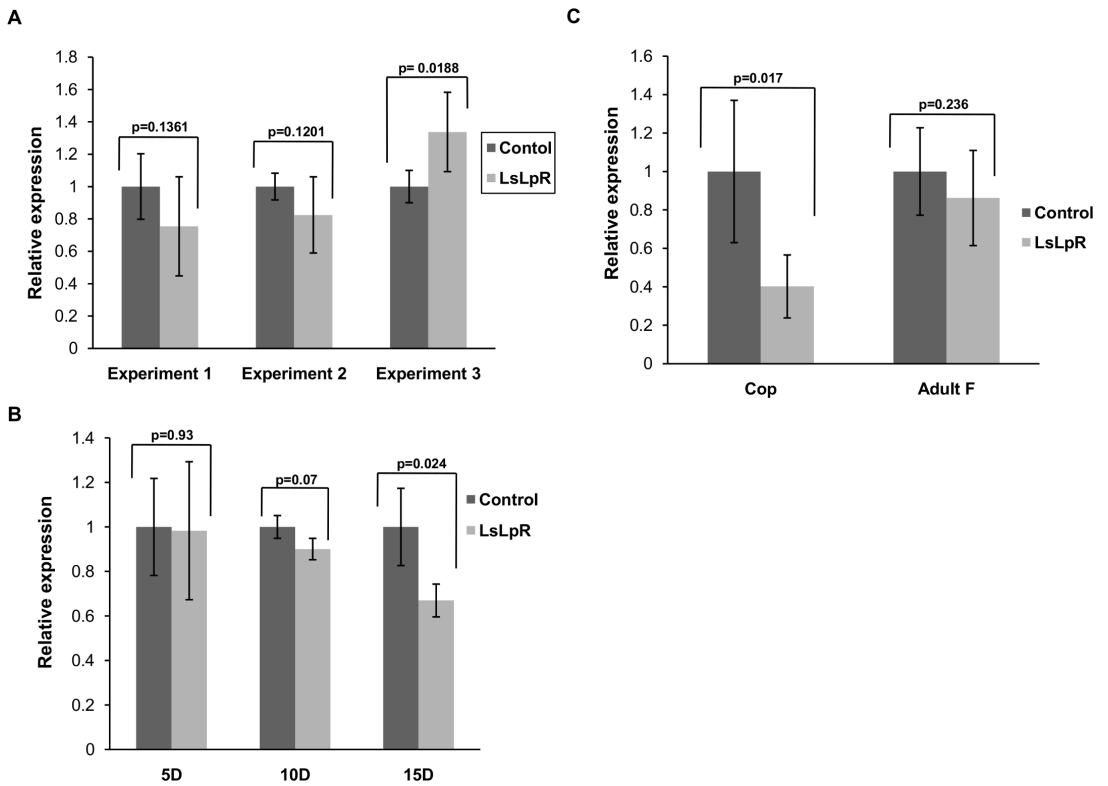
Fig 7. Treatment with dsRNA against *LsLpR*. (A) Relative expression of *LsLpR* in the adult females after injection of dsRNA in pre-adult females (30–32 days post injection). (B) Relative expression of *LsLpR* after injection of dsRNA (fragment 1 + 3) in adult females and measured at days 5, 10 and 15 (post injection). Expression PCR was carried out on 5 female lice from control and knock-down group at each time point. (C) Relative expression of *LsLpR* in copepodids (n = 5 × 20) after knock down (fragments 1 + 3) in nauplii I, assayed before the infection of a host and in adult female lice at the time of termination of the experiment. Error bars show standard deviation and P-values for independent-samples T-test analysis are shown, expression levels of LsLpR in control versus LsLpR dsRNA-treated group.

15 introns that span about 122 kbp. Whereas, other isoforms such as LpR2, 3 and 4 contained 15 exons separated by 14 introns. The second intron of *Bm*LpR was the largest that span >65 kbp similar to LsLpR where second intron span 44.2 kbp. BLAST searches showed that LsLpR

Table 2. Summary of the RNAi experiments.

Experiment #	Fragment #	Total Lice injected	Total Lice recovered	No of females carrying Eggs	Average no of copepodids hatched per louse	No of female lice analyzed in RT-qPCR
1	Fragment 1	40	11	11	415±42.5	9
	Control	37	5	5	370.5±55.8	5
2	Fragment 2	30	20	20	145±104	6
	Control	30	18	17	516±166.8	6
3	Fragments 1+ 3	31	23	23	500±105	7
	Control	30	28	28	520±95	6

Table 3. Summary of the infection trial experiment.

Fragment #	No of female lice recovered	No of Male recovered	Female lice which produced egg-strings	Average no of copepodids hatched	No of female lice analyzed in RT-qPCR
Fragments 1+ 3	16, 12	7, 9	16, 12	248±74	5, 5
Control	22, 22	11, 8	22, 17	259±56	5, 5

Nauplii I larvae treated with dsRNA (fragments 1 + 3) from *LsLpR* were sampled as copepodids and used to infect Atlantic salmon, counted as adults (male and female) and if females produced eggstings and finally if the eggs hatched and produced normal copepodid larvae. The numbers represent recovered larvae and adult sea lice from two fish.

shared the highest amino acid identity and similarity with LpR of decapods and insects. Phylogenetic analysis placed the LsLpR along with other crustacean and insect LpRs and showed that vertebrate VLDLRs/VgRs and LDLRs were closely related to each other and appeared as a sister group of the decapod/insect LpRs. The VgRs of vertebrates did not group together with decapod/insect VgRs indicating that they have evolved independently.

Structural analysis revealed that LpR shared the same structural domains as found in other members of LDLR superfamily. The LBD of LpR usually consist of several cysteine-rich repeats, eight in LsLpR (Fig 1) which was identical to LBD of several insect LpRs such as *B. mori*, *L. maderae* and *L. migratoria*, LpR1 of crustacean *P. Japonica* and vertebrates VLDLRs/ vitellogenin receptors. However, LBD of some insect LpRs contains seven cysteine-repeats and are structurally identical to LDLRs (Fig 1) [32, 61, 62]. The existance of these repeats in the LBD is imporant for their binding to ligand and the acquisition of cellular lipids but the importance of the numbers of cysteine-repeats in the LBD is not known [62]. LsLpR also contains an EGF-precursor domain which is involved in the acid-dependent displacement of the ligand from the LBD as observed in LDLR-LDL complex at endosomal pH [54, 63, 64]. However, the insect LpR-HDLp complex is not dissociated under an acidic environment, which supports the concept of ligand recycling [65]. The structures of extracellular (LBD and EGF-precursor) domains of human LDLR have been solved by X-ray [54]. The LsLpR shared similar structures when modelled against LDLR. Similar results were found as seen in LsLpR when Locust LpR were modelled against LDLR and it was suggested that despite their high structural similarity, the specificity of both receptors (LDLR and LpR) for their ligands is mutual exclusive [66, 67]. In LsLpR, the EGF-precursor domain followed the 69 residues long *O*-linked sugar domain (OLSD). All insect LpRs contain OLSD, however, the length varies in different insect species [28]. For example, OLSD of *L. maderae* is consisting of 70 residues whereas the length of OLSD of *A. aegypti* is over 250 residues. Moreover splice variants have been reported that affects this region of OLSD in LpR from other insect species including *B. germanica*, A. *aegypti*, *G. mellonella* and *B. mori* [29, 32, 68, 69]. A single copy of well conserved NPXY internalization motif was found in the cytoplamsic domain of *LsLpR*. The three-dimensional structure prediction and multiple protein sequence alignment both revealed that the sequence of LsLpR contained all structural motifs which are common in LpRs and in other members of LDLR family.

In insects, lipids are transported by the Lp from the fat body to oocytes through receptor mediated endocytosis [18, 33]. Generally, the expression of *LpR* transcripts was observed throughout the ovarian development and increased during vitellogenesis of several insect species including *A. aegypti*, *L. maderae*, *B. germanica*, *S. ricini*, *B. mori* and *D. melanogaster* [28, 31–34, 62]. Here in salmon lice, high levels of mRNA and protein was found in the ovaries and vitellogenic oocytes of female. Accumulation of neutral lipids was also found in vitellogenic oocytes and ovaries of adult female lice. These results suggest that lipids may be transferred

directly from the intestine to growing oocytes and ovaries, where the receptor might be involved in the up-take of lipids to the developing oocytes.

Lipids are the major source of energy for the developing embryos in oviparous animals and 90% of the energy utilized by the developing embryos of *Culex quinquefasciatus* originates from lipids [70]. Similarly, maternally deposited lipids in the larvae of salmon lice are also major source of energy before their settlement to a new host. In larvae, lipids are transported as lipoproteins from their site of storage to different tissues during development. The mechanism of lipoprotein uptake by receptor-mediated endocytosis has been suggested in the fat body tissue of larval and young adult locusts [13, 27]. In salmon lice, the levels of expression and localization of *LsLpR* transcripts in larvae reached its highest level in copepodids where mRNAs of the receptor were found in the intestine and neuronal somata of the brain. These results are in agreement to the expression of *LpR* in the larvae of insect species. In larva of *S. ricini*, the expression of *srLpR7-1* was detected in fat body, brain, malpighian tubule, whereas low expression was observed in adult individuals [62]. Similarly in *B. mori*, the isoform *LpR-4* was expressed in the brain and central nervous system of larvae along with other developmental stages [31]. In adults of *L. migratoria* and *A. mellifera*, the expression of LpR was reported in the midgut [27, 35]. The distribution of maternally deposited neutral lipids in the larvae of salmon lice were found in the yolk of hatched nauplii, which were reduced after moulting into copepodids (7 dph) and complete depletion was noted in the aged copepodids (after 10 dph). Notably, the expression of *LsLpR* was highest in the 7 dph copepodids and therefore reflected the transfer of lipids from the yolk to different tissues to secure rapid growth and development.

To further elucidate the function of *LsLpR* in the salmon lice, RNA interference was performed to knock down the *LsLpR* in salmon lice. Three independent RNAi experiments were conducted in the larvae and a significant reduction in *LsLpR* transcripts was noted. However, no change in survival or swimming performance of copepodids were noted and utilization of lipids from yolk were similar in both control and *LsLpR* dsRNA treated groups. It is possible that the levels of knockdown achieved for LsLpR may not be sufficient to disrupt the mobilization of lipids from yolk to other tissues of larvae. Secondly, it is also possible that protein levels were still high within the time frame of these RNAi experiments for the supply of lipids to tissues during larval development. Similar lack of abnormal development was also achieved in the Tsetse fly where the LpR (*GmmLpR*) receptor was significantly knocked down. Here, lipid levels in hemolymph remained unchanged, and oocytes developed normally [71]. Likewise, three independent RNAi experiments were conducted in preadult II female salmon lice. No significant silencing of *LsLpR* was found with any of the three different RNAi fragments and all the adult females produced normal egg-strings. In all RNAi experiments normal development to the copepodid stages was observed; however, in one of the three experiments the numbers of hatched copepodids were reduced from females injected with *LsLpR* (fragment 2) as compared to control. Similar RNAi results were found in *S. ricini* [62]. The female pupae of *S. ricini* were injected with *LpR* dsRNA along with controls, but no considerable reduction in the mRNA level was found and no abnormalities in ovaries or egg production were noted. Furthermore, RNAi was conducted in *B. germanica* and reduction in Lp levels was noted in the ovary but no significant effect on the ovarian development and fertility was noted [32]. Moreover, in the fat body of *B. germanica*, the effects of RNAi began to disappear after three days and levels of *LpR* mRNA, and lipophorin contents increased. In salmon lice during infection trial, the *LsLpR* was knocked down in copepodids by 60%. No significant knock-down was observed in adult females that developed from these copepodids (approximately 60 days after infection). Moreover, RNAi experiment in adult females showed that the maximum knock down of *LsLpR* (30%) was only observed at days 15. Hence, it appears that *LsLpR* is difficult to

knockdown in adults while in larvae effect of knockdown is not achieved to a level where any obvious abnormal phenotype is observed. Further RNAi studies are needed in the future in different insect and crustacean species to explain the sensitivity of RNAi towards LpRs.

Supporting information

S1 Fig. Sequence alignment of ligand binding domain of LpR, VLDR, LDLR and Vtg receptor. Ligand binding domain of LsLpR, LpR from insects and crustacean (LpRs) and vertebrates and crustacean and VLDR, LDLR and Vtg receptors from vertebrates are aligned. Ligand binding domain of LsLpR is consisting of total eight ligand binding repeats (R1-R8) and each repeat contains six cysteine residues and marked with Asterisks.
(TIFF)

S2 Fig. Sequence alignment of EGF-precursor domain of LpRs VLDR, LDLR and Vtg receptors. Sequences of EGF-precursor domain of LsLpRs has been aligned to LpRs from insects and crustacean and vertebrates VLDR, LDLR and Vtg receptors sequences. EGF-precursor domain is consisting of three EGF repeats (EGF-1 to EGF-3) and each repeat contains six cysteine residues which are marked with Asterisks. Five (YWXD (F/Y)) motifs are also present in the EGF-precursor domain which are required for the formation of β–propeller structure.
(TIFF)

S3 Fig. Sequence alignment of cytoplasmic domain from LpRs VLDR, LDLR and Vtg receptors. Sequence alignment of cytoplasmic domain of LsLpR, LpRs of insects and crustacean and vertebrates VLDR, LDLR and Vtg receptors. The cytoplasmic domain of LsLpR contain one copy NPXY (X/V) motif which is required for the clathrin-mediated internalization of receptor-ligand complex.
(TIFF)

S4 Fig. Expression of *LsLpR* in various tissues of adult female lice. Equal amounts of total RNA from various tissues were reverse transcribed, and RT-PCR was carried out to determine the quantitative variations of *LsLpR* transcripts among samples as analysed on agarose gel. *Ef1a* was used as an internal control. Abbreviations: SQT, sub-cuticular tissue; IN, intestine; OV, Ovaries; OO, Oocytes.
(TIFF)

S1 Table. Predicted calcium binding sites in LsLpR. LBD, ligand binding domain: R, repeat: EGF, EGF-precursor domain.
(DOC)

Acknowledgments

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