Hematophagy in the salmon louse (*Lepeophtheirus salmonis*)

Characterization of genes and proteins involved in parasite blood-feeding

Erna Irene Heggland

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

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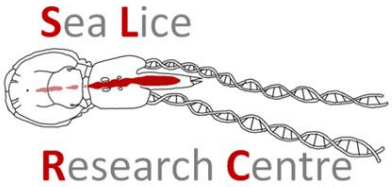

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Bergen, October 2019

Erna Irene Heggland

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List of publications

This thesis is based on the following papers, which are from here on out referred to by their Roman numerals.

Paper I

Erna Irene Heggland, Christiane Tröße, Christiane Eichner & Frank Nilsen (2019). «Heavy and light chain homologs of ferritin are essential for blood-feeding and egg production of the ectoparasitic copepod *Lepeophtheirus salmonis*». Molecular & Biochemical Parasitology 232, pp. 1-10. DOI: 10.1016/j.molbiopara.2019.111197

Paper II

Erna Irene Heggland, Christiane Eichner, Svein Isungset Støve, Aurora Martinez, Frank Nilsen & Michael Dondrup (2019). «A scavenger receptor B (CD36)-like protein is a potential mediator of intestinal heme absorption in the hematophagous ectoparasite *Lepeophtheirus salmonis*». Scientific Reports 9, pp. 1-14. DOI: 10.1038/s41598-019-40590-x

Paper III

Erna Irene Heggland, Michael Dondrup, Frank Nilsen & Christiane Eichner (2019). «Host gill attachment enables blood-feeding by the salmon louse (*Lepeophtheirus salmonis*) chalimus larvae, and alters parasite development and transcriptome». Manuscript, posted on the preprint server bioRxiv: https://doi.org/10.1101/815316

Abbreviations

AB	Abdomen
ALAD	Aminolevulinic acid dehydratase
ALAS	Aminolevulinic acid synthase
Blast	Basic local alignment search tool
CPOX	Coproporphyrinogen oxidase
CT	Cephalothorax
dsRNA	Double stranded RNA
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FECH	Ferrochelatase
GS	Genital segment
HCH	Heavy chain homolog
Heme	Iron protoporphyrin IX
HMBS	Hydroxymethylbilane synthase
HO	Heme oxygenase
LCH	Light chain homolog
PPOX	Protoporphyrinogen oxidase
RNAi	RNA interference
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative PCR
SLRC	Sea Lice Research Centre
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen synthase
w/w	Weight by weight

List of figures and tables

Summary

The salmon louse (*Lepeophtheirus salmonis*) is an obligate, hematophagous ectoparasite infecting salmonid fish such as the Atlantic salmon (*Salmo salar*). The parasite is at present the number one threat to the Norwegian salmon farming industry. There is a high density of hosts in salmon farms, and due to the high mutation rate and fecundity of the parasite, the salmon louse is currently ahead of the development of effective pest controls. Resistance has been reported against several available chemotherapeutants, and non-therapeutic interventions are as per now not sufficient to treat salmon louse infestations. Particularly adult female lice have blood as a major dietary component. This is a high-risk, high-reward strategy which the parasite is well-adapted to. Blood is highly nutritious and constantly renewed in the vertebrate host, but also contains toxic, yet necessary, compounds such as iron and heme (iron protoporphyrin IX). The salmon louse is likely a heme auxotroph, as many homologous enzymes of the conserved heme biosynthetic pathway are not found within its genome. As such, the salmon louse is innately dependent on its host for iron and heme supply. Blood-feeding and subsequent iron and heme trafficking are thus essential to study in the salmon louse, and could potentially reveal candidate proteins for pest management by e.g. vaccination or nutritional deprivation.

Ferritin is a well-conserved multi-subunit iron storage and distribution protein that can be either cytosolic or secreted. In the present study, several ferritin-encoding genes were identified in the salmon louse genome (*LsFer1*, *2*, *3* and *4*). Secreted heavy chain homolog (*LsFer1*) and secreted light chain homolog (*LsFer2*) transcripts were found to be expressed in the salmon louse intestine, where the precursor protein is probably loaded with iron intracellularly before it is secreted to the hemolymph. RNA interference-mediated knockdown of these two transcripts resulted in severe phenotype alterations for the adult female salmon louse. In both knockdown groups, it was unable to produce proper egg strings, and with an almost complete egg hatching failure. Histological sections of knockdown animal genital segments revealed that the developing oocytes lacked the structure and integrity seen in normal developing oocytes. Furthermore, the knockdown animals lacked the ability to fully engorge in

salmon blood. Additionally, in wildtype lice, the transcript levels of *LsFer1* and *LsFer2* were decreasing during starvation, further indicating the importance of these genes in relation to the blood meal.

Further, an investigation of a CD36-like protein named *L. salmonis* heme scavenger receptor class B (LsHSCARB) was performed. The transcript and protein were located to the salmon louse intestine. The receptor has an extracellular domain likely facing the intestinal lumen. The postulation was that the domain could scavenge host-derived heme. The domain was able to bind to heme *in silico* as demonstrated by ligand docking using bioinformatical tools. The receptor was also able to bind heme *in vitro*, as shown using a recombinantly expressed protein in a hemin-agarose pull down assay. During a starvation period, the transcript levels of *LsHSCARB* in the adult female salmon louse decreased steadily, suggesting that the receptor is down-regulated by the absence of a food source. RNAi mediated receptor ablation *in vivo* led to a decrease in absorbed heme levels in salmon louse tissues, and knockdown animals had shorter egg strings with a lower hatching success.

Because having blood as a diet requires proper handling of blood-components, a hypothesis was that initiating in blood-feeding would to a shift in the salmon louse transcriptome. Results in this thesis indicate that the salmon louse normally starts feeding on blood in the mobile preadult I stage, but that those lice that were attached to the vascular fish gills were feeding on blood already in the chalimus I stage. The lice attached to the gills also developed at a slower pace than those lice elsewhere on the host. Chalimus larvae located on gills were therefore sampled for RNA-sequencing and subsequent gene expression analyses, and compared to chalimus larvae from host fins and general body surfaces, that were of similar instar age. Several transcripts were found differentially expressed in chalimus larvae on gills, among these ferritins, digestive enzymes, genes of unknown functions and genes with FNII domains, to mention some. These genes could be vital for blood-feeding in the salmon louse.

Combined, these results stress the importance for the salmon louse to maintain proper ways of handling the blood meal, particularly the micronutrients iron and heme. This

thesis builds on the little knowledge on iron and heme biology in *L. salmonis* from earlier, and provides a further understanding of salmon louse hematophagy. The results of this thesis could possibly be used to lay the groundwork for future methods of controlling the salmon louse infestations in the salmon aquaculture.

1. General introduction

This thesis investigates hematophagy (blood-feeding habit), with a special focus on genes and proteins involved in the trafficking and storage of iron and heme in the ectoparasitic arthropod *Lepeophtheirus salmonis* (Krøyer, 1837). The following sections will provide background information on the parasite and its host and why salmon louse infestations are of major concern in the aquaculture sector. Further details on the parasite lifestyle will eventually lead the reader to understand the significance of hematophagy. Finally, benefits and risks regarding the diet of *L. salmonis* will be stressed, alongside the knowledge as to how this can potentially lay the foundation for future pest control.

1.1 *Lepeophtheirus salmonis* (Krøyer, 1837)

The marine ectoparasitic copepod of the phylum Arthropoda, *L. salmonis* (see Table 1 for full taxonomic classification), is otherwise known by its common name "salmon louse". Its hosts are namely salmonid species within the genera *Salmo* (*S. salar* (Linnaeus, 1758) and *S. trutta* (Linnaeus, 1758)), *Salvelinus* (*S. alpinus* (Linnaeus, 1758)) and *Oncorhynchus* (*O. mykiss* (Walbaum, 1792)), and it has a circumpolar distribution in the Northern Hemisphere (Kabata, 1979; Pike and Wadsworth, 1999). Salmon louse subspecies include the Atlantic (*L. salmonis salmonis*) and the Pacific (*L. salmonis oncorhynchi*) variant (Skern-Mauritzen et al., 2014). The Atlantic subspecies has been the organism subjected to investigation in this thesis, and is for simplification referred to as "salmon louse" from here on out.

Table 1: Salmon louse taxonomic classification*

Rank	Taxa
Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea (Brünnich, 1772)
Class	Maxillopoda (Dahl, 1956)
Subclass	Copepoda (Milne-Edwards, 1840)
Infraclass	Neocopepoda (Huys & Boxshall, 1991)
Superorder	Podoplea (Giesbrecht, 1882)
Order	Siphonostomatoida (Thorell, 1859)
Family	Caligidae (Burmeister, 1834)
Genus	*Lepeophtheirus* (von Nordmann, 1832)
Species	*salmonis* (Krøyer, 1837)

*Retrieved (March 2019) from the Integrated Taxonomic Information System (ITIS) (https://www.itis.gov).

1.1.1 Occurrence in salmon aquaculture

Aquaculture, the farming of aquatic organisms, is the fastest growing food producing sector worldwide, which is necessary to meet the nutritional demand of the growing population in a sustainable way (FAO, 2018). In Norway, particularly farming of Atlantic salmon (*S. salar*) contributes significantly to the labor and economy. In 2017, 1.22 million tons salmon were produced, which amounts to a first-hand value of NOK 61.4 billion (Statistics Norway 2018), making Norway the largest producer of Atlantic salmon in the world. However, as the industry of fish farming increases, directly related problems such as diseases, fish welfare and ecological issues increase alongside it. Among these problems is the occurrence of ectoparasitic salmon lice (Fig. 1). Amounting to an annual cost of around NOK 5 billion, sea louse (including salmon louse) infestations are seen as the culprit of inhibited economic growth in the salmon farming industry in Norway (Iversen et al., 2017). This is however not a new phenomenon; even before the salmon aquaculture rise in the 1960's, wild salmonids have co-existed with parasitizing salmon lice. However, infestations were not a major issue under natural conditions, as the distance between hosts was significant, resulting in a low infestation rate. Furthermore, wild salmonids migrate up rivers to spawn. The infestation pressure then drops partly because the parasite falls of its host in freshwater

as it is isoosmotic with the marine environment (1000 mOsm/l) (Hahnenkamp and Fyhn, 1985), and also because of a mechanical delousing when travelling up rivers with strong currents. Today, however, a high density of fish in sea cages in farms provide a high number of possible hosts for the parasite all year round, resulting in a high reproductive success and thus an epidemic of high infestation pressures by elevated concentrations of salmon louse larvae. This leads to negative impacts regarding economic, environmental and animal welfare issues.

As the parasite has a short generation time with a continuous production of a high number of eggs and because there is a high usage of anti-salmon louse medicaments, the selection pressure favors individuals with mutations that cause decreased sensitivity towards various chemotherapeutants, yet with a fitness that allows for proliferation. A decrease in sensitivity toward treatments has been reported for e.g. organophosphates (Fallang et al., 2004; Jones et al., 1992; Kaur et al., 2015), avermectins (Carmichael et al., 2013; Espedal et al., 2013) and hydrogen peroxide (H_2O_2) (Helgesen et al., 2015; Treasurer et al., 2000) to mention a few. In order to minimize usage of chemicals in farms, alternative non-medical methods of managing salmon louse infestations have emerged. Cleaner fish such as the ballan wrasse (*Labrus bergylta* (Ascanius, 1767)) and lumpfish (*Cyclopterus lumpus* (Linnaeus, 1758)) are used as biological control in sea cages alongside salmon to aid in pest management by feeding on the parasites and thereby mitigating the infestation pressure (Hjeltnes et al., 2019; Treasurer, 2002). The wrasse industry is however criticized, as fish catching causes unwanted bycatching (Mortensen et al., 2013), and there is reported reduced fish welfare in the pens (Treasurer and Feledi, 2014). Other preventative actions against salmon lice include e.g. creating a physical barrier for the host-parasite interaction by using closed cages in farms supplied with seawater intake from 25 meter depth (Nilsen et al., 2017), thermal delousing by treating the fish with seawater heated above the preferable habitat of the parasite (Grøntvedt et al., 2015), and delousing with freshwater treatments as reviewed by Powell et al. (2015). Despite all efforts, the salmon louse problem seemingly appears here to stay. Consequently, an increased focus on researching the basic parasite biology is needed in order to find tools for pest control.

Figure 1: Salmon lice infestation. A group of mature adult female lice with egg strings, feeding off a salmon. Photo: Lars Are Hamre.

1.1.2 Salmon louse life cycle

The salmon louse life cycle consists of eight stages, each separated by a molt where a new exoskeleton is grown and the old one is shed. An illustrated life cycle overview is given in Figure 2. The stages are in chronological order as follows: nauplius I and II (both planktonic), copepodid (infective), chalimus I and II (both immobile parasitic), preadult I and II (both mobile parasitic) and adult (mobile parasitic and reproductive) (Hamre et al., 2013; Johnson and Albright, 1991a). At 10 °C, a new pair of egg strings is extruded from the mature adult female about every ten days, with the capacity of producing up to 1200 eggs with each cycle (Costello, 2006; Hamre et al., 2009). Developmental and reproductive rates are temperature dependent. The following details on development is based on a temperature of 10 ˚C. Development from fertilization to the adult stage is completed in approximately 40 (♂) to 52 (♀) days (Johnson and Albright, 1991b), but can be as short as 38 (♂) to 44 (♀) days for the fastest developers (Hamre et al., 2019). Nauplius I larvae (~0.5-0.6 mm) hatch directly from the mature adult female louse egg strings. As of the first molting, after approximately one day, the louse has developed to the nauplius II stage, which is

morphologically highly similar to the previous nauplius stage. After the second molt, 4-5 days later, the salmon louse is in the copepodid stage (~0.7 mm). This is the infectious stage when the louse converts from a planktonic to a parasitic lifestyle. During its planktonic stages, the louse disperses with ocean currents, and infestations of lice in one farm can, given the right conditions, spread to neighboring farms and wild salmon up to 30 kilometers away (Salama et al., 2013). A range of stimuli affect the copepodid ability and specificity of host recognition, such as chemosensation (Devine et al., 2000; Fields et al., 2007; Komisarczuk et al., 2017), photosensation and mechanosensation (Bron et al., 1993a). The copepodid attaches to its host by a stabbing action with hook-like structures on its second antennae (Bron et al., 1991), before extruding a frontal filament directly before molting. Further molting occurs on the host, both chalimus stages last for about 4-5 days, first to the immobile chalimus I (~1.2 mm) and II (~2.2 mm) stages, which are attached to the host by the elongated frontal filament (Bron et al., 1991; Gonzalez-Alanis et al., 2001). At the chalimus II stage, sexual distinguishing can be completed by measuring size differences (Eichner et al., 2015a). The preadult stages both last about one week. Upon reaching the pre-adult I and II (♀= ~3.6 - 5.2 mm, ♂= ~3.4 - 4.3 mm) and adult stages (♀ without egg strings= 8 - 11 mm, ♂= 5-6 mm), the louse is mobile and can be sexually distinguished by visual inspections. Mature adults are reproductive, and produce eggs (hatching within ~9-10 days) with larvae that have the potential of infecting new hosts and completing yet another life cycle.

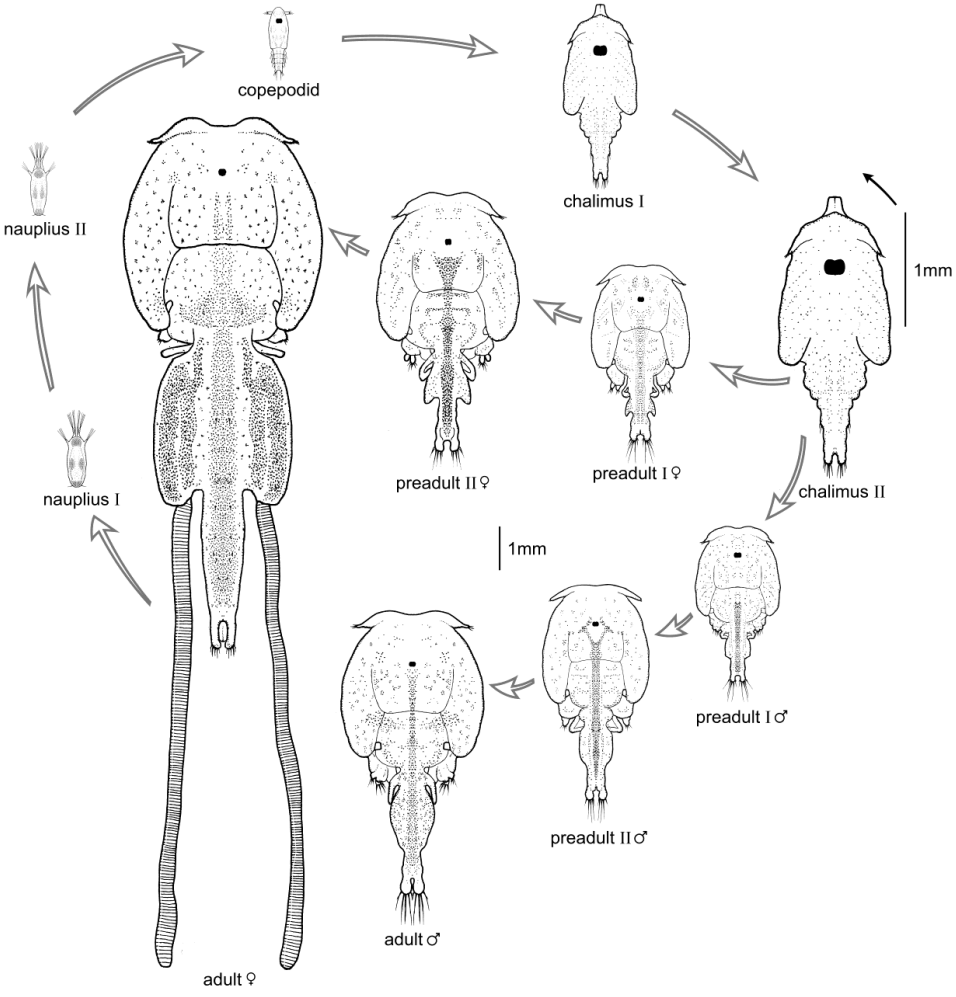

Figure 2: Salmon louse life cycle. The salmon louse life cycle consists of eight developmental stages. These stages are the planktonic nauplia I and II, the infectious copepodid, the immobile chalimus I and II, and the mobile preadult I, II and adult louse. Animal sizes of various stages are indicated in the figure. Planktonic and attached stages are in scale, and mobile stages are in scale. The reader is referred to the text for further information on the salmon louse life cycle. Illustration: "SLRC *Lepeophtheirus salmonis* life cycle" by Sea Lice Research Centre is licensed under a Creative Commons Attribution-ShareAlike 4.0 International License.

1.1.3 Salmon louse biology and anatomy

The gross anatomy of the salmon louse body can be divided into a cephalothorax, genital segment and abdomen (Fig. 3) (Johnson and Albright, 1991a). The body of arthropods is covered by an exoskeleton, in which the major component is chitin, a polymer which is subjected to a tightly regulated synthesis and degradation process in the salmon louse (Eichner et al., 2015b; Harðardóttir et al., 2019). The rigid cuticle protects the vital louse body parts against external forces, but also exists to provide anchorage for muscles and epidermis (Bron et al., 2000). The tissue located underneath the cuticle is called the sub-epidermal tissue, and is assumed to perform liver-like functions with e.g. the presence of several types of glandular tissues (Øvergård et al., 2016). Being a crustacean, the salmon louse has an open circulatory system containing hemolymph, which is a tissue fluid analogous to the blood of vertebrates. The sub-epidermal tissue produces hemocoel, which is the body cavity that holds the hemolymph.

The first three salmon louse life stages are lecithotrophic (Pike and Wadsworth, 1999), meaning they depend on maternal yolk sac reserves for nutrition. When the louse reaches the infectious copepodid stage, yolk nutrients are limited and the copepodid needs to attach to a host to complete its life cycle (see section 1.1.2). The salmon louse feeds on the salmonid skin and blood after it has attached to a suitable host (Brandal et al., 1976; Pike, 1989) which provides it with nutrients. As the louse molts into the mobile parasitic stages, it grazes on larger parts of its host and may cause greater damage. Fish skin is a metabolically active organ with living cells in all layers (unlike other vertebrates), and is vital for normal physiological functions. A louse feeding on a salmonid causes a mechanical disruption of this barrier, which can have detrimental effects for the fish, such as fatality due to osmoregulatory imbalance (Grimnes and Jakobsen, 1996; Pike and Wadsworth, 1999) and an increased susceptibility to secondary infections by opportunistic pathogens (Mustafa et al., 2000).

Salmon louse reproductive organs include the ovaries (♀) (Fig. 3) and testes (♂). Both ovaries and testes are located on each side of the louse coalesced eyes (see

Komisarczuk et al. (2019) for more details on organ locations). Ovaries produce oocytes that are transported through the oviducts where they mature by increasing in size and convoluting (Ritchie et al., 1996), and also undergoing yolk maturation by vitellogenesis (Dalvin et al., 2009). Testes produce spermatozoa that are transported through vas deferens to spermatophore sacs in the genital segment and stored until copulation (Ritchie et al., 1996). The male salmon louse reaches the mature adult stage prior to the female salmon louse. The adult male precopulates with immature preadult II females, guarding the female until it has molted to the reproductive adult stage. At this point, the male will deposit its spermatophores onto the female genital segment, providing the female with spermatozoa that fertilizes its eggs as they are extruded from the genital segment (Ritchie et al., 1996). A knockdown of genes encoding mucin-like spermatophore wall proteins found expressed in the male salmon louse resulted in halted reproduction of female lice cultivated alongside the knockdown males (Borchel and Nilsen, 2018), highlighting the importance of spermatophore proteins for the parasite reproductive success.

In the non-feeding, planktonic larval stages, the parasite does not have a functional alimentary canal; this first becomes present in the infectious copepodid stage (Bron et al., 1993b). As the salmon louse is semi-transparent, its intestine is clearly visible, particularly in the blood-feeding stages, as a red line when filled with salmon blood (Fig. 3). The intestine is stretched continuously from the oral cone ventrally on the cephalothorax, further through the cuticle-lined foregut, or the esophagus, followed by the midgut, and finally the cuticle-lined hindgut ending in a short rectum (Bron et al., 1993b; Nylund et al., 1992). Peristaltic movement in the gut facilitates efficient food bolus homogenization (Nylund et al., 1992). A peritrophic membrane has not been described in the salmon louse alimentary canal (Bron et al., 1993b; Nylund et al., 1992). The salmon louse gut has been shown to be a site for expression of various genes related to the blood meal. Here, one finds e.g. digestive enzymes such as trypsin-like peptidases (*LsTryp1-5*) (Johnson et al., 2002; Kvamme et al., 2004), and the microsomal triglyceride transfer protein (*LsMTP*) (Khan et al., 2017).

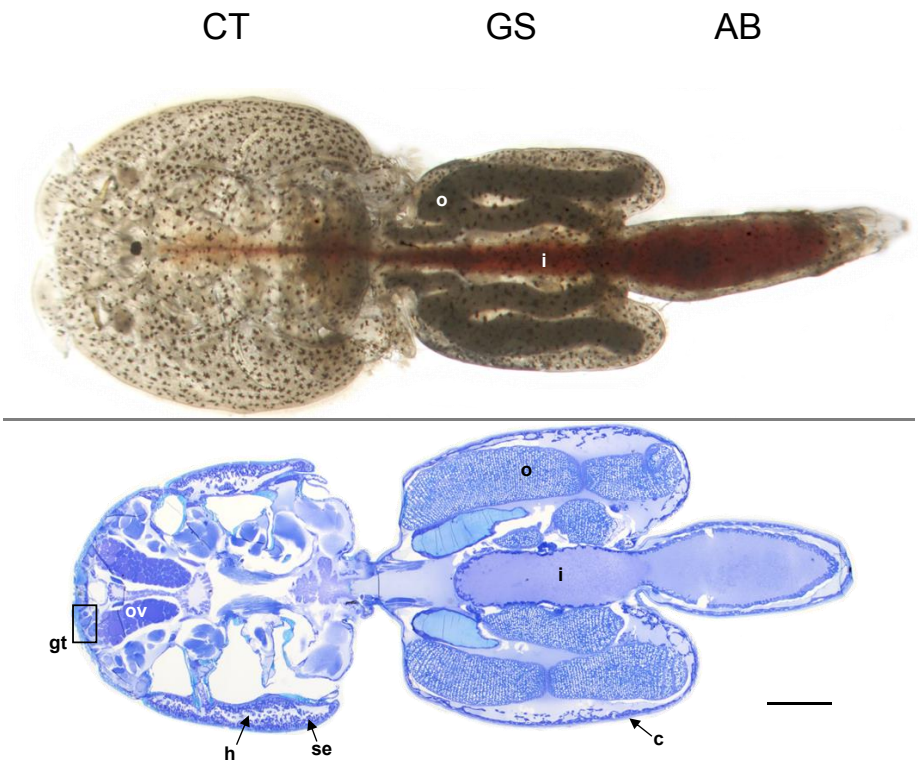

Figure 3: Morphology of a mature adult female salmon louse (dorsal view). Photograph of live animal (top panel) and toluidine blue dyed histological section (bottom panel) showing the anatomy of a salmon louse body. The gross anatomical division includes a cephalothorax (CT), genital segment (GS) and abdomen (AB). The ovaries (ov) are located adjacent to the coalesced eyes. The GS contains unfertilized eggs, or oocytes (o). The blood-filled intestine (i) is seen as a red line stretching throughout the body from the mouth cone on the cephalothorax, through the genital segment, and finally through to the rectum in the posterior abdomen. Other tissues marked are glandular tissue (gt), hemocoel (h), sub-epidermal tissue (se) and cuticle (c). Scale bar = 1 mm. The figure is adapted from **Paper II**.

1.2 Hematophagy

Blood is a tissue fluid that consists of several nutrients, and can provide a great source of energy for many animals. Blood is also continuously renewed in vertebrates through hematopoiesis, allowing blood-feeding (hematophagous) organisms an ongoing supply of feed. Hematophagy has occurred in a broad range of taxa and several parasites (like various worms and arthropods) have adapted such a special feeding approach to

facilitate their parasitic lifestyle. The nature of hematophagy has been described for several species, including the nematode *Schistosomiasis mansoni* (Hall et al., 2011), ticks (Mans and Neitz, 2004), mosquitos such as *Aedes aegypti* (Harrington et al., 2001), and the salmon louse (Brandal et al., 1976) to mention a few. Blood is particularly rich in protein, with iron-proteins being among the most abundant (including in salmon (Røed et al., 1995; Sandnes et al., 1988)). Ingested proteins are digested to dipeptides and amino acids by intestinal enzymatic catalysis. These are further used as substrate for lipid, carbohydrate and protein synthesis, which are necessary for normal cellular processes. However, a consequence of intestinal protein degradation is the liberation of micronutrients such as iron and heme. Iron and heme both behave as double-edged swords as they are cofactors that are essential for aerobe life, yet they have potentially cytotoxic effects as described in the sections to follow.

1.3 Iron

Iron (Fe) is an elemental transition metal with atomic number 26 in the periodic system. It is one of the most abundant elements on Earth and has a wide range of industrial uses as an alloy ingredient of steel. However, in addition to being important for inorganic structures, iron also plays a vital role in biology. It is a mineral with many physiological purposes, as it contributes as a cofactor to the active center of several metalloproteins, enabling various biochemical pathways on a cellular level. Iron is necessary for non-heme iron-proteins involved in e.g. DNA synthesis. As an example, iron is required as the enzyme ribonucleotide reductase metal center, which is involved in the formation of deoxyribonucleotides (Furukawa et al., 1992). Iron is also part of the inorganic cofactor iron-sulfur (Fe-S) cluster, which has many functions, such as being in the respiratory chain mitochondrial complexes I, II and III (Hatefi et al., 1962). While several oxidation numbers are possible for iron, +2 and +3 are the most common. Iron(II) compounds (Fe^{2+}) are known as ferrous, whereas iron(III) compounds (Fe^{3+}) are known as ferric. The nature of iron in being able to undergo cyclic reduction and oxidation (redox) enables the element to cause a wide range of cellular injury through the generation of reactive oxygen species (ROS) (Stohs and Bagchi, 1995). Ferric iron

reduction by superoxide anion can be seen in formula (1). Oxidation in the Fenton reaction (Fenton's reagent was first described over a century ago (Fenton, 1894)), which can be seen in formula (2), shows that ferrous iron can react with H_2O_2 and produce hydroxyl radical ($OH\cdot$) (Koppenol, 1993), a ROS able to initiate e.g. lipid peroxidation and cause oxidative stress. In brief, iron is an essential mineral, and must be obtained through the diet. However, as a consequence of the highly reactive nature of iron, free cellular iron is generally held at a minimum, and rather bound to proteins such as the iron storage protein ferritin, iron transporter transferrin, or contained to the heme group of hemoglobin in red blood cells circulating in vertebrate blood.

(1) $Fe^{+3} + O_2^{-}\cdot \rightarrow Fe^{+2} + O_2$

(2) $Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{-} + OH\cdot$

1.3.1 Iron storage and trafficking

In complex multicellular organisms, transportation mechanisms are vital to distribute the essential mineral iron to all cells, yet still avoiding toxic effects of iron excess. Not considering heme iron, vertebrate ferric iron is kept in a nontoxic state and for the most part transported in the circulation within blood serum (Holmberg and Laurell, 1945). The serum protein responsible for this was later named *transferrin* due to its ability of transporting iron between sites of absorption, storage and usage (Holmberg and Laurell, 1947). The major cellular storage protein for iron is ferritin, which is a protein with highly conserved features. Ferritin was initially isolated from horse spleen as early as in 1937, and in the following years, its properties and functions were steadily investigated (Granick, 1942). A few decades later, testing of serum ferritin was implemented clinically as an index of iron stores in humans (Lipschitz et al., 1974). Ferritin sequesters iron in the nonreactive, ferric state. The iron pool within ferritin is thus available for proteins that require the metal as cofactor. Ferritin is a large spherical and hollow protein, and the highly conserved three-dimensional structure is evident as

seen in similarities of crystal structures of ferritins from e.g. the Japanese tiger prawn (*Penaeus japonicus*) (PBD id: 6A4U) (Masuda et al., 2018), the American bullfrog (*Lithobates catesbeiana*) (PDB id: 1MFR), (Ha et al., 1999), human (*Homo sapiens*) (PDB id: 2FHA) and horse (*Equus ferus caballus*) (PBD id: 1AEW) (Hempstead et al., 1997) to mention a few. The typical ferritin protein is cytosolic and consists of 24 subunits of two types of chains; the heavy chain (HC) and the light chain (LC) subunit in various proportions, and can harbor approximately 29% (w/w) iron (Mann et al., 1986), or roughly 4500 iron molecules. In arthropods, ferritin subunits are often secretory and are homologous to the vertebrate subunits. The subunits in arthropods are the heavy chain homolog (HCH) and the light chain homolog (LCH). HCH subunits have ferroxidase activity, which oxidizes ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron (Pham and Winzerling, 2010). LCH subunits, on the other hand, are annotated as LCH because of lacking ferroxidase activity, and is rather implicated in the nucleation of iron (Pham and Winzerling, 2010). As many arthropod homologs of ferritin contain a signal peptide, directing it for the secretory pathway, they also act as a vehicle for iron transportation to recipient tissues, as well as for storage and detoxification. Secreted hemolymph ferritin has been reported in several arthropods, such as in the Brazilian skipper *Calpodes ethlius* (Nichol and Locke, 1989), the tobacco hornworm *Manducta sexta* (Huebers et al., 1988), the hard ticks *Haemaphysalis longicornis* (Galay et al., 2014b) and *Ixodes ricinus* (Hajdusek et al., 2009). In ticks, RNAi-mediated silencing of ferritin has resulted in impaired egg production, a stop in their blood-feeding behavior and higher levels of oxidative stress (Galay et al., 2014b, 2013; Hajdusek et al., 2009). Ferritin synthesis is regulated on a transcriptional and/or post-transcriptional (translational) level. Post-transcriptional regulation is mediated by interaction of an iron-responsive element with its binding proteins in the untranslated region of the mRNA (Muckenthaler et al., 2008).

1.4 Heme

The word “heme” is derived from the Greek *haima* which means blood (Medical Dictionary, 2012), and has been called one of the key “pigments of life” as it causes

the characteristic red coloration of vertebrate blood. Heme is a cofactor found in hemoproteins, enabling a wide range of biochemical processes that are essential for the normal cell physiology. More specifically, heme is the iron-containing pyrrole ring of protoporphyrin IX (Fig. 4 and 5). Various types of heme exist, and the end-product of the synthesis pathway is heme B. Other types of heme include e.g. heme A and heme O (Fig. 4). Heme is herein used as a generic term, including various valence states of iron and heme types, unless otherwise stated. Heme B is largely bound to the gas transportation protein hemoglobin, which is the protein (alongside the oxygen storage heme-protein myoglobin) that was the very first atomic-level protein structure to be determined (Kendrew et al., 1960; Perutz et al., 1960). The work on this horse-derived hemoglobin structure laid the foundation for further elucidation of hemoglobin in other species, and the heme B cofactor is also seen in crystal structures of e.g. human hemoglobin (PDB id: 1GZX) (Paoli et al., 1996) and the teleost fish *Leiostomus xanthurus* hemoglobin (PDB id: 1SPG) (Mylvaganam et al., 1996). Other key cellular processes where heme is essential include electron transfer by cytochromes (Reid et al., 1984), gas and redox sensing (Shimizu et al., 2015), and DNA transcription (Hira et al., 2007). Heme is also a key element in processes related to oxygen metabolism in peroxidases (Ator and Montellano, 1987), and circadian clock control in mammals (Yin et al., 2007). The necessity of and adaptations to heme in blood-feeding arthropods are thoroughly reviewed (Graca-Souza et al., 2006; Whiten et al., 2018). E.g. in the blood-feeding arthropods *Rhodnius* and *Rhipicephalus*, heme is found bound to hemolymph proteins *Rhodnius* heme-binding protein (RHBP) (Oliveira et al., 1995) and Hemelipoprotein (HeLp) (Maya-Monteiro et al., 2000). Prior to this study, several genes encoding proteins with predicted heme moieties have been described in the salmon louse, however none of these have offered particular focus to the heme group (Helgesen et al., 2017; Øvergård et al., 2017, 2016; Sandlund et al., 2018). Despite all cellular events that are dependent on heme as cofactor, heme acts cytotoxic by ROS generation and following oxidative stress. Lipid peroxidation is mainly induced by conversion of organic hydroperoxides (ROOH) into highly reactive alkoxyl (RO·) and peroxyl (ROO·) radicals (formulas (3) and (4)) (Graca-Souza et al., 2006). It has also been suggested that free heme in solution or bound to hemoproteins may produce

hydroxyl radicals by a reaction similar to the Fenton reaction (Graca-Souza et al., 2006; Sadrzadeh et al., 1984). Heme is thus, alike iron, important to maintain bound by appropriate metalloproteins in order to avoid cellular demise.

(3) $\text{Heme-Fe}^{+2} + \text{ROOH} \rightarrow \text{Heme-Fe}^{+3} + \text{OH}^{-} + \text{RO}\cdot$

(4) $\text{Heme-Fe}^{+3} + \text{ROOH} \rightarrow \text{Heme-Fe}^{+2} + \text{H}^{+} + \text{ROO}\cdot$

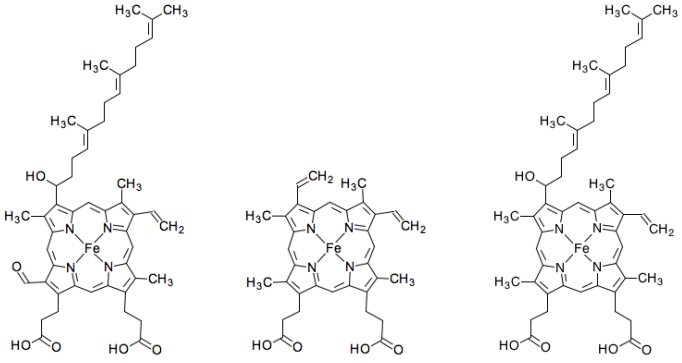

Figure 4: Various heme types. Heme exists in various forms. Heme B is the most common, as the heme biosynthetic pathway product, and can be converted to e.g. heme A and O. The figure is assembled using KEGG COMPOUND Database illustrations.

1.4.1 Heme biosynthesis and degradation

Metazoan heme biosynthesis is a conserved and well-established biochemical pathway that is present in all aerobic branches in the tree of life and is described numerous times in literature (Ajioka et al., 2006; Donegan et al., 2019; Hamza and Dailey, 2012; Koreny et al., 2013; Ponka, 1999). Briefly, synthesis of heme involves eight enzymes, four cytoplasmic and four mitochondrial (Fig. 5). The pathway begins in the mitochondria with the synthesis of δ-aminolevulinic acid (δ-ALA) from the

condensation of glycine and succinyl-CoA catalyzed by the enzyme ALA-synthase (ALAS: EC 2.3.1.37). The four steps that follow take place in the cytoplasm. Two δ-ALA molecules are converted to a monopyrrole porphobilinogen (PBG) by dehydration catalyzed by ALA-dehydratase (ALAD: EC 4.2.1.24) (also known as PBG synthase or PBGS). Next, four PBG molecules combined make up the cyclic tetrapyrrole uroporphyrinogen III in two steps, catalyzed by hydroxymethylbilane synthase (HMBS: EC 2.5.1.61) and uroporphyrinogen synthase (UROS: EC 4.2.1.75) subsequently. The cyclic tetrapyrrole uroporphyrinogen III is then decarboxylated by uroporphyrinogen decarboxylase (UROD: EC 4.1.1.37) to make up coproporphyrinogen III. This concludes the cytoplasmic residency of the pathway, with the remaining synthesis steps occurring in the mitochondria again. Coproporphyrinogen oxidase (CPOX: EC 1.3.3.3 or EC 1.3.98.3) converts coproporphyrinogen III to protoporphyrinogen III, which is then further converted to protoporphrin IX (PPIX) by protoporphyrinogen oxidase (PPOX: EC 1.3.3.4 or EC 1.3.5.3). The heme B (also known as protoheme) molecule is finally completed by addition of ferrous iron (Fe^{2+}) to PPIX by the pathway terminal enzyme, ferrochelatase (FECH: EC 4.99.1.1). Heme B can be further converted to various kinds of heme (Fig. 4) by cytochrome c oxidases (COX, EC 1.9.3.1) (Michel et al., 1998), depending on the protein it will be incorporated into (e.g. cytochromes). Heme B conversion to heme O is catalyzed by heme O synthase (COX10). Heme O can be further modified to heme A catalyzed by cytochrome c oxidase assembly protein subunit 15 (COX15).

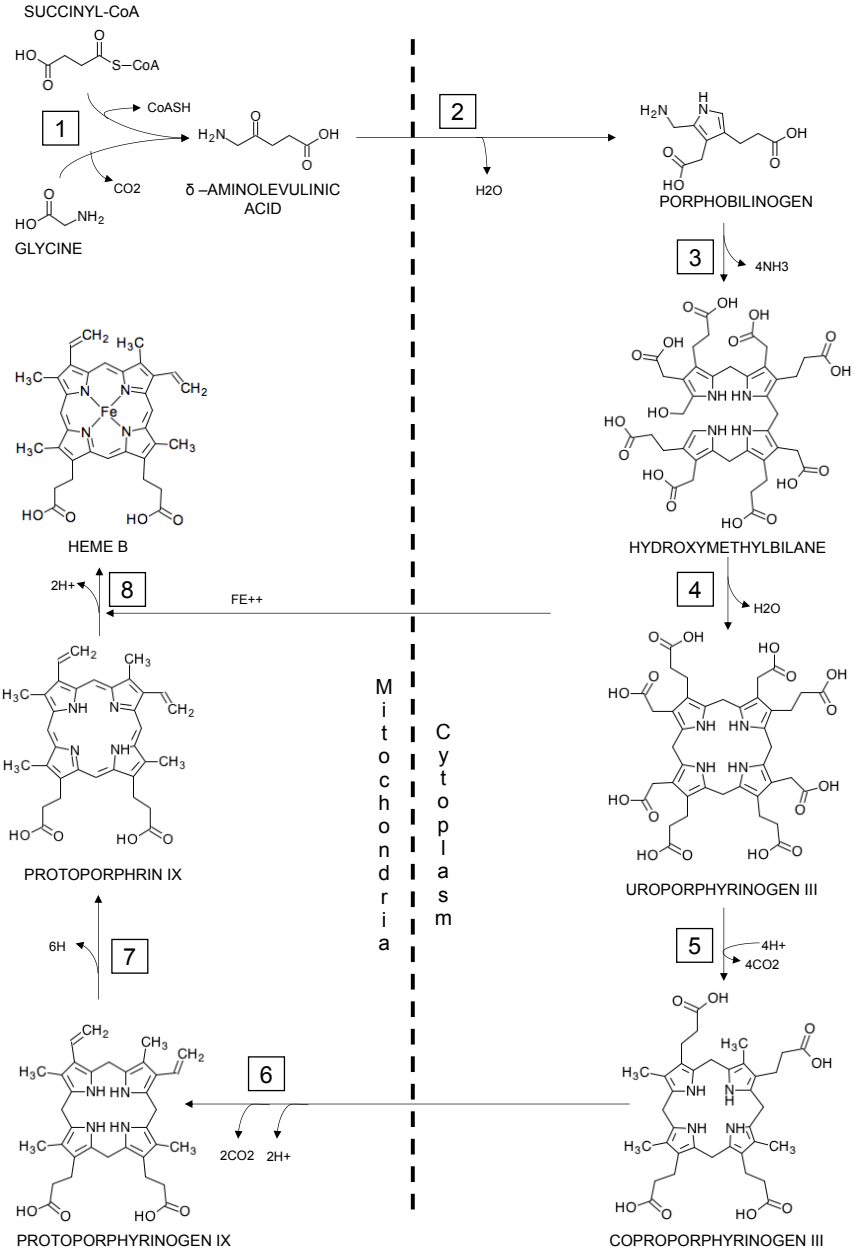

Figure 5: Heme biosynthetic pathway. Biosynthesis of heme from amino acid glycine and succinyl-CoA as precursors. Mitochondrial and cytoplasmic pathway locations are indicated. Boxed numbers represent pathway enzymes. 1 = δ-aminolevulinic acid synthase (ALAS), 2 = ALA-dehydratase (ALAD), 3 = hydroxymethylbilane synthase (HMBS), 4 = uroporphyrinogen synthase (UROS), 5 = uroporphyrinogen decarboxylase (UROD), 6 = coproporphyrinogen oxidase (CPOX), 7 = protoporphyrinogen oxidase (PPOX), 8 = ferrochelatase (FECH). More details on the pathway is found in the main text. The figure is assembled using KEGG COMPOUND Database illustrations.

Heme catabolism in eukaryotes is mediated by the enzyme heme oxygenase (HO, EC 1.14.14.18) which uses heme as substrate, and cleaves the ring structure into ferrous iron, biliverdin-IX-α and carbon monoxide (CO) intracellularly (Tenhunen et al., 1969). The liberated iron may further be stored in ferritin or used in metalloproteins or transported to the mitochondria to e.g. be recycled as heme-iron (Fig. 6). Both synthesis and degradation of heme within a cell is tightly regulated as the availability of heme promotes feedback loops. Elevated levels of available heme inhibits new synthesis of endogenous heme (Furuyama et al., 2007), whereas it induces expression of HO (Shibahara et al., 1978). In this way, the cell balances heme levels to avoid excess heme and maintain homeostasis. Although this degradation pathway mediated by HO is highly conserved, the kissing bug *Rhodnius prolixus* pathway slightly deviates from it. Here, two cysteinylglycine residues are found to be added to the heme group prior to oxidative cleavage (Paiva-Silva et al., 2006). However, further modification and oxidative cleavage of degradation products are likely mediated through HO-like enzymes, and a conserved HO homolog has been identified in the *R. proxilus* genome (Walter-Nuno et al., 2013).

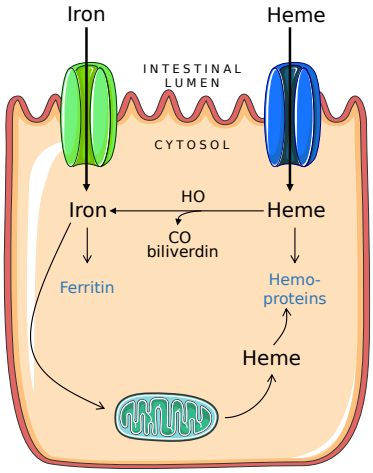

Figure 6: Iron and heme trafficking intertwined. Simplified graphical illustration of how iron and heme trafficking are intertwined. HO = heme oxygenase, CO = carbon monoxide. It should be noted that in this simplified illustration, further trafficking of ferritin and hemoproteins, and incorporation of hemoproteins into the mitochondria are not shown. The illustrated cell components are downloaded from Servier Medical Art by Servier (https://smart.servier.com/) (Attribution 3.0 France: CC BY 3.0 FR).

1.4.2 Heme auxotrophy

Until the late '90s, the consensus in communities within iron and heme research was that all nucleated cells in multicellular aerobe organisms have the ability to make up the iron-containing porphyrin ring structure *de novo*, as heme is a cofactor that is essential for every aerobe cell (Ponka, 1997). After this, however; the dogma on the universality of heme biosynthesis had to be reconsidered, as it turned out not all animals have the opportunity to rely on endogenous heme production. In 1999, the hematophagous parasitic cattle tick *Rhipicephalus (Boophilus) microplus* was the first reported animal that was not able to synthesize heme on its own, but rather being an obligate heme auxotroph (Braz et al., 1999). Following this, free-living worms such as the soil-nematode *Caenorhabditis elegans* and parasitic helminths were also found to be unable to make heme *de novo* (Perner et al., 2019; Rao et al., 2005). With more genomes being sequenced and annotated, more organisms are added to the list of natural heme auxotrophs, one being the tick vector of Lyme disease *I. scapularis* (Gulia-Nuss et al., 2016). Ticks appear to lack several orthologues of enzymes within the heme biosynthetic pathway, with only the three enzymes last in the synthesis still present, leaving ticks with no other option than to scavenge exogenous heme for survival. In addition to being heme auxotrophs, ticks and nematodes have been found to not possess a traditional heme degradation pathway as they lack HO activity (Perner et al., 2019, 2016; Rao et al., 2005) The malaria parasite *Plasmodium spp.*, unlike the aforementioned species, is not an obligate heme auxotroph. The protozoan parasite encodes a full genetic apparatus for endogenous heme biosynthesis, yet does not rely on this during its blood-stage growth (Goldberg and Sigala, 2017), suggesting that a period with an abundance of exogenous heme from the host blood leads to a redundancy of the heme biosynthetic pathway in this parasite.

1.4.3 Heme trafficking

The ability of intestinal absorption of exogenous heme from the diet has early on been recognized in mammalia (Björn-Rasmussen et al., 1974; Gräsbeck et al., 1979; Tenhunen et al., 1980). Dietary heme works as a source of iron, as heme-iron is much more bioavailable

than non-heme iron (Carpenter and Mahoney, 1992; Lynch et al., 1989). Still, the mechanism behind the movement of heme across cellular membranes has to a great extent remained an unanswered question. It has been suggested that, due to its amphipathic nature, heme may passively diffuse unassisted over membranes, a phenomenon which has been observed in model intracellular lipid bilayers *in vitro* (Rose et al., 1985; Thöny-Meyer, 2009). However, considering the highly reactive nature of free heme, and the fact that free heme is prone to self-aggregation in aqueous solutions as well as aggregation with lipid membranes, this has been deemed an unlikely event to occur *in vivo* (Hamza and Dailey, 2012). Instead, highly regulated trafficking of heme across membranes, facilitated by committed protein(s) is probably a more accurate scenario. The first detailed characterization of an intestinal heme transporter was published in 2005, when Shayeghi et al. (2005) isolated the heme carrier protein 1 (HCP1) from mouse duodenum. Nevertheless, fast forward one year, HCP1 is renamed as the proton-coupled folate transporter/HCP1 (PCFT/HCP1) as further protein characterization uncovered that it is a transporter with approximately two orders of magnitude greater affinity for folate than heme (Qiu et al., 2006). In *C. elegans*, the heme responsive gene 1 (HRG-1) is situated in the endosome and lysosome, and is postulated to regulate heme homeostasis (Rajagopal et al., 2008). The HRG-1 paralogue HRG-4 is on the other hand expressed in the cytoplasm of intestinal cells of *C. elegans*, and has been implicated in heme uptake in the intestine of worms (Rajagopal et al., 2008). However, there are no reported arthropod or vertebrate homologs of HRG-4. Intestinal heme absorption in arthropods lack consensus, but an ATP binding cassette subtype B10 (ABCB10) was found to be implicated in heme transportation from digestive vacuoles to hemosomes in the tick *R. microplus* midgut (Lara et al., 2015, 2003). Further investigation is needed to elucidate the trafficking of heme in arthropods, and using heme auxotroph organisms could contribute to increased knowledge of this by taking advantage of an organism lacking an endogenous heme pool. Further details on inter- and intracellular trafficking of iron and heme are not included here, but can be found in a thorough review by Muckenthaler et al. (2016).

2. Aims of the study

The overall aim of this research was to gain knowledge about salmon louse hematophagy, with a special focus on iron and heme trafficking. Ferritin is an iron-storage protein, and ferritin-encoding genes were of interest to investigate in the blood-feeding louse, which is exposed to substantial amounts of dietary iron. Heme trafficking is much more scarcely described throughout the available literature, and an aim was to search for a heme receptor in the parasite intestine. The silencing of a heme receptor should result in a reduction of absorbed heme levels, and this should ultimately have negative impacts on parasite physiology. *In silico* examination of the heme synthesis and degradation pathways is included as extra results in this thesis to understand iron and heme trafficking in the louse. Finally, further insight into the salmon louse transcriptome upon initiating hematophagy could provide several candidate genes to investigate and characterize in the future. The knowledge created in this thesis could give a better understanding of salmon louse weaknesses as a result of its hematophagous, parasitic lifestyle, and could contribute to propose new targets for pest management.

Key objectives of this thesis were:

- Characterize and annotate ferritins in the salmon louse genome.
- Perform functional studies of secreted ferritins in the salmon louse.
- Establish a reproducible assay for quantifying heme in the salmon louse.
- Identify and characterize a heme receptor in the salmon louse intestine.
- Perform putative heme receptor knockdown studies and assess absorbed heme levels in salmon louse tissues.
- Investigate the onset of hematophagy in the salmon louse.
- Study the transcriptome of chalimus larvae with a premature onset of blood-feeding while attached to host gills.

3. Methodology

3.1 Fish experiments and lice infestations

When performing *in vivo* experiments, mimicking natural conditions of salmon lice infesting salmon is essential for studying the host parasite interaction and the parasite biology. However, the benefit of using defined conditions in a laboratory includes minimization of natural variations, which could otherwise disturb the experimental setup. For this thesis, biological experiments with salmon lice have been performed both at the wet laboratory facilities at the Institute of Marine Research (IMR) in Bergen, Norway, and at the LiceLab at the Institute of Biological Sciences at the University of Bergen, Norway. The facilities have specialized units for cultivating salmon lice required for biological experiments. Salmonids are cultivated according to ethical guidelines determined by the Norwegian Food Safety Authority (Norwegian Food Satefy Authority, 2015) and the Norwegian legislation on the care and use of animals for scientific procedures (Lovdata, 2015). Seawater is pumped from 105 meter depths and particle filtered (20 µm) to maintain proper and relatively steady environmental conditions for the fish at all times. Parameters such as oxygen saturation, water temperature, water salinity and fish welfare were monitored daily. Salmon louse eggs collected from in-house laboratory strains of salmon lice were hatched in continuous flow incubators (Fig. 7) and allowed to develop to the copepodid stage (Hamre et al., 2009). The copepodids were used to infect Atlantic salmon in common tanks (500 l), and the lice were collected from the fish when reaching the desired life stage, e.g. preadult II females for dsRNA injections. When re-attaching preadult or adult lice to the host fish, lice were placed on their back on a wet piece of paper. The paper with lice was then placed on the side of an anesthetized salmon (mixture of 60 mg/l benzocaine and 5 mg/ml methomidate), before removing the paper and placing the salmon back in an array of single fish tanks (50 l) with a continuous supply of fresh seawater (6 l min^{-1}) (Fig. 8) (Hamre and Nilsen, 2011).

Figure 7: Salmon louse incubators. Incubators with individual hatching wells used to keep and observe egg strings and the developing salmon louse larvae. A continuous supply of sea water is obtained through the green tubes. Photo: Lars Are Hamre.

Figure 8: Single fish tank arrays. Wet-lab facility at the SLRC used in salmon louse experiments. Each tank holds one salmon with salmon lice. Photo: Lars Are Hamre.

3.2 Genome browsing

The salmon louse genome has been sequenced and is publicly available at LiceBase (https:/licebase.org) and Ensembl Metazoa (http://metazoa.ensembl.org/Lepeophtheirus_salmonis/). In addition to the sequenced genome in LiceBase, there are among other things an Atlantic salmon louse genome annotation, a genome browser, a Basic local alignment search tool (Blast) (Altschul et al., 1990) function and access to related high-throughput genomics and transcriptomic data. These data allow for analyses across salmon louse developmental stages and also across various salmon louse tissues, such as intestine, ovaries, testis etc. RNA-sequencing (RNA-seq) data reveals the transcript quantity and presence in a biological sample (Wang et al., 2009).

To investigate the heme biosynthetic and degradation pathways in the salmon louse, orthologue protein sequences from *Drosophila melanogaster* (described in a review by

Perner et al. (2019)) and *I. scapularis* were downloaded in FASTA format from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). These sequences were applied as queries in the Blast function with default settings in LiceBase against the genome of the Arctic-endemic copepod *Tigriopus kingsejongensis*, which is the only other copepod (in addition to the salmon louse) with a publicly available genome upon finishing this thesis (Kang et al., 2017). *T. kingsejongensis* is free-living, and not a hematophagous parasite, and ought to therefore have a complete heme biosynthetic pathway. The primary search was BlastP (protein → protein), and if no hits were found, a tBlastN (6 frames, protein → translated nucleotide) search was conducted. The obtained hits were fed into the InterProScan 5 database (https://www.ebi.ac.uk/interpro/; Jones et al. (2014)), for protein sequence analysis and classification to obtain protein families (Pfam) and gene ontology (GO) terms. Sequences of likely bacterial origin (minimum 10 best NR Blast hits were bacteria) were not used further. The Blast hits from *T. kingsejongensis* and *D. melanogaster* were then used as query for Blast searches against the salmon louse genome, with the same approach of downstream sequence analyses. Only hits with an E-value below 1E-6 were considered.

3.3 RNA interference

RNA interference (RNAi) was first reported in the flower *Petunia* (Napoli et al., 1990), and later unraveled by Fire et al. (1998) as a genetic interference by double stranded RNA (dsRNA) in the nematode worm *C. elegans*. RNAi is a conserved biological process in cells that can be provoked by introduction of dsRNA into an organism with the purpose of gene expression manipulation. RNAi can also be triggered naturally e.g. in response to foreign dsRNA of viral origin to protect against pathogens, as first described in plants (Hamilton and Baulcombe, 1999). The gene silencing mechanism of RNAi is described in a review by Sen and Blau (2006). Briefly, the silencing is initially driven by Dicer-mediated cleavage of the trigger (e.g. dsRNA) into small interfering RNAs (siRNAs). These siRNAs will then become part of the RNA-induced silencing complex (RISC) that interacts with the target mRNA, leading to the cleavage

of it and thus its degradation. By that, mRNA translation is inhibited, which leads to a decreased protein expression.

RNAi has proven to be a powerful tool in investigating physiological functions and phenotype alterations by a loss-of-function manner in the salmon louse. Lice may be subjected to dsRNA at various life stages in different formulas. In newly hatched nauplius I, dsRNA is introduced to the larvae in a bath treatment where they are left to soak in the dsRNA until they have molted to the nauplius II stage (approximately 24 hours at 10 °C) (Eichner et al., 2014). After this, the larvae are placed in incubators in running seawater until they reach the copepodid stage. At this point, phenotype alterations (e.g. inhibited molting, decreased swimming capacity, increased mortality etc.) can be observed, if present, and lice may be sampled for assessment of transcript down-regulation by real time quantitative PCR (RT-qPCR), or used in infestation trials and sampled at a later life stage. In the preadult II and adult stages, lice (male or female) are injected in the cephalothorax using thin capillaries with a dsRNA solution to provoke the RNAi mediated knockdown (Dalvin et al., 2009) (Fig. 9). Upon injection, the lice are placed back on their host and left until they reach the mature adult stage with egg string production. In this thesis, louse fitness was monitored with a special focus on the parasite fecundity. This because one of the main goals of studying the salmon louse is to obtain more knowledge about the parasite biology and its specific protein functions, and ultimately to discover targets for therapeutic intervention by identifying its Achilles heel(s).

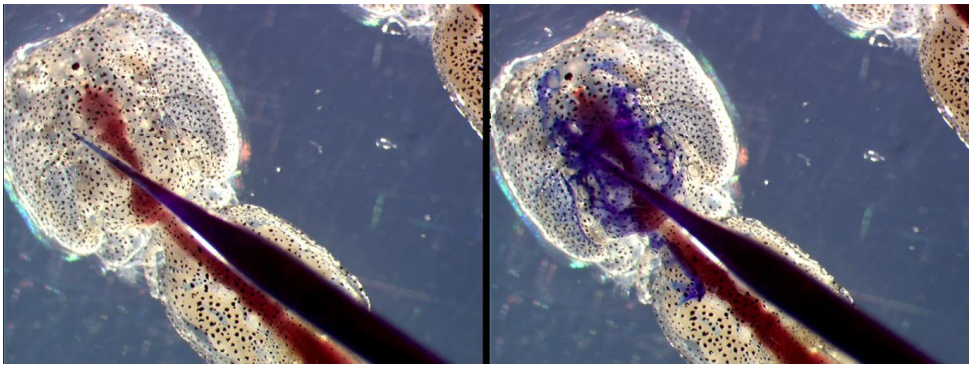

Figure 9: dsRNA injection. An adult female salmon louse is injected with borosilicate glass capillaries containing dsRNA in order to provoke a transcript knockdown. The dsRNA is mixed with bromophenol blue in order to observe the success of an injection. Photo: Lars Are Hamre.

3.4 Heme quantification

Heme is a molecule with various biochemical properties with characteristic spectrums that can be quantitatively analyzed with e.g. spectrophotometry measuring the absorbance, high-performance liquid chromatography (HPLC) or fluorescence (Sinclair et al., 1999). Heme is not fluorescent in itself. Therefore, in order to quantify heme by fluorescence, one must reduce the heme molecule to the ring structure protoporphyrin IX without iron. This can be accomplished by boiling a sample in e.g. saturated oxalic acid. The resulting porphyrin ring structure without iron may be emitted with light around λ 400 nm and the emission spectrum peak is read at around λ 600 nm using a spectrofluorometer (Morrison, 1965; Sinclair et al., 1999; **Paper II**). Results may then be compared to a standard curve of heme where there is a linear relationship of arbitrary fluorescence levels with a known set of concentrations. This approach was in this thesis adapted to quantify heme in the salmon louse. However, only absorbed heme levels were of interest, and not heme in the still digesting blood meal. Blood contains an excess of heme, and measuring heme from the ingested fish blood could lead to false positive results, possibly masking the actual absorbed heme levels. This was a vital point in designing the experiment, as the heme quantification assay was to be used on RNAi knockdown salmon lice where a gene encoding a possible mediator of intestinal heme absorption was silenced. In order to avoid

intestinal blood in the analyses, the adult female salmon louse intestine was dissected out and used to check for knockdown success by RT-qPCR. To normalize heme levels, a protein quantification assay was used to ensure that differences in heme concentrations are not due to an overall protein reduction in the salmon louse caused by the knockdown (**Paper II**).

4. Summary of papers

4.1 Paper I

«Heavy and light chain homologs of ferritin are essential for blood-feeding and egg production of the ectoparasitic copepod *Lepeophtheirus salmonis*»

Here, the goal was to study the importance of ferritin in the salmon louse. Ferritin is the major known iron storage protein in animals, and is well characterized and much described in literature. Vertebrate ferritin is mostly made up of two subunits, the heavy chain and the light chain, which assemble into a three dimensional protein shell. Together, these two subunits oxidize, detoxify and store iron, which otherwise could harm the cell by generating reactive oxygen species. Arthropods have ferritin subunits that are homologous to the vertebrate ones. The salmon louse has several predicted ferritin subunits (LsFer1-4). These include intracellular heavy chain homologs (LsFer3-4), a secreted heavy chain homolog (LsFer1) and a secreted light chain homolog (LsFer2). Secreted ferritins are common in arthropods, where they are found to be involved in transportation of iron in addition to its storage and detoxification. The transcripts of *LsFer1* and *LsFer2* are both expressed in the salmon louse midgut, both decrease under starvation, and both yield similar phenotypic alterations during RNAi knockdown experiments, and they both have a similar temporal expression pattern. These similarities indicate that the two subunits may be heterodimers of one ferritin protein. The knockdowns of *LsFer1* and *2* caused the adult female lice to cease blood-feeding and almost completely halted egg production. Histological analyses of knockdown animals revealed that the silencing of *LsFer1* and *2* caused oocytes to not develop properly, as they lacked the structure seen in control oocytes. Combined, these results stress the importance of these genes for the normal parasite biology, and could potentially be targets for pest management.

4.2 Paper II

«A scavenger receptor B (CD36)-like protein is a potential mediator of intestinal heme absorption in the hematophagous ectoparasite *Lepeophtheirus salmonis*»

In this project, the aim was to learn more about intestinal heme absorption, using the salmon louse as a model organism. Heme, or iron protoporphyrin IX, is a protein cofactor most commonly found in the hemoglobin of red blood cells where it acts as a vehicle for gas transportation. Heme is also, alike iron, potentially cytotoxic if not bound and detoxified by appropriate metalloproteins. The intestinal absorption of heme has remained an enigmatic process, even though dietary heme provides the most bioavailable form of iron. The hypothesis was that the scavenging of exogenous heme is essential for the normal parasite biology, and that there should be an intestinal protein in the salmon louse that facilitates heme absorption. A scavenging CD36-like receptor is a potential mediator of heme absorption in the salmon louse. Homologs of CD36-like receptors in other species have not been suggested to be implicated in the absorption of heme previously. The receptor was named “heme scavenger receptor class B” or LsHSCARB. The receptor is expressed in the adult female salmon louse midgut, and the protein was found to bind heme *in silico* as well as *in vitro*. Adult female louse starvation led to a decrease in the receptor transcript levels. Moreover, a functional knockdown study mediated by RNAi caused the adult female lice to have significantly lowered absorbed heme levels, as measured by fluorescence. The knockdown animals also produced shorter egg strings with a worsened egg hatching success. These results provide the first clue of a heme trafficking pathway not described in any other species to date.

4.3 Paper III

«Host gill attachment enables blood-feeding by the salmon louse (*Lepeophtheirus salmonis*) chalimus larvae, and alters parasite development and transcriptome»

The main objective of this project was to investigate the salmon lice transcriptome connected to blood-feeding, and also to investigate when, during the louse development, blood-feeding starts. Hematophagy (blood-feeding habit) is a common trait in parasitizing arthropods as blood is a highly nutritious tissue fluid which is constantly renewed in the vertebrate host. Blood also contains micronutrients such as iron and the iron-containing pyrrole ring, heme. Iron and heme are both molecules that the salmon louse needs to obtain from its diet, yet they are also potentially highly toxic if not properly detoxified. Initiating in hematophagy should thus require a shift in the salmon louse transcriptome. In order to study this, Atlantic salmon were infected with salmon louse copepodids, and lice were sampled at 10 and 18 days post infestation (dpi). Lice developmental state (stage and instar age), settlement sites, and whether the louse guts were filled with blood, were determined. Lice mostly settled on the host fins, and moved towards the host body upon becoming mobile preadults. The lice found on gills were slower developed than lice elsewhere on the host, and these lice often had a blood-filled intestine already at the chalimus I stage. Aside from lice on the gills, the observation was that blood-feeding would start only when the lice reach the mobile preadult I stage. Chalimus larvae on gills and on skin (fins or body) sampled at 10 and 18 dpi were therefore used for RNA-sequencing. The expression of several genes is differently regulated comparing lice from the gills versus those from the skin. Many genes are up-regulated in lice sampled from gills, and among these are ferritins (iron storage/transportation), peptidases (digestion), genes of unknown functions, FNII-encoding genes etc. In conclusion, ingesting blood should normally start when the louse reaches the mobile stages, and a premature onset of this diet causes the lice to develop at a slower pace. A shift in gene expression is evident in the lice that have been attached to the vascular host gills.

5. General discussion

The major aim of the present study was to obtain more knowledge about hematophagy in the salmon louse. Hematophagy is a common trait in parasitism, as blood from a vertebrate host is highly nutritious, as well as continuously renewed through hematopoiesis. Blood is heavily biased towards proteins containing iron and heme, which are micronutrients that work as cofactors of certain proteins, enabling several biochemical events in cells of all animals. However, the potential cytotoxic effects caused by the blood meal stress the need for tightly regulated processes in handling its components. The salmon louse is an obligate marine ectoparasite feeding off the skin and blood from its salmon host. Elucidating blood as a diet, with a particular focus on iron and heme trafficking pathways in the salmon louse, has yielded new insight about the biology of the hematophagous arthropod, and the results of **Paper I**, **II** and **III** are here linked together and discussed.

5.1 Characterization of studied genes and proteins

Throughout the course of this study, genes and proteins analyzed have been characterized by bioinformatical and functional studies, and have been annotated accordingly.

5.1.1 Ferritin subunits LsFer1-4

In **Paper I**, salmon louse ferritin homologs were characterized. Due to sequence similarities throughout investigated phyla and the conservation of ferritin motifs, the hypothesis that LsFer1, 2, 3 and 4 have the function of iron storage and detoxification and belonging to the ferritin-like superfamily (PF00210) is sustained. Of the cytosolic ferritins, there were identified HCHs (*LsFer3* and *LsFer4*), whereas of the secreted ferritins one HCH (*LsFer1*) and one LCH (*LsFer2*) were identified. All HCH ferritins investigated here, however, have the predicted ability of nucleating iron (in addition to HCH specific conserved motifs such as ferroxidase activity) which is normally reported as being LCH specific (Pham and Winzerling, 2010). The intracellular HCH subunits may therefore make up an iron storage molecule alone. Hybrid type ferritins, having

both HCH and LCH features, are common in crustaceans (Maiti et al., 2010; Masuda et al., 2018; Qiu et al., 2008). As *LsFer3* was mainly found to be expressed in testis, it most likely has a male specific function. There was no sign of post-transcriptional control of ferritin in the salmon louse, as no iron-responsive element motifs were predicted in the mRNA untranslated regions. The results here rather suggest that LsFer1 and 2 are primarily regulated at a transcriptional level, as transcripts are responsive to starvation (**Paper I**) and blood-feeding (**Paper III**).

5.1.2 Heme scavenger LsHSCARB

In **Paper II**, a gene encoding a scavenger receptor class B (SCARB) was identified and subjected to bioinformatical and functional investigation. The characteristics of SCARB (PF01130) includes transmembrane domains in the N- and C-terminuses in the amino acid sequence, with ends located intracellularly, and an extracellular ligand binding domain, which are criteria that LsHSCARB fulfill. Scavenger receptors make up a heterogeneous protein group, which has been reported to scavenge various ligands. In Mammalia, SCARB homologs are reported to scavenge e.g. oxidized and non-oxidized low-density lipoprotein (Endemann et al., 1993), β-carotene (van Bennekum et al., 2005) and very long chain fatty acids (Drover et al., 2008). In arthropods, SCARB homologs have been found to mediate tick systemic RNAi (Aung et al., 2011b), and a gene knockdown caused negative effects on egg weight, hatching rate and tick survival (Aung et al., 2011a). Furthermore, a scavenger receptor in the crustacean freshwater shrimp *Macrobrachium nipponese* was up-regulated by various dietary lipid sources (Ding et al., 2016), suggesting that scavenger receptors may be important in nutrient trafficking in other crustacean species in addition to the salmon louse. Further analyses of LsHSCARB all pointed in the direction of it being able to scavenge heme from the blood ingested by the salmon louse. The hypothesis was strengthened by *in silico*, *in vitro* as well as *in vivo* experiments (**Paper II**), however further recombinant protein characterization should be conducted. LsHSCARB ligand specificity is not known, as only heme was investigated as a potential ligand here. Other proteins that can mediate heme absorption in the salmon louse intestine may also exist.

5.1.3 Transcripts identified by RNA-sequencing

Genome annotation underlying transcriptomics is done automatically and transcripts found in RNA-seq (as in **Paper III**) are mapped to genes of this model. Annotations of predicted amino acid sequences are conducted by blasting against the NCBI GenBank NR (https://www.ncbi.nlm.nih.gov/) and SwissProt (Bairoch and Apweiler, 2000) databases. Due to large datasets, automatic annotation is necessary, but the drawbacks may be that genes in the reference genome (http://metazoa.ensembl.org/Lepeophtheirus_salmonis) could have been wrongly assembled and in that way, protein domains could be lost or fusion proteins could be produced. Proteins could be annotated with erroneous functions due to similarity with proteins sharing a conserved region, yet not having the same functional domains. An incorrect assembly could also result in inaccurate transcript predictions in the datasets if e.g. two differently regulated transcripts are annotated as one. Nevertheless, specific protein families are predicted for the output transcripts from the experiments, which adds more confidence to the analyses. Additionally, in transcriptomics, the purpose is often to identify unexpected groups of transcripts that are regulated in an experiment. Important transcripts that emerge in the datasets are investigated further manually to evaluate the sequence quality.

5.2 Missing genes in the salmon louse

Because comprehending salmon louse heme biology is vital in order to understand and discuss the results of this thesis, extra genome analyses are included here. In order to investigate the salmon louse heme biosynthetic and degradation pathways, the first goal was to find homologous sequences in the free-living copepod *T. kingsejongensis* (Kang et al., 2017). This copepod had homologs for all synthesis and degradation pathway enzymes, however no homologs for COX10 were found, the enzyme needed for the conversion of heme B to heme O (Table 2). Sequences from *T. kingsejongensis* and *D. melanogaster* were further used as query for Blast searches against the salmon louse genome. Here, homologs for only one synthesis enzyme were found; CPOX (Stable ID: EMLSAP00000008964 and EMLSAP00000009295) (Table 3). CPOX catalyzes

the synthesis of protoporphyrinogen III from coproporphyrinogen III. Assuming that the salmon louse could obtain coproporphyrinogen III from its diet, any further modifications of protoporphyrinogen III to PPIX and insertion of an iron atom to the porphyrin ring center by FECH are still unlikely events as the enzymes able to catalyze these reactions are seemingly missing. The parasite has retained both the oxygen dependent and independent version of CPOX. Additionally, the two genes are not only found in the genome, they are also found in the salmon louse transcriptome, suggesting that translated CPOX homologs in the salmon louse are functional. Publicly available RNA-seq data in LiceBase report that the aerobe CPOX homolog transcript, EMLSAT00000008964, is expressed somewhat evenly in all salmon louse life stages and tissues (https://licebase.org/). Anaerobe CPOX homolog transcript, EMLSAT00000009295, is expressed mostly in testis, but is also found in ovaries and eggs (https://licebase.org/). CPOX homolog function in the salmon louse remains an unanswered question. The presence of homologs for COX10 and COX15 indicates that the salmon louse could convert heme B to heme O and heme A, required in various hemoproteins. The heme B molecule is probably mostly scavenged from host hemoglobin, as the salmon has a complete heme biosynthetic pathway (https://www.kegg.jp/kegg-bin/show_pathway?sasa00860). Lack of HO indicates that the salmon louse cannot break down the heme molecule to obtain iron, at least not according to current knowledge about heme catabolism. Iron is likely scavenged from circulating host transferrin. Based on the lack of stated enzymes, the proposed postulation is that the salmon louse is among the natural heme auxotrophic organisms (alongside e.g. ticks (Braz et al., 1999) and worms (Rao et al., 2005)), and to our knowledge this is the first detailed report of a Crustacean species in this group. The co-evolution of the hematophagous parasitizing salmon louse with its host seem to have rendered the heme biosynthetic pathway redundant in this species and likely promoted gene loss. Genes not being transcribed are prone to accumulate mutations, be truncated and eventually lost from the genome (Albalat and Cañestro, 2016). An excess of exogenous heme might have repressed ALAS transcription (Furuyama et al., 2007), and thus eliminated the synthesis of endogenous heme in the louse with neutral effects on parasite fitness. However, the suggestion that salmon lice lack means of heme

biosynthesis and degradation are based on *in silico* genomic analyses alone. Furthermore, this raises a new question: how does the salmon louse cope with excess heme obtained from the diet? Alternative heme detoxification, catabolic mechanisms and cellular heme excretion events should be studied further.

Table 2: Genome mining in *Tigriopus kingsejongensis.* Predicted enzymes of the heme biosynthesis and degradation pathway in *T. kingsejongensis* found by Blast searches using *D. melanogaster* and *I. scapularis* sequences as query. Green = positive hits, white = no hits with an E-value less than 1E-6.

Protein	Blast search	Query	Best hit	Score (bits)	E-value	PFAM
ALAS	BlastP	NP_477281.1 (a)	Tk02602	618	0	PF00155*
ALAD	BlastP	NP_001261752.1 (a)	Tk04959	369	2E-128	PF00490 (ALAD)
HMBS	BlastP	NP_612103.1 (a)	Tk00609	276	8E-87	PF03900 (HMBS)
UROS	tBlastN	NP_572507.1 (a)	maker-scaffold1394_size43558-snap-gene-0.7	89	9E-19	PF02602 (UROS)
UROD	BlastP	NP_610501.1 (a)	Tk06395	433	3E-146	PF01208 (UROD)
CPOX	BlastP	NP_524777.1 (a)	Tk12349	484	2E-171	PF01218 (CPOX)
		EEC11362.1 (b)	Tk08114	192	1E-56	PF04832**
PPOX	BlastP	NP_651278.2 (a)	Tk10742	354	9E-118	PF01593***
FECH	BlastP	NP_524613.1 (a)	Tk06128	512	0	PF00762 (FECH)
HO	BlastP	NP_524321.1 (a)	Tk11351	134	2E-38	PF01126 (HO)
COX10	tBlastN	NP_609382.1 (a)	No hit	N/A	N/A	N/A
COX15	BlastP	NP_611855.1 (a)	Tk00231	397	3E-137	PF02628 (COX15)

a) *D. melanogaster*
b) *I. scapularis*
*Aminotransferase class I and II (GO:0003870 5-aminolevulinate synthase activity)
**SOUL heme-binding protein (GO:0003824 catalytic activity/IPR034505: Anaerobic CPOX)
***Flavin containing amine oxidoreductase (GO:0004729 oxygen-dependent protoporphyrinogen oxidase activity)

Table 3: Genome mining in the salmon louse. Predicted enzymes of the heme synthesis and degradation pathway in the salmon louse found by Blast searches using *T. kingsejongensis* and *D. melanogaster* sequences as query. Green = positive hits, grey = only sequence similarity, white = no hits with an E-value less than 1E-6.

Protein	Blast search	Query	Best hit	Score (bits)	E-value	PFAM
ALAS	BlastP	Tk02602 (a)	EMLSAP00000001120	183	7E-52	PF00155*
	BlastP	NP_477281.1 (b)	EMLSAP00000001120	179	1E-50	PF00155*
ALAD	tBlastN	Tk04959 (a)	No hit	N/A	N/A	N/A
	tBlastN	NP_001261752.1 (b)	No hit	N/A	N/A	N/A
HMBS	tBlastN	Tk00609 (a)	No hit	N/A	N/A	N/A
	tBlastN	NP_612103.1 (b)	No hit	N/A	N/A	N/A
UROS	BlastN	maker-scaffold1394_size43558-snap-gene-0.7 (a)	No hit	N/A	N/A	N/A
	tBlastN	NP_572507.1 (b)	No hit	N/A	N/A	N/A
UROD	BlastP	Tk06395 (a)	EMLSAP00000006932	142	7E-37	PF00001**
	tBlastN	NP_610501.1 (b)	No hit	N/A	N/A	N/A
CPOX	BlastP	Tk12349 (a)	EMLSAP00000008964	466	4E-165	PF01218 (CPOX)
	BlastP	Tk08114 (a)	EMLSAP00000009295	259	3E-81	PF04055***
PPOX	BlastP	Tk10742 (a)	EMLSAP00000003284	47.4	7E-06	PF01593†
	tBlastN	NP_651278.2 (b)	No hit	N/A	N/A	N/A
FECH	tBlastN	Tk06128 (a)	No hit	N/A	N/A	N/A
	tBlastN	NP_524613.1 (b)	No hit	N/A	N/A	N/A
HO	tBlastN	Tk11351 (a)	No hit	N/A	N/A	N/A
	tBlastN	NP_524321.1 (b)	No hit	N/A	N/A	N/A
COX10	BlastP	NP_609382.1 (b)	EMLSAP00000000335	334	1E-112	PF01040††
COX15	BlastP	Tk00231 (a)	EMLSAP00000010869	512	0	PF02628 (COX15)

a) *T. kingsejongensis*
b) *D. melanogaster*
* Aminotransferase class I and II (GO:0008890 glycine C-acetyltransferase activity)
**7 transmembrane receptor (rhodopsin family)
***Radical SAM superfamily (GO:0004109 coproporphyrinogen oxidase activity/ IPR034505: Anaerobic CPOX)
†Flavin containing amine oxidoreductase
††UbiA prenyltransferase family (GO:0048034 heme O biosynthetic process)

5.3 Iron and heme trafficking in the salmon louse

In the traditional view of iron and heme trafficking, the two pathways are linked together as heme degradation by HO liberates one iron molecule per heme molecule. This iron molecule may be further incorporated into iron-storage proteins, iron-binding proteins, or it can be recycled into the heme biosynthetic pathway by insertion of iron to PPIX by FECH in the mitochondria (Fig. 6 and 10A). As no homologs of HO and an incomplete heme biosynthetic pathway are found in the salmon louse, the proposed systems of iron and heme trafficking here would be separate events, with no donation of heme-iron to the intracellular iron pool (Fig. 10B). This would imply that the salmon louse is innately dependent on scavenging of both host iron and heme, without the option of using heme as an iron source or using absorbed iron in endogenous heme synthesis. This could be a weakness in the blood-feeding parasite, as it would imply that there exists at least two vital pathways that could be targeted by novel louse interventions. Absence of HO upon the heme biosynthetic pathway loss could reassure that cells are not harmed by excess levels of iron liberated by heme degradation. In this thesis, the putative absorption of heme in the salmon louse (by LsHSCARB) is described (**Paper II)**. Here, both LsHSCARB transcript and protein are detected in cells lining the adult female salmon louse intestinal tract, supporting the functional hypothesis that LsHSCARB is a nutrient-scavenging protein. In **Paper I**, an iron storage and transportation mechanism is proposed mediated by the ferritin subunits LsFer1 and LsFer2. The transcripts of these subunits were located also in cells lining the intestinal tract of the adult female salmon louse. In **Paper I**, the mature protein localizations have not been analyzed, and hence conclusions about its spatial distribution cannot be drawn. However, based on the signal peptides in both sequences, and knowledge from literature on other secreted ferritins in arthropoda (Galay et al., 2014b; Hajdusek et al., 2009; Huebers et al., 1988; Nichol and Locke, 1989), the postulation is that the mature protein is secreted to the salmon louse hemolymph.

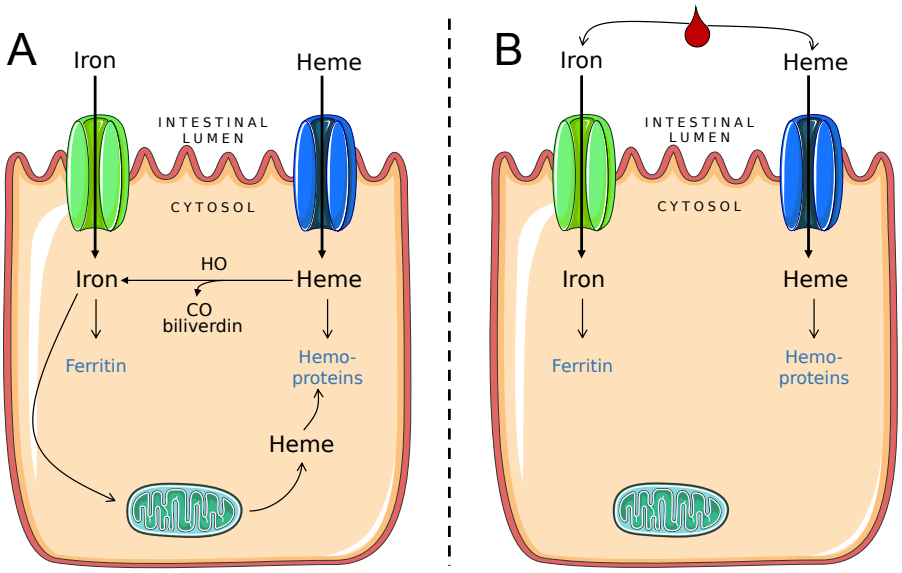

Figure 10: Iron and heme trafficking. Simplified graphical illustration of iron and heme trafficking in (**A**) common intestinal epithelial cell and (**B**) proposed salmon louse intestinal epithelial cell exposed to a blood meal. See main text for further explanation of the two different systems. It should be noted that in this simplified illustration, further trafficking of ferritin and hemoproteins, and incorporation of iron (other than for heme biosynthesis) and hemoproteins into the mitochondria are not shown. HO = heme oxygenase, CO = carbon monoxide. The illustrated cell components are downloaded from Servier Medical Art by Servier (https://smart.servier.com/) (Attribution 3.0 France: CC BY 3.0 FR).

5.4 Further insight into salmon louse hematophagy

One of the conclusions of this thesis so far is that blood is an important dietary component for the salmon louse, as it provides the parasite with nutrients it cannot synthesize on its own. Learning more about the louse blood-feeding behavior can therefore provide new knowledge about basic salmon louse biology, and more generally about hematophagy in arthropods. In **Paper III**, a finding is that the salmon louse starts actively feeding on blood in the mobile preadult I stage. In an artificial infestation, as the results of this thesis are based on, the vascular fish gills are a common settlement site. Lice attached to gills have visual evidence of a blood-filled gut already at the chalimus I stage (also observed in attached copepodids, Dr. Christiane Eichner,

pers. comm.). This was the rationale behind examining the transcriptomes of chalimus lice on gills versus lice on other salmon parts. As such, chalimus larvae presumably developing on two different diets, one hematophagous and one on a diet of skin epidermis, could be compared. The higher expression of *LsFer1* and *LsFer2* in chalimus larvae on gills give support to the suggestion that these lice have been exposed to blood, as not obtaining adequate iron storage mechanisms upon ingestion of host blood would have detrimental effects for the lice (**Paper I**). The vast increase in digestive enzyme transcripts further supports the assumption that the chalimus lice on gills have another nutritional access than those elsewhere on the host. Thus, transcripts emerging in **Paper III** might be important for the salmon louse during hematophagy. Transcripts with e.g. putative anti-coagulative, heme detoxification, digestive and unknown functions could be at the center for new topics to be studied. Fibronectin type II (FNII) domains are widely distributed in the salmon louse genome, yet their functions here are still enigmatic (Harasimczuk et al., 2018; Øvergård et al., 2016). The FNII-encoding genes found to have a higher expression in chalimus larvae attached to gills should be further studied to elucidate the possible role of FNII in salmon louse hematophagy.

Another interesting observation is that being attached to the host gills caused the lice to develop at a slower pace than those on skin (**Paper III**). There have been contradicting results about this in the past (Johnson, 1993; Johnson and Albright, 1992). However, in this study, lice instar ages were determined (Eichner et al., 2018, 2015a), and not only developmental stages, adding more confidence to the results of this thesis. Johnson and Albright (1992) suggested that reduced growth of lice developing on gills could be due to non-specific humoral factors of the host, which the parasite is more exposed to at vascular attachment sites. A continued hypothesis for the reduced growth of lice on gills is that blood exposure causes the lice to alter their transcriptome to sustain homeostasis upon ingesting a blood meal. This could be at the expense of cellular proliferation and thus organismal growth.

5.5 Considerations of methodology

Several of the methods applied during this thesis are well-established within the field of salmon louse biology. Among these are e.g. *in situ* hybridization, salmon louse genome analyses with bioinformatical tools, immunohistochemistry and salmon louse infestation studies, and will not be considered to a deep extent. The heme quantification assay was established in order to conduct measurements needed for **Paper II**. This allowed the measurement of the lowered heme levels in *LsHSCARB* knockdown animals. However, it did not allow differentiation between the various kinds of heme in the louse. In order to study this, heme levels by e.g. mass spectrometry could be assessed. The starvation experiments conducted in both **Paper I** and **II** showed that the transcript levels of *LsFer1*, *LsFer2* and *LsHSCARB* decreased with the time lice were absent from the host and thus food source. While this adds an interesting story to the publications, especially since it was believed that a heme receptor should be up-regulated during heme shortage (Rajagopal et al., 2008), in retrospect it would be more convincing if a rescue experiment was included where starved lice were placed back on fish and transcript levels were subsequently analyzed. This would allow a stronger conclusion that ferritin and the putative heme scavenger receptor are regulated by the dietary status of the louse. RNAi knockdown experiments have been a significant driving force in this thesis, and are therefore thoroughly considered in the following section.

5.5.1 RNAi experiments

Although RNAi is a well-established methodology in the salmon louse and is routinely performed at the SLRC, discarding pest control candidates because of *"invisible phenotype alterations"* may occur. A routine RNAi experiment initiated in preadult II female lice is terminated upon the extrusion of the second pair of egg strings of the adult females (approximately 40 days after dsRNA injection). The knockdown animals are at this time point visually inspected, and sampled for assessment of knockdown efficacy and histological analyses. If there are no visible phenotype alterations (e.g. shorter egg strings or other abnormalities) at this time point, the knockdown target may be labeled as non-vital for the salmon louse, and therefore not inspected further. A pilot

knockdown of *LsHSCARB* was terminated when the control lice extruded their second egg string pair. At termination, knockdown animals appeared to have a normal morphology, with normal egg string lengths and hatching success, and also the knockdown animals did not have any visible negative effects as seen in histological sections (data not shown), even though the knockdown was 99% efficient. However, as the fluorescent heme quantification assay had been established for salmon louse tissues during the course of this PhD, there was a possibilty to measure absorbed heme levels in the louse, which were found to be significantly decreased (**Paper II**). As the salmon louse does not encode HO, it could be speculated that instead of excreting degradation products of heme, the louse recycles heme that is already absorbed. In that sense, a decreased heme absorption capacity may not manifest in detrimental consequences at an early stage after knockdown. Additionally, it has to be taken into consideration that free-living salmon louse larval stages are non-feeding, dependent on maternal yolk sac reserves and unable to synthesize heme *de novo* (as the louse is likely a heme auxotroph), even though they require the cofactor in their cells. Nauplius larvae and copepodids probably require maternally derived heme until they are attached to a host themselves. An extended period of *LsHSCARB* ablation should therefore in theory in time have a negative impact on the salmon louse fecundity, as there is a wide recognition for the importance of heme for development (Gaughan and Krassner, 1971; Perner et al., 2016; Toh et al., 2015; Walter-Nuno et al., 2013), illustrating that a portion of heme of maternal origin is probably deposited into the oocytes. By this reasoning, the second experimental knockdown of *LsHSCARB* was prolonged, and terminated 69 days after dsRNA injection, when the control animals had extruded their fifth pair of egg strings. Upon the second RNAi experiment termination, the *LsHSCARB* silencing was still successful and knockdown animals had a 60% decrease in absorbed heme levels. There were also significantly shorter egg strings with a significantly lowered hatching success, likely due to the lowered heme levels (**Paper II**). Prolonging the experimental knockdown study design and assessment of heme levels hence allowed further studies of the scavenger receptor.

The knockdowns of secretory ferritin subunits *LsFer1* and *LsFer2*, on the other hand, resulted in severe phenotype alterations for the salmon louse already at the extrusion

of the second pair of egg strings of the control animals (**Paper I**). Knockdown animals produced little to no viable eggs, and their intestines were not filled with blood, as was observed in the engorged control animals. A possible explanation for the early visible phenotype alteration in *ferritin* knockdowns compared to the *LsHSCARB* knockdown could be attributed to different protein functions. LsHSCARB is, as already discussed, suspected to be related to heme absorption (**Paper II**), whereas ferritin is commonly related to iron storage and transportation (**Paper I**). If there is a limited possibility for the salmon louse to store iron upon ingestion of its blood meal, the louse is prone to the many cytotoxic effects of excess free iron. A strategy for the parasite to avoid this toxicity could be to cease the blood-feeding behavior, which would certainly have negative downstream effects such as under-nutrition (including, but not limited to, iron deficiency), which would further lead to a reduced parasite fecundity. Similar results were also obtained in the blood-feeding tick after ferritin knockdown (Galay et al., 2013; Hajdusek et al., 2009). Ablation of an absorption mechanism, on the other hand, would not lead to the same immediate negative effects. Therefore, when designing an RNAi experiment, one should consider what effects to expect from silencing the selected target, and plan the experiment termination, and downstream analyses, accordingly. Another aspect to consider upon conducting knockdown experiments is that RNAi mediated knockdown targets mRNA, and not protein. A slow turnover of a protein could lead to a later onset of a phenotype alteration, even though mRNA levels are downregulated. Finally, herein gene *knockdowns* have been performed, as opposed to gene *knockouts* where gene expression is eliminated. Remaining transcripts may still be translated and cause a continued mature protein production, which may postpone phenotype alterations in the salmon louse. Gene *knockout* is per today not an established methodology in the salmon louse.

5.6 Iron, heme and lice control

As resistance towards a wide range of anti-sea lice medicaments is a common and growing problem, new therapeutic interventions for the aquaculture industry are sought after. One SLRC vision is to develop a vaccine for the salmon, with a significant

protection against the salmon louse. However, the nature of the host-parasite interaction between the salmon and the salmon louse causes challenges. Salmon lice are ectoparasites that are scavenging their host for nutrients from the external environment, creating a bottleneck in identifying antigens for a vaccine that initiates an immune response targeting the parasite. As till today, only one vaccine is commercially available against an ectoparasite. This is the Bm86 antigen vaccine against the tick *R. microplus* (Willadsen et al., 1989). Bm86 is a plasma membrane glycoprotein expressed on the microvilli of digestive cells of the tick midgut (Gough and Kemp, 1993). An RNAi mediated silencing of *R. microplus Bm86* caused a reduced tick fitness, as knockdown animals had a lower survival rate and produced eggs with a decreased mass (Bastos et al., 2010). Membrane proteins with an extracellular domain facing the parasite midgut lumen could thus be potential targets for pest management by vaccination. LsHSCARB fulfils these criteria, as it is a protein within the CD36 superfamily (PF01130) with two transmembrane regions, one extracellular ligand-binding domain, and N- and C-terminal tails located intracellularly, and is expressed in the salmon louse midgut, and with knockdown effects already discussed (**Paper II**). Ferritin subunits LsFer1 and LsFer2 also fulfil these criteria to a certain degree as the transcripts are located in the salmon louse gut, and the knockdowns cause detrimental effects for parasite egg production **(Paper I)**. However, as discussed, the secreted ferritin protein is likely in the salmon louse hemolymph. The recombinant secreted HCH IrFER2 (GenBank ID: ACJ70653) (Hajdusek et al., 2009) and HlFER2 (GenBank ID: BAN13552) (Galay et al., 2013) have been tested in pilot studies as vaccine candidates in cattle and rabbits against tick infestations with promising results (Galay et al., 2014a; Hajdusek et al., 2010). However, a more recent vaccine trial could not reproduce these results (Knorr et al., 2018). Secreted ferritins are therefore worth testing as vaccine candidates in the salmon against the salmon louse, however results should be interpreted carefully. How well antibodies from the vaccinated salmon maintain their integrity in the salmon louse gut, and whether salmon antibodies may be transported across cellular membranes are unknown. Obtaining more knowledge about the salmon louse digestive tract is a key requirement.

An alternative to targeting the aforementioned proteins by vaccination could be receptor agonistic or antagonistic targeting. This is more relevant for LsHSCARB than for ferritin, as LsHSCARB has a predicted ligand-binding domain in the salmon louse intestinal lumen. In order to achieve this, the binding pocket structure and shape of LsHSCARB should be further investigated. A screen for various molecules, in addition to heme, that could bind to it need to be tested. This can be achieved using recombinant protein *in vitro*, but also by utilizing bioinformatical tools *in silico*. In order for a competitive binding to occur, the receptor should have a higher affinity for the tested molecule than for heme. The molecule should also be possible to add to the salmon feed, without compromising the nutritional status, and without accumulating in the salmon fillet as it is used for human consumption. Furthermore, the molecule should be LsHSCARB specific, and it needs to be able to avoid degradation in the salmon louse intestine. It also needs to avoid aggregation, as this could have detrimental effects for the salmon. This kind of nutritional immunity is depicted in Figure 11.

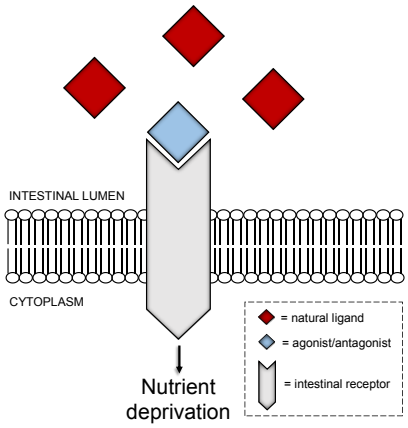

Figure 11: Pest control by nutrient deprivation. Nutritional immunity may be achieved by agonistic or antagonistic binding to an intestinal nutrient receptor, blocking the natural ligand uptake from the intestinal lumen and into the cytoplasm.

Vaccination and nutritional deprivation experiments that target salmon louse antigens related to hematophagy, such as ferritin (**Paper I**) and the heme scavenger receptor

(**Paper II**), should last until the louse population reaches the mobile, blood-feeding stages (**Paper III**). However, mobile lice can alternate between hosts (Ritchie, 1997), and in that way an effect (other than infestation rate) of sitting on a treated versus a control host could be camouflaged. A solution for this could be to avoid a common garden setup, and rather use single fish tanks in pilot tests (Hamre and Nilsen, 2011).

6. Conclusion

This thesis has contributed to the understanding of the importance of hematophagy in the ectoparasitic salmon louse, *L. salmonis*. The work presented herein has provided details on the trafficking of iron and heme in the parasite. An insight into the salmon louse transcriptome while being attached to the vascular host gills has demonstrated that the salmon louse alters its gene expression drastically depending on its nutrient access. Perhaps most strikingly, this thesis has proposed a novel candidate for the intestinal heme absorption in an arthropod.

Main conclusions of this thesis are:

- The salmon louse has both intracellular and secreted homologs of the iron storage protein ferritin.
- The secreted ferritins are vital for reproduction and hematophagy in the adult female salmon louse.
- The salmon louse is likely among the natural heme auxotrophic organisms, rendering heme an essential micronutrient for the parasite.
- A reproducible heme quantification assay has been established for salmon louse tissues, enabling functional knockdown studies of candidate heme receptors, and evaluating the total absorbed heme levels in the louse.
- The CD36-like scavenger receptor, LsHSCARB, was characterized in the salmon louse, and was predicted to have heme as a ligand by a 3D model with *in silico* docking studies. This binding was further supported *in vitro* by a recombinant protein and heme-binding assay.
- *LsHSCARB* knockdown resulted in decreased absorbed heme levels in the adult female salmon louse. The knockdown animals produced fewer eggs with a worsened hatching success.
- Hematophagy does not begin until the mobile preadult I stage, unless lice are attached to the vascular host gills.
- Chalimus larvae feeding of blood from the host gills, have an altered transcriptome. These larvae differentially express e.g. various digestive enzymes, transcripts related to iron and heme trafficking, gene transcripts of unknown functions and FNII-encoding genes etc. compared with chalimus larvae attached to host avascular sites.

7. Future perspectives

The essence of this thesis has been in exploring the salmon louse hematophagous nature and emphasizing the importance of this. Several new questions have been raised here. Ferritin regulation, the role of a male-specific ferritin, the role of CPOX and FNII-encoding genes in the louse should be further investigated. Future studies should also further focus on the trafficking of iron and heme from being protein-bound in salmon blood and until being utilized in metalloproteins in the salmon louse tissues. This include enzymatic breakdown of salmon iron- and hemoproteins such as hemoglobin and transferrin in the salmon louse intestine, movement across cellular membranes, distribution to various tissues, including shuttling to developing oocytes, and storage and detoxification mechanisms. Unravelling that both iron and likely heme are essential molecules for the salmon louse, and that heme oxygenase is seemingly lost from the parasite genome, reveal that iron and heme follow pathways that are both important, and are probably independent of each other. A heme quantification assay has already been established during the course of this thesis, and could be further applied when studying hemoproteins in the salmon louse. In parallel with establishing more knowledge of iron and heme in the salmon louse, the work of testing ferritin and the heme scavenger receptor (in addition to other possible proteins characterized in the future) as candidates for therapeutic interventions would be of interest. Pest control by vaccination or nutritional deprivation could help provide a protective effect for the salmon against the salmon louse. However, this is challenging work, as ectoparasites are experts of concealing their antigens from their hosts. In addition, in line with the 3Rs principle (Replacement, Reduction, Refinement) (Russell and Burch, 1959), the use of experimental animals should be kept to a minimum. Future work should therefore be interdisciplinary and strongly include bioinformatical tools, in addition to biological tools, in order to analyze candidate vaccine targets, or receptor agonists and antagonists, *in silico* and *in vitro* prior to testing them experimentally.

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Heavy and light chain homologs of ferritin are essential for blood-feeding and egg production of the ectoparasitic copepod *Lepeophtheirus salmonis*

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ABSTRACT

The salmon louse, *Lepeophtheirus salmonis*, is a hematophagous ectoparasite of salmonid fish. Due to its blood-feeding activity, the louse is exposed to great amounts of iron, which is an essential, yet potentially toxic mineral. The major known iron storage protein is ferritin, which the salmon louse encodes four genes of (*LsFer1-4*). Two of the ferritins are predicted to be secreted. These are one of the heavy chain homologs (*LsFer1*) and the light chain homolog (*LsFer2*). Here, we perform functional studies and characterize the two secreted ferritins. Our results show that knocking down *LsFer1* and *LsFer2* both negatively affect the parasite's physiology, as it is not able to properly feed and reproduce. In a starvation experiment, the transcript levels of both *LsFer1* and *LsFer2* decrease during the starvation period. Combined, these results demonstrate the importance of these genes for the normal parasite biology, and they could thus potentially be targets for pest management.

1. Introduction

Iron is an essential element necessary for many cellular processes in all animals. Iron-containing proteins are present in several key biochemical pathways, such as DNA synthesis, energy metabolism and electron transfer reactions [1]. Free intracellular iron can rapidly be reduced to the ferrous (Fe^{2+}) form, and may catalyze the generation of reactive oxygen species (ROS) which are highly cytotoxic reactive radicals that can cause harm to cellular compounds such as lipids, DNA and proteins [2]. In order to avoid cell harm, iron storage and distribution must be implemented by tightly regulated mechanisms, using designated proteins in the cell.

Ferritin is the major intracellular iron storage protein. Here, ferric iron (Fe^{3+}) is sequestered and kept in a nontoxic, soluble and biologically non-reactive state [3]. In that state, the iron can be recruited to various proteins that require the metal as cofactor to perform their proper biological function. Typically, ferritin is composed of 24 subunits which can harbor up to 4000 Fe atoms [4]. Vertebrate ferritins are made up of the heavy (H) chain and the light (L) chain subunits, whereas arthropod ferritins are made up of homologs to these (heavy-chain homolog, HCH, and light-chain homolog, LCH) which are often secretory [4]. Characteristics for HCH ferritin are amino acid (aa) residues that contribute to a ferroxidase center, which oxidizes Fe^{2+} to Fe^{3+}, tyrosine residues that cause rapid biomineralization of iron, and a pore opening that allows for iron passage. LCH ferritin is characterized by aa residues that induce nucleation of iron. LCH ferritin often has a larger sequence variety, with less similarity to its vertebrate orthologue, and it is often annotated as LCH because of the lacking ferroxidase center [5]. In contrast to cytoplasmic ferritins, secreted ferritins contain cysteine residues that promote inter- and intrasubunit disulfide bonds [6].

With the exception of intracellular iron within iron storage proteins, the majority of vertebrate iron is found in blood, complexed within the porphyrin ring of heme as a cofactor of hemoglobin used for gas transportation, but also in other forms such as bound to the iron transfer protein transferrin [7]. Blood-feeding parasites ingest high levels of iron and thus need to protect their cells from iron overload upon feeding and absorption. The salmon louse, *Lepeophtheirus salmonis*, is an obligate marine ectoparasite, feeding off the skin and blood of its salmonid fish host [8]. With an increasing resistance towards various chemical treatments [9], the salmon louse is considered to be an economically and ecologically important parasite to combat. The parasite's life cycle is divided into eight stages which are as follows: the planktonic nauplius I and II, the infectious copepodid, the attached parasitic chalimus I and II, and the mobile parasitic preadult I and II and adult lice [10,11]. At 10 °C, the development of *L. salmonis* from fertilization to mature adult lice is completed in approximately 40 (male) to 52 (female) days [12]. Once the female louse has reached the mature adult

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stage, it will continuously produce pairwise egg strings. The hatching larvae have the potential to further infect hosts upon reaching the copepodid stage. In order to be able to successfully produce viable offspring, the louse needs to absorb several macro- and micronutrients from its blood meal. One of the many micronutrients required for oocyte development is the mineral iron, which the salmon louse requires proper storage for until it is needed in iron-proteins.

Here, we report the presence of four ferritin homologs (*LsFer1-4*) in the genome of *L. salmonis* and analyze the sequences. Further, we perform functional studies of the two ferritins with signal peptides (*LsFer1* and *LsFer2*) by *in situ* hybridization, starvation as well as RNA interference (RNAi) to evaluate the importance of these genes in reproduction and blood-feeding of the adult female salmon louse. Results from the knockdown study illustrate the detrimental effects for the hematophagous parasite lacking *LsFer1* or *LsFer2*, as normal development and fecundity is negatively impacted upon RNAi mediated knockdown of the genes.

2. Material and methods

2.1. Sequence analyses

Initial data for searching transcript and protein sequences and primer design were extracted from the *L. salmonis* genome annotation in Ensembl Metazoa (https://metazoa.ensembl.org/Lepeophtheirus_salmonis). All following analyses are based on the GenBank sequences: BT121711 (*LsFer1*), BT121232 (*LsFer2*), MK887318 (*LsFer3*), BT077723 (*LsFer4a*) and BT121164 (*LsFer4b*). Glycosylation prediction was performed using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and the NetOGlyc 4.0 Server [13]. Protein BLAST searches were conducted against the Genbank and SwissProt databases [14]. Conserved domain search was conducted using InterProScan [15]. Signal peptides and cleavage sites in the aa sequences were predicted using Phobius [16]. Searches for iron-responsive elements (IREs) in the untranslated regions were performed using SIREs Web Server 2.0 [17].

2.2. Sequence alignment and phylogeny

Aa sequences of LsFer1-4 were used to obtain ferritins of various species from a BLASTp search [14] or from searching The UniProt Consortium [18] for ferritins of model organisms. Alignment was performed in UGENE using MUSCLE alignment under default conditions [19,20]. Grey shading indicates percentage identity. For alignment, following sequences annotated with heavy chain were chosen: *Homo sapiens* (UniProt: P02794), *Salmo salar* (UniProt: P49946), *Xenopus laevis* (UniProt: P49948), *Caenorhabditis elegans* (NP_491198.1), *Penaeus monodon* (ABP68819.1), *Haemaphysalis longicornis* (AAQ54713.1), *H. longicornis* (BAN13552.1)*, *Trichoplusia ni* (XP_026744300.1)*, *Manduca sexta* (AAK39636.1)*, *Anopheles gambiae* (XP_312474.4)*. Light chain sequences chosen were: *H. sapiens* (UniProt: P02792), *X. laevis* (UniProt: Q7SXA5), *S. salar* (NP_001134896.1), *T. ni* (XP_026744299.1)*, *M. sexta* (AAF44717.1)*, *A. gambiae* (XP_001237468.3)*. Sequences with signal peptides are marked with *. For alignment however, the signal sequence was removed. From sequences without signal peptides, the start methionine was removed for alignment.

Sequences used for phylogenetic analysis were: *Caligus clemensi* (ACO15165.1 and ACO14799.1), *Caligus rogercresseyi* (ACO11534.1), *Drosophila melanogaster* (AAF07876.1, NP_524873.1 and NP_572854.1), *Aedes aegypti* (AAO41698.1 and AQY17412.1), *Ixodes ricinus* (ACJ70653.1), *Rhipicephalus microplus* (UniProt: A0A034WXT0), *S. salar* (UniProt: P49947 and P49946, *H. sapiens* (UniProt: P02792 and P02794), *Mus musculus* (UniProt: P29391 and P09528), *C. elegans* (NP_504944.2 and NP_491198.1) and *H. longicornis* (BAN13552.1 and AAQ54713.1). The website http://www.phylogeny.fr was used to construct the phylogenetic tree with default settings [21]. More specifically, sequences were adjusted and aligned by MUSCLE with defaults [20]. The phylogenetic tree was reconstructed by maximum likelihood in PhyML using WAG + G model [22]. The graphical tree was obtained using TreeDyn [23].

2.3. Animals

L. salmonis salmonis was raised on Atlantic salmon (*S. salar*) under laboratory conditions in tanks with seawater (salinity 34.5 ppt and 10 °C) as described before [24]. Fish were daily handfed commercial dry pellets and kept according to Norwegian animal welfare regulations. Experiments were approved by the governmental Norwegian Animal Research Authority (ID8589). Fish carrying lice from RNAi experiments were kept in single-fish tanks [25]. Fish were anesthetized with a mixture of methomidate (5 mg/l) and benzocaine (60 mg/l) upon samplings, or setup of salmon louse infections. Egg string pairs with larvae were incubated and hatched in single incubators in a seawater flow through system [24].

2.4. Ontogeny

The genomic location for the validated transcript sequences (RACE-PCR) were determined using the software GMAP [26] with the Atlantic salmon louse genome (LSalAtl2s, https://metazoa.ensembl.org/Lepeophtheirus_salmonis) and the raw reads of existing RNA-sequencing data were recounted by *featureCounts* [27] at these regions. Available RNA-sequencing data are used as found in LiceBase (http://licebase.org, Dondrup et al., in prep) and from data of a time-series previously published [28]. Data from LiceBase are bulk samples of different developmental stages (nauplius I, nauplius II, planktonic copepodids, attached copepodids at three different sampling time points, chalimus I and II, female and male of preadult I and II and adult lice, as well as eggs (unfertilized extracted from the genital complex of the adult female, immature fertilized eggs (0–24 hours after extrusion) and more mature fertilized eggs (2–7 days after extrusion)). In addition, samples of different tissues (intestine, ovaries, testis feet and antenna) are shown. The data from the time series [28] are RNA-sequencing samples of attached parasitic stages (chalimus I and II and preadult I), which were divided into different instar ages (called young (directly after previous molt), middle and old (directly before next molt)) as well as planktonic copepodids. In addition, unpublished RNA-sequencing data (Eichner et al., unpublished) for the transcript regions from nauplius I and II, taken at three different time points (directly after hatching or molting respectively, in the middle of the stage, and before the next molt) as well as attached copepodids, sampled one, three and five days after infection, are included. RNA-sequencing and data processing for the nauplius and attached copepodid samples was done using the same protocols as in Eichner et al. [28]. Six biological replicates were sequenced for each time point. Normalization was done by edgeR [29] after combining all data sets using the same protocol as in Eichner et al. [28].

2.5. Isolating RNA, synthesis of cDNA, qRT-PCR and RACE

Tissues for RNA isolation were stored in RNAlater (Ambion), at 4 °C overnight and then at −20 °C until usage. Total RNA extraction from adult female lice was executed using TRI Reagent (Sigma-Aldrich) as instructed by the manufacturer. Samples were homogenized using 5 mm stainless steel beads and a TissueLyser II (Qiagen) for 1 min at 30 Hz. Genomic DNA digestion was done by DNase I after manufacturer's instructions (Invitrogen). Quantification of RNA and purity check were done using NanoDrop ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies). DNase-treated RNA was reverse transcribed using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene). qRT-PCR was performed on a QuantStudio 3 qPCR machine using PowerUp SYBR Green Master Mix (Applied Biosystems) on duplicate samples

Table 1
Primers used for RACE, *in situ* hybridization (ISH), RNA interference (RNAi) and qPCR. T7 promoter extension is underlined. Primer efficiency (E%) is given for qPCR assays.

Primer name	Sequence (5'→3')	Application/E%
LsFer1 5' RACE	TCCAGTCACCAAGAGCCTCATG	5' RACE
LsFer1 3' RACE	GCAAGATTATCTCTCTGGAGATTGGC	3' RACE
LsFer2 5' RACE	GTATTTGGTCTTGTCATTCCGGGC	5' RACE
LsFer2 3' RACE	GGTGCTCACACTGATGTACAATGTGG	3' RACE
LsFer3 5' RACE	CACGCCTGGTCCAACACGTTTCATTC	5' RACE
LsFer3 3' RACE	CAAGCATGGCTCAACAAATCCGAC	3' RACE
LsFer4 5' RACE	ACGAGTTAAGAGGGTTCCAA	5' RACE
LsFer4 3' RACE	ATGAGTTCCCAAATCCGTCA	3' RACE
LsFer1 T7 fwd	TAATACGACTCACTATAGGGACAGGAGCCCATGGATCTTGTA	ISH/RNAi
LsFer1 rev	TCCAGTCACCAAGAGCCTCATG	ISH/RNAi
LsFer1 T7 rev	TAATACGACTCACTATAGGGTCCAGTCACCAAGAGCCTCATG	ISH/RNAi
LsFer2 fwd	GGTGCTCACACTGATGTACAATGTGG	ISH/RNAi
LsFer2 T7 fwd	TAATACGACTCACTATAGGGGGTGCTCACACTGATGTACAATGTGG	ISH/RNAi
LsFer2 rev	GTATTTGGTCTTGTCATTCCGGGC	ISH/RNAi
LsFer2 T7 rev	TAATACGACTCACTATAGGGGTATTTGGTCTTGTCATTCCGGGC	ISH/RNAi
Cod_*CPY185* fwd	TAATACGACTCACTATAGGGATAGGGCGAATTGGGTACCG	RNAi
Cod_*CPY185* rev	TAATACGACTCACTATAGGGAAAGGGAACAAAAGCTGGAGC	RNAi
SYBR *LsEF1α* fwd	GGTCGACAGACGTACTGGTAAATCC	qPCR/93%
SYBR *LsEF1α* rev	TGCGGCCTTGGTGGTGGTTC	qPCR
SYBR *LsFer1* fwd	GCAAGATTATCTCTCTGGAGATTGGC	qPCR/93%
SYBR *LsFer1* rev	TCCAGTCACCAAGAGCCTCATG	qPCR
SYBR *LsFer2* fwd	GGTGCTCACACTGATGTACAATGTGG	qPCR/92%
SYBR *LsFer2* rev	CAAATGTCTGTTGATAAGAACCGAGAG	qPCR

(PCR: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and finally a melt curve analysis at 60–95 °C). Duplicate samples had a difference in Ct-values less than 0.35. The reference gene *LseEF1α* [30] was used to calibrate the *LsFer1* and *LsFer2* transcript levels. A five-point standard curve of twofold dilutions was prepared to test the efficiency of the qPCR assays: E% = $(10^{1/\text{slope}} - 1) \times 100$ [31]. Relative differences (ΔΔCt) in threshold were calculated and transformed by the formula $2^{-\Delta\Delta Ct}$ [32]. Rapid Amplification of cDNA Ends (RACE) was conducted by using the SMARTer RACE cDNA Amplification Kit (Clontech).

2.6. RNA interference

LsFer1 and *LsFer2* were separately knocked down by RNAi in preadult II female lice as described before [33] in two independent experiments. Briefly, double stranded (ds) RNA fragments were synthesized by the Megascript RNAi kit (Ambion) using adult female louse cDNA as template. Atlantic cod (*Gadus morhua*) trypsin (*CPY185*) fragment was used as negative control. All primers are listed in Table 1. Fragments were diluted to 600 ng/μl and 2% (v/v) bromphenol blue was added to enable visualization of the injections. Preadult II female lice were injected with thin capillaries and left to recover for four hours before being placed on fish in individual tanks (three fish per group with a total of 30–39 female lice). Additionally, an equal number of uninjected adult male lice (in relation to the number of female lice per fish) were also placed on each fish to assess the fecundity of the knockdown animals. Experiments were terminated upon the extrusion of the control lice's second pair of egg strings (approximately 40 days after injection). Upon termination, egg strings were collected and incubated in hatching wells. Any hatching of the egg strings was noted. Adult female lice were sampled in RNAlater for knockdown assessment or in Karnovsky's fixative for histological analyses.

2.7. Histology

Salmon lice destined for histological analyses were fixed in Karnovsky's fixative and sectioned as earlier described [34]. Briefly, 2 μm thick plastic sections were stained with filtered toluidine blue and rinsed with tap water. Ready slides were mounted with DPX mounting solution (Sigma-Aldrich) and covered with glass cover slips.

2.8. In situ hybridization

The localizations of *LsFer1* and *LsFer2* mRNAs were detected in the adult female salmon louse by using *in situ* hybridization (ISH) as earlier described [35,36] with the DIG RNA Labelling Kit (Roche) on two sections per probe. RNA antisense probes of 568 bp (*LsFer1*) and 576 bp (*LsFer2*) were made from a target specific cDNA template (see Table 1 for primer sequences). Sense probes acted as negative control for transcript localizations.

2.9. Starvation

Adult female lice were removed from their host, the Atlantic salmon, and either sampled directly (zero days starved) in RNAlater or placed in individual incubators with running sea water [24]. The lice in the incubators were sampled one, two, four and eight days after being separated from their food source as described before [37]. RNA isolation and cDNA synthesis were performed as described in Section 2.5 on five replicates of each sampling time point. The transcript levels of *LsFer1* and *LsFer2* were finally assessed by qPCR.

2.10. Statistics

Statistics were performed by using IBM SPSS Statistics 23 for Windows. Data sets were first tested by Shapiro-Wilk's test of normality and Levene's test for equality of variances. An independent *t*-test was used to evaluate the mean between two groups. For non-normal data sets, a Mann-Whitney U test was conducted. A p-value ≤ 0.05 was considered statistically significant. Data are presented as mean values ± standard deviations (SD) or as individual values.

3. Results

3.1. Sequence analyses

cDNA sequences of ferritins *LsFer1-4* in the salmon louse were obtained by 5′ and 3′ RACE using sequence specific primers (Table 1). Main results of sequence analyses are summarized in Table 2. No IREs were predicted for any of the genes. *LsFer1* consists of an open reading frame (ORF) of 639 bp. A protein BLAST search of the *L. salmonis* full

Table 2
Sequence summary of ferritin genes *LsFer1-4* in the salmon louse.

Gene	Signal peptide	Predicted subunit	ORF (including signal peptide)	GenBank ID
LsFer1	Yes	HCH	213 aa	BT121711
LsFer2	Yes	LCH	224 aa	BT121232
LsFer3	No	HCH	180 aa	MK887318
LsFer4a	No	HCH	171 aa	BT077723
LsFer4b	No	HCH	173 aa	BT121164

length LsFer1 protein showed 78% identity with a Ferritin subunit precursor from *Caligus rogercresseyi* (Genbank accession: ACO11534.1), 54% identity with Ferritin, lower subunit-like from *Eurytemora affinis* (Genbank accession: XP_023345228.1), and 40% identity with Ferritin 1-like protein A from *Daphnia pulex* (Genbank accession: ABK91577.1). *LsFer2* consists of an ORF of 672 bp. A protein BLAST search of the *L. salmonis* full length LsFer2 protein showed 69% identity with a Ferritin light chain, oocyte isoform from *Caligus clemensi* (Genbank accession: ACO15165.1), and 29% identity with 32 kDa ferritin subunit from *Galleria mellonella* (Genbank accession: AAL47694.1). The ORFs of *LsFer1* and *LsFer2* were analyzed in Interpro-Scan, and both proteins were characterized as containing a ferritin-like domain (PF00210). In LsFer1 there is a predicted ferroxidase site, a ferrihydrite nucleation center and an iron ion channel, which are not predicted in LsFer2. LsFer2 has a predicted dinuclear metal binding motif. A scan in Phobius shows that LsFer1 has a signal peptide from aa 1 → 21 with a cleavage site from aa 17 → 21, and a non-cytoplasmic region from aa 22 → 213. A Phobius scan for LsFer2 shows that the protein has a predicted signal peptide from aa 1 → 24 with a cleavage site from aa 17 → 24, and a non-cytoplasmic region from aa 25 → 224. LsFer1 has two predicted O-linked glycosylation sites at aa 29 and 36 and no predicted N-linked glycosylation sites. LsFer2 has no predicted O-linked glycosylation site, and one predicted N-linked glycosylation site at aa 55.

LsFer3 has an ORF of 540 bp. A protein blast shows a 59% identity with a ferritin of *Calanus sinicus* (Genbank accession: APC62655.1) and 58% identity with *Pseudodiaptomus annandalei* (Genbank accession: AGT28487.1). *LsFer4a* and *b* encode similar ORFs with only slight variations in their C-terminals affecting 3 aas only as well as 2 additional aas in *LsFer4b*. *LsFer4a* has an ORF of 513 bp whereas *LsFer4b* has an ORF of 519 bp. Both LsFer4a and 4b have 60% identity with a ferritin of *Calanus sinicus* (Genbank accession: APC62655.1) and a 58% identity with a ferritin heavy chain A-like of *Eurytemora affinis* (Genbank accession: XP_023344839.1). According to Phobius scans, LsFer3-4 lack signal peptides. Interpro-Scan predicts that LsFer3-4 have a ferritin-like domain (PF00210) and have ferroxidase activity, a ferrihydrite nucleation center and an iron ion channel.

3.2. Sequence alignment and phylogeny

Sequence alignment (Fig. 1) shows that LsFer1, 3, 4a and 4b contain all conserved aas of the ferroxidase site. In addition, the conserved aspartic acid of the iron entry pore is found in all these *L. salmonis* ferritins, while the conserved glutamic acid is not found in LsFer1. Like the insect sequences shown in the alignment, it is replaced by threonine. There are only minor differences in the aa sequence between LsFer4a and LsFer4b, as the nucleotide sequence at the C-terminal end shows differences. LsFer2 contains only two of the conserved aas making up the functional ferritin motif. However, all of the conserved cysteins found in the light chain of insects responsible for disulfide bridges are also found in LsFer2. Two cysteine residues common in disulfide bridges are also conserved in LsFer1.

To investigate the relationship between LsFer1-4 and other vertebrate and invertebrate ferritins, a maximum likelihood phylogenetic analysis was performed (Fig. 2). The analysis shows that LsFer1 clusters together with secretory invertebrate HCH ferritins. LsFer2 clusters together with secretory invertebrate LCH ferritins. Both LsFer1 and LsFer2 have their closest relation to ferritin sequences of the parasitic copepod *C. clemensi*. LsFer3-4 cluster together in a separate clade, close with intracellular ferritins (HC and LC) of both vertebrates and invertebrates.

3.3. Ontogeny

To determine spatial and temporal expression patterns of the ferritin subunits, RNA-sequencing data were used to count the transcripts (Fig. 3). The two secreted ferritins *LsFer1* (Fig. 3a) and *LsFer2* (Fig. 3b) have similar absolute expression counts and pattern, and they are highly expressed in the intestine. *LsFer3* (Fig. 3c) is nearly exclusively found in male lice and transcripts are highly expressed in testis. *LsFer4a* and *LsFer4b* are differing at the 3′ end only, and the total count of LsFer4 is shown (Fig. 3d). *LsFer4* (Fig. 3d) is expressed in the intestine, but is also found in all other tissues investigated, it is highly expressed already in immature eggs.

3.4. In situ hybridization

To localize and confirm where the secretory ferritin transcripts (*LsFer1* and *LsFer2)* are expressed, we utilized *in situ* hybridization. Both transcripts were found to be expressed in cells lining the intestinal tract of the adult female louse (Fig. 4).

3.5. Starvation

To evaluate the effect starvation has on the expression of *LsFer1* and *LsFer2*, transcript levels were measured by qPCR in a time series after removing the lice from their host. With increased time absent from the food source, decreased levels of both transcripts were found in the adult female *L. salmonis* (Fig. 5). More specifically, the expression of *LsFer1* is significantly down-regulated from day 2 and throughout the starvation period (*t*-test: day 1: $p = 0.132$, day 2: $p = 0.001$, day 4: $p = 0.005$, day 8: $p = 0.0000213$, $n = 5$). At eight days of starvation, the expression of *LsFer1* is decreased with on average 67% (Fig. 5a). *LsFer2* shows a similar profile, but shows a significant decrease already from day 1 of starvation (*t*-test: day 1: $p = 0.007$, day 2: $p = 0.0000219$, day 4: $p = 0.0000493$, day 8: $p = 0.00000669$, $n = 5$) and at day 8 of starvation, *LsFer2* has decreased with on average 82% (Fig. 5b).

3.6. RNAi phenotype, histology and knockdown effect of LsFer1 and LsFer2

In order to study the functions of *LsFer1* and *LsFer2 in vivo*, two independent RNAi knockdown experiments were conducted. In both experiments, the knockdown animals' recovery rates (lice sampled at the end of the experiment/lice injected at the start of the experiment) are similar to the control animals', ranging from 17%–59% (Table 3). However, the fecundity of the parasite is affected by knocking down *LsFer1* or *LsFer2*. In both experiments, larvae from control animals hatched from all egg strings. However, knockdown of *LsFer1* resulted in one hatched out of ten egg string pairs (exp. 1), and when repeated, no egg strings (out of 6 pairs) hatched (exp. 2). Knockdown of *LsFer2* resulted in egg strings present in 10 of 23 animals, and only 5 pairs of these hatched (exp. 1). In experiment 2 of *LsFer2* knockdown, all animals (7 out of 7) had egg strings upon termination, but none of these hatched.

In all control adult female salmon lice, a salmon blood-filled intestinal tract is seen as a red line stretching from the mouth part in the cephalothorax, through the genital segment with maturing oocytes, and finally through the abdomen ending in the rectum (Fig. 6a). When knocking down *LsFer1* (Fig. 6b) and *LsFer2* (Fig. 6c), an alteration in phenotype is seen. In both knockdown groups, the animals have little or no blood in their intestines, as seen by visual inspection (Table 3 and Fig. 6). While all lice in the control groups had a high amount of blood

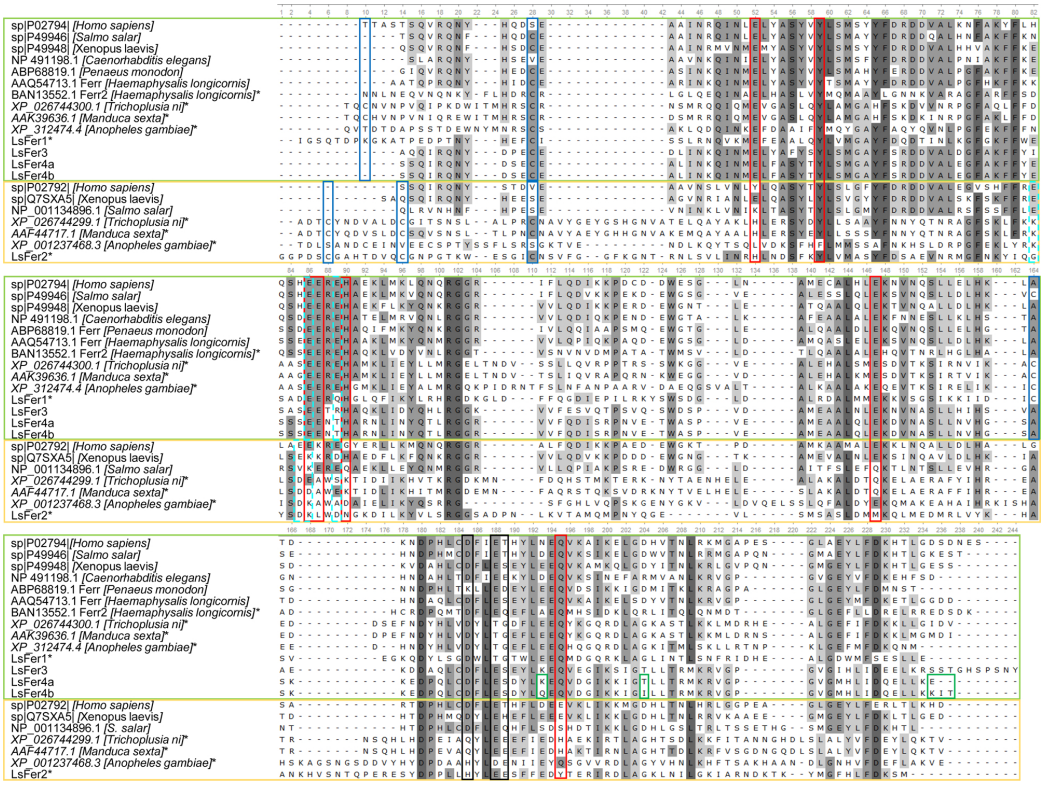

Fig. 1. Ferritin sequence alignment. Sequences found in *L. salmonis* (LsFer1-4) with known ferritin sequences annotated as heavy (green outline) and light chain (yellow outline). Amino acids that make up the ferroxidase center of the heavy chain in human are marked with a red box, the conserved pore for iron entry black and the aa making up the human ferrihydrite nucleation center are marked with cyan (dashed line). The conserved cysteines, which are involved in inter and intra disulphide bridges in insects are marked with blue. Differences in the aa sequence between Fer4a and Fer4b are marked with green. Sequences with signal sequences are marked with*. For alignment, all signal sequences were removed. All other sequences were depleted for the start methionine.

in their intestine, this was not the case in the *LsFer1* and *LsFer2* knockdown animals (Table 3). In order to more closely examine the knockdown effects, sections were made for histology (Fig. 6). In the control louse (Fig. 6a), oocytes are stacked and well-structured with lipid-droplets (seen as white dots) and with chorions that define boundaries between individual oocytes. In the *LsFer1* knockdown animal, oocytes appear as a homogenous mash and without visible chorions subdividing individual oocytes (Fig. 6b). Histology of the *LsFer2* knockdown louse reveals a genital segment with little and highly under-developed oocytes present (Fig. 6c). Lice shown in Fig. 6 are representative of both experiments. Photographs of all lice from exp. 2 are shown in Supplementary Fig. S1.

The efficacies of the knockdowns of *LsFer1* and *LsFer2* were assessed by qPCR. Knockdowns were successful for both treatments in both experiments, and experiment 2 is presented with individuals' levels of transcripts (Fig. 7). In experiment 1, *LsFer1* is on average 85% down-regulated (Mann-Whitney U: $p = 0.008$, $n = 5$), and *LsFer2* is on average 78% down-regulated (*t*-test: $p = 0.005$, $n = 5$). In experiment 2, knockdown of *LsFer1* resulted in an on average 93% down-regulation (*t*-test: $p = 0.000171$, $n = 5$) (Fig. 7a). Knockdown of *LsFer2* resulted in a down-regulation of 60–86% for four out of five animals tested, whereas up-regulation of 41% was found in the last one (Fig. 7b). This individual had a similar phenotype and morphology as the rest of the knockdown group, and is therefore not omitted from the dataset or statistical analysis (Mann-Whitney U: $p = 0.076$, $n = 5$).

4. Discussion

In this study, we present the characterization and first functional studies of ferritin in the obligate hematophagous ectoparasitic salmon louse, *Lepeophtheirus salmonis*. We report the presence of four ferritin homologs (*LsFer1-4*) in the genome of the salmon louse, and find that two of these (*LsFer1-2*) have predicted signal peptides in the N-terminus of their aa sequences. Functional studies show that *LsFer1-2* are expressed in the midgut, and both are essential for the blood-feeding and reproduction of the adult female salmon louse.

Results from the sequence analysis tools used here give support to the hypothesis that *LsFer1-4* are homologs of ferritin as there are significant sequence similarities with ferritins of other species found by BLAST searches. Since *LsFer1,3* and *4* have the highly conserved motifs for the ferroxidase site, whereas *LsFer2* does not, *LsFer1,3* and *4* are likely heavy chain homologs (HCH), whereas *LsFer2* is likely a light chain homolog (LCH). This is further supported by the phylogenetic analysis, which shows that LsFer1 mainly clusters together with other HCHs and LsFer2 mainly with other LCHs (Fig. 2). LsFer3-4 clusters phylogenetically closer together with ferritin subunits without a signal peptide. Classically secreted ferritins are commonly found in arthropods where they are reported to be involved in detoxification, storage and distribution of iron. As a well-studied phenomenon in insects, ferritin is loaded with absorbed iron in the endoplasmic reticulum (ER), and is then secreted to the hemolymph, a tissue fluid analogous to

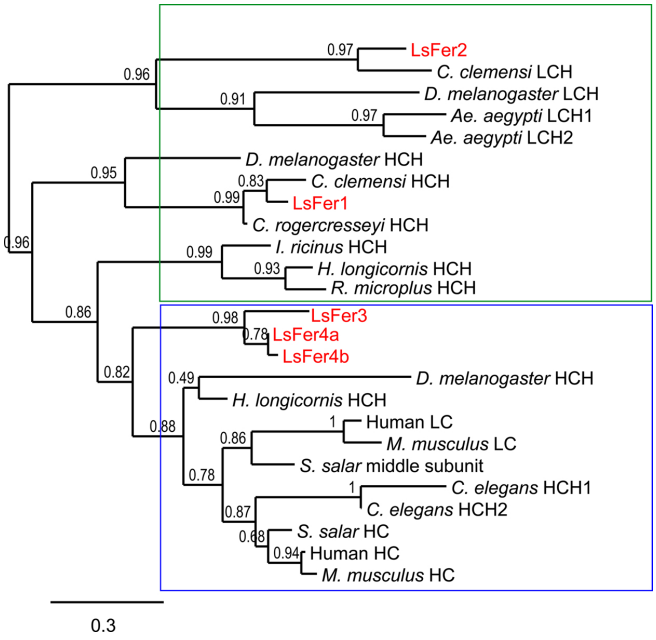

Fig. 2. Unrooted phylogenetic tree of vertebrate and invertebrate ferritin subunits. The phylogenetic analysis was performed on the Phylogeny.fr platform. Secreted ferritins are marked with the green box and intracellular ferritins with the blue box. Salmon louse ferritins (LsFer1-4) are written in red letters. LsFer1 clusters together with secreted invertebrate HCH ferritins, and LsFer2 clusters together with secreted invertebrate LCH ferritins. LsFer3-4 cluster together and are close to branches of intracellular heavy and light chain ferritins of both vertebrate and invertebrate origin. Numbers at branches represent support values (approximate likelihood-ratio). HC(H) = heavy chain (homolog), LC(H) = light chain (homolog). Scale bar: 0.3 amino acid substitutions per site.

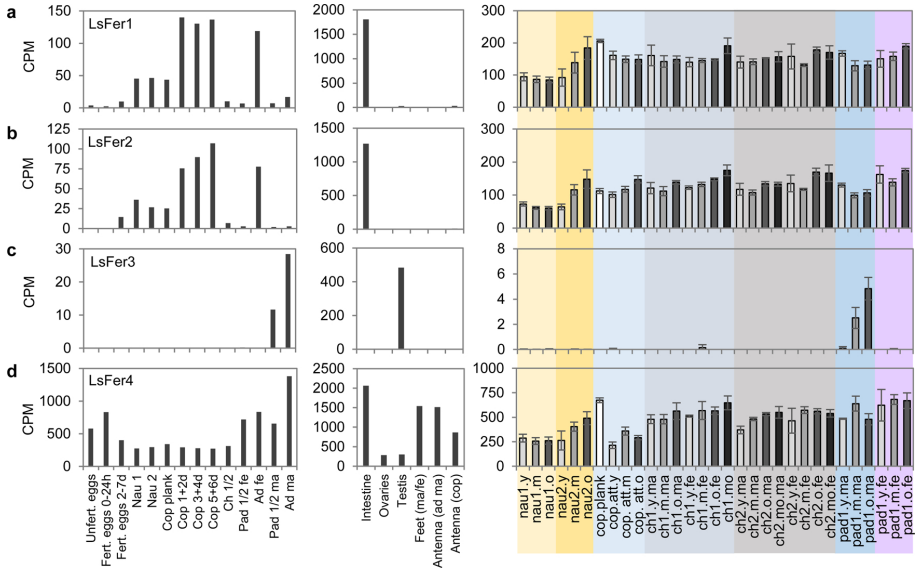

Fig. 3. Ontogeny. RNA-sequencing data (left and middle column: LiceBase, right column: Eichner et al., 2018 [28] with additional data from nauplius and attached copepodids (unpublished data)) for *LsFer1* (a), *LsFer2* (b), *LsFer3* (c) and *LsFer4* (d). Available reads were recounted for the transcribed regions of the ferritin genes found by RACE-PCR. See Section 2.4 "Ontogeny" for explanations of the abbreviations used in the figure.

blood in vertebrates, and subsequently used as a vehicle for iron transportation to various tissues [38]. In the current study, we find that both LsFer1-2 ferritin subunits are predicted to have signal peptides, and are thus likely destined for the secretory pathway. Since the salmon louse lacks the iron transfer protein transferrin (unpublished, http://licebase.org), it may utilize secreted ferritin to shuttle iron to recipient tissues and still avoiding iron-related cytotoxicity. The localizations of the precursor and mature LsFer1-2 proteins are however not determined, as only transcript localization is assessed here. Further work is required to determine if ferritin acts as an iron transporter in the salmon louse. Also, whether LsFer1 and LsFer2 are subunits of the same protein, or if they make up two different proteins remains an

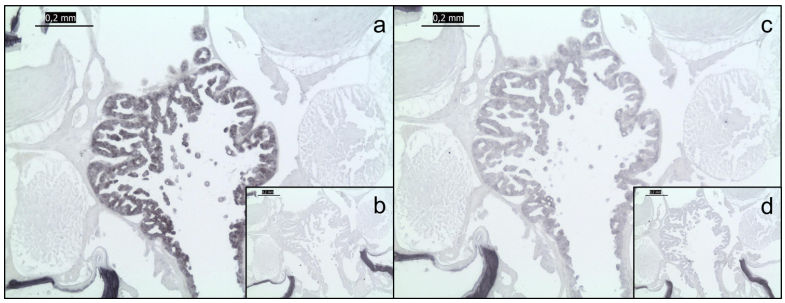

Fig. 4. *In situ* hybridization. The transcripts of both *LsFer1* (a) and *LsFer2* (c) are only found in cells lining the gut of *L. salmonis* by *in situ* hybridization. Negative controls (sense probes) of *LsFer1* (b) and *LsFer2* (d) show no signal.

unanswered question. Since LsFer2 lacks conserved aa residues for ferroxidase catalytic activity, it is highly unlikely that the LCH subunit may constitute an iron storage molecule on its own. Also, LsFer1 and LsFer2 contain cysteine residues reported to form intersubunit disulfide bonds in other secretory ferritins [6].

Characterization of the *LsFer1-4* genes by RNA-Seq showed that *LsFer1-2* have similar expression patterns. Both secreted ferritin subunits are highly expressed in the intestine of the salmon louse, consistent with having an iron storage and/or transportation function. Additionally, *LsFer1-2* are expressed in the larval, non-feeding stages of the salmon louse, which could indicate extra functions of the ferritin subunits, or they could be necessary for detoxification of iron from the turnover of maternally derived iron-proteins. The similar expression patterns of *LsFer1-2* suggests that the two subunits could assemble into a complete ferritin shell. *LsFer3*, which is primarily expressed in testis, must have a male specific function. *LsFer4* has a more uniform expression pattern, and probably has an intracellular iron storage function in several tissues of the salmon louse. Further analysis by *in situ* hybridization confirmed that the transcripts of *LsFer1* and *LsFer2* are both expressed in cells lining the intestinal tract of the adult female salmon louse. Epithelial cells in the gut are the entry site for iron absorbed from the blood of the parasite's host, and is where iron storage proteins need to be ready and available. In the starvation experiment, the expression of *LsFer1-2* is decreasing with the increased starvation time. A reason for this could be that the salmon louse is an ectoparasite that is either on or off its host. It does not experience a gradient in iron availability, other than remnants of blood in the gut after feeding (seen as a fainter red color with increased starvation time). Expressing a nutrient storage molecule when there is an absence of nutrients available is unnecessary and requiring energy. Similar to our results, an HCH ferritin in *A. aegypti* was found in the midgut of the mosquito and was regulated by blood-feeding [39]. Also, Wu et al., found that a ferritin homolog in abalone *Haliotis discus hannai* was up-regulated by dietary iron [40]. Walter-Nuno et al., also found that blood-feeding increased the expression level of several genes involved in heme and iron metabolism, including ferritin [41]. These findings indicate that ferritin gene expression is a consequence of the dietary iron status. A common way of controlling ferritin transcript translation in a cell is by iron regulatory proteins (IRPs) that inhibit ferritin translation during iron shortage by binding to an IRE upstream of the ferritin ORF [42]. Yet, no IREs were predicted in any of the ferritin sequences in the salmon louse, even though the louse does have an IRP-IRE complex [37]. We do not know if uptake of iron is controlling ferritin translation here as it does post-transcriptionally in vertebrates [43] and other various invertebrates [44,45]. Not all organisms depend on translational control by IRE however, *e.g.* plants regulate ferritin levels on a transcriptional level in response to iron and oxidants [46]. How ferritin expression is regulated in the salmon louse remains an unanswered question.

Table 3
Overview of RNA interference knockdown experiments of *LsFer1* and *LsFer2*.

Experiment	Treatment	Animals recovered/ injected	Fully engorged[a]	Hatching success[b]
1	Control	19/39	19/19	19/19
	LsFer1	18/38	5/18	1/10
	LsFer2	23/39	10/23	5/10
2	Control	5/30	5/5	5/5
	LsFer1	12/30	0/12	0/6
	LsFer2	7/30	1/7	0/7

[a] Determined by visual inspections of blood-filled intestines.
[b] Number of successfully hatched egg string pairs and number of females bearing egg strings upon termination of the experiments.

The functional study by knocking down *LsFer1* and *LsFer2* in the salmon louse mediated by RNAi had negative consequences for the fecundity of the parasite. Fewer of the adult female lice had egg strings, and when present, they had an overall much lower hatching success in both knockdown groups compared with the control group. Histological analyses demonstrated that oocyte development was impaired, and this is probably why eggs hatching was highly unsuccessful. Knockdown of genes involved in iron metabolism is already known to limit reproductive success of other hematophagous parasites, such as the tick's reproduction and development [44,47,48]. Furthermore, knockdown of a ferritin in the vector *Rhodnius proxilus* resulted in drastically reducing (95%) viable eggs for the hematophagous insect [41]. As the salmon louse is semi-transparent, its red, blood-filled gut can easily be observed

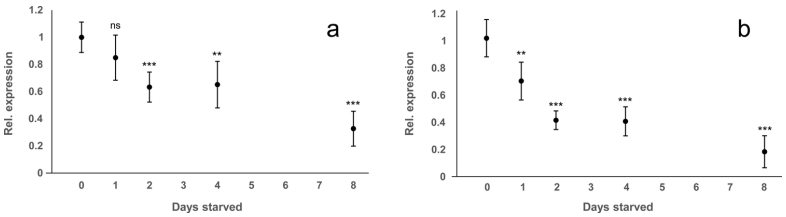

Fig. 5. Effect of starvation. Transcripts of both *LsFer1* (a) and *LsFer2* (b) were assessed in adult female lice by qPCR. The mRNA levels of both transcripts were calibrated against unstarved lice (day 0 post start of starvation), and showed increased down-regulation with increasing starvation time. Data are presented as mean values ± SD. Asterisks indicate statistical significance against day 0: ** = $p \leq 0.01$, *** = $p \leq 0.0001$, ns = not significant. $n = 5$.

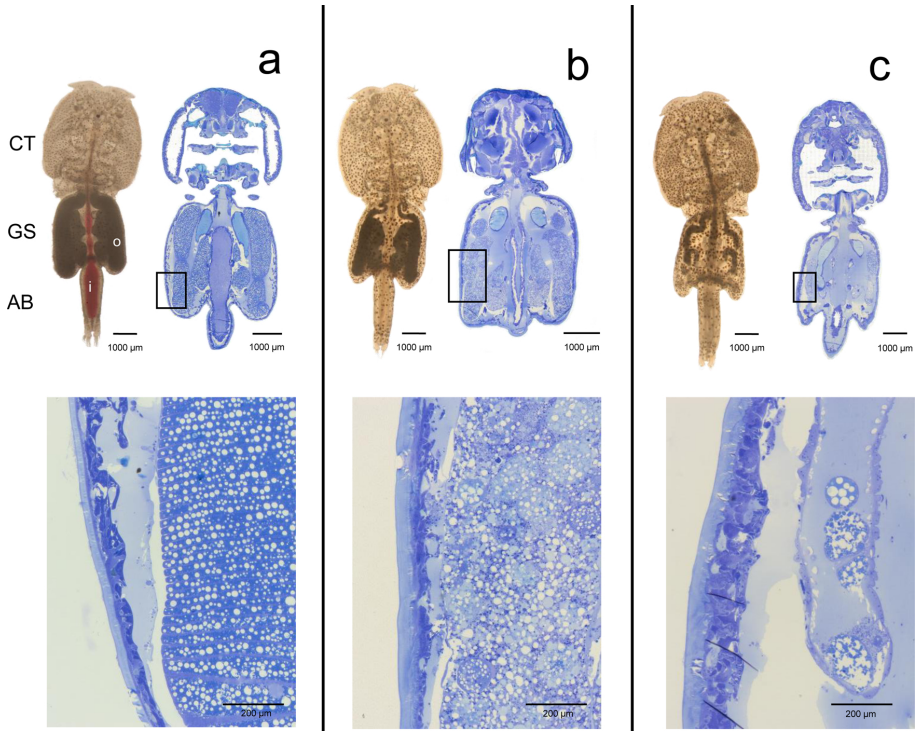

Fig. 6. Phenotype and histology. Phenotype and toluidine blue stained histological sections of an untreated control adult female louse (a), a *LsFer1* RNAi knockdown louse (b) and a *LsFer2* RNAi knockdown louse (c). Of every histological section, a magnified photo of the genital segment is shown in the lower panel. All three lice are representative of their group. CT = cephalothorax, GS = genital segment, AB = abdomen, i = intestine, o = oocyte.

by visual inspection. Animals in the knockdown group had less blood in their intestines than animals in the control group, which were fully engorged. This phenomenon has also been reported in ticks upon knockdown of ferritin [48]. An explanation for this could be as free iron generates ROS, having a reduced capacity of sequestering and thus detoxifying iron could lead to detrimental effects for the parasite upon blood-feeding. A solution for the salmon louse could be to cease the blood-feeding behavior. Downstream effects of not ingesting blood would include severe undernutrition and consequently cause egg production to be halted.

In conclusion, this report on four ferritin homologs in the salmon louse is the first where we have functionally characterized the two secretory subunits (*LsFer1-2*). *LsFer1* and *LsFer2* were both found to be vital for blood-feeding and reproduction in the hematophagous parasite

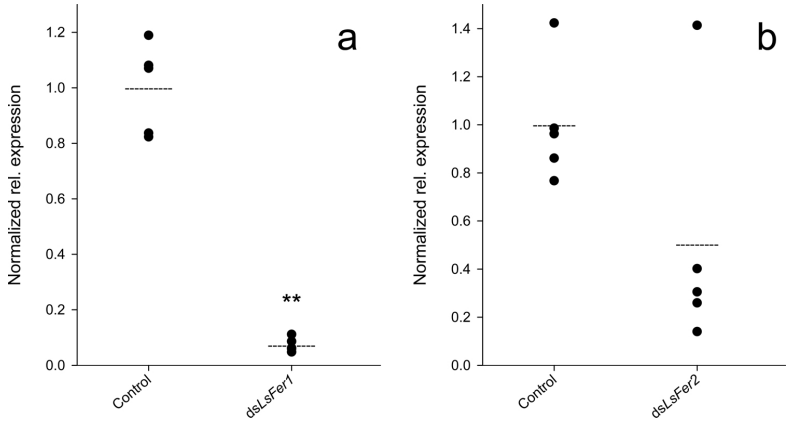

Fig. 7. Knockdown efficacy. RNAi mediated knockdown effect of (a) *LsFer1* and (b) *LsFer2* measured by qPCR from experiment 2. Results are displayed as individual animals' values, and the mean value for each group is indicated by a dotted line. ** = $p \leq 0.01$. $n = 5$.

in a knockdown study and starvation experiment. Our results confirm several previous findings on the importance of ferritin throughout arthropods. Further work on these genes and proteins could provide a deeper knowledge of the iron metabolism of *L. salmonis* and should be studied further. Considering the detrimental effects the knockdown of *LsFer1-2* had on the egg production in the salmon louse, these results could find their application as treatment targets in the aquaculture sector in order to combat the infamous parasite.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2019.111197.

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II

SCIENTIFIC REPORTS

OPEN

A scavenger receptor B (CD36)-like protein is a potential mediator of intestinal heme absorption in the hematophagous ectoparasite *Lepeophtheirus salmonis*

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Intestinal absorption of heme has remained enigmatic for years, even though heme provides the most bioavailable form of iron. The salmon louse, *Lepeophtheirus salmonis*, is a heme auxotrophic ectoparasite feeding on large quantities of blood from its host, the salmon. Here we show that a scavenging CD36-like receptor is a potential mediator of heme absorption in the intestine of the salmon louse. The receptor was characterized by a heme binding assay using recombinantly expressed protein, *in situ* hybridization and immunohistochemistry, as well as functional knockdown studies in the louse. A computational structural model of the receptor predicted a binding pocket for heme, as also supported by *in silico* docking. The mRNA and protein were expressed exclusively in the intestine of the louse. Further, knocking down the transcript resulted in lower heme levels in the adult female louse, production of shorter egg strings, and an overall lower hatching success of the eggs. Finally, starving the lice caused the transcript expression of the receptor to decrease. To our knowledge, this is the first time a CD36-like protein has been suggested to be an intestinal heme receptor.

Iron is a transition metal, which is essential for many proteins present in all branches of the phylogenetic tree of life and must be obtained through the diet. The most bioavailable form of iron is heme, the iron-containing pyrrole ring of protoporphyrin IX[1]. Heme is a prosthetic group found as cofactor in many metalloproteins and is known to contribute to essential cellular processes, such as electron transport, signal transduction, detoxification, gas transport and sensing[2–4]. Although heme is necessary for many purposes in the cell, it may also exert cytotoxic effects by generation of reactive oxygen species (ROS) and cause damage to DNA, proteins and lipids[5–7].

The classical heme biosynthetic pathway is an evolutionarily conserved multi-step enzymatic reaction that in eukaryotic cells takes place partially in the mitochondria and partially in the cytoplasm. Heme biosynthesis begins with the synthesis of δ-aminolevulinic acid (ALA) by δ-aminolevulinate synthase 1 (ALAS1) as the rate-limiting reaction, and ends with the addition of an iron atom to the center of the protoporphyrin IX ring by ferrochelatase (FECH)[8]. Even though heme is essential for aerobe cells, some organisms are unable to produce this cofactor on their own. Natural heme auxotrophic organisms depend upon exogenous heme through their diet for survival. In this group we find, among others, the hematophagous parasitic cattle tick *Rhipicephalus (Boophilus) microplus* feeding off cattle blood[9], the soil-nematode, *Caenorhabditis elegans*, and the parasitic nematode *Brugia malayi*[10].

By analyzing its genomic sequence, we uncovered that the Atlantic salmon louse, *Lepeophtheirus salmonis*, is likely among the natural heme auxotrophs as well, because it lacks homologs for most enzymes of the classical heme biosynthetic pathway, including ALAS1 and FECH (https://licebase.org, unpublished). The salmon louse is an obligate ectoparasite of salmonid fish. It is considered a major problem for both farmed and wild salmon

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Primer name	Sequence (5′→3′)	Application
LsHSCARB 5′ RACE	CCTCCTTCCACTTCCACTTCGGACTCA	5′ RACE
LsHSCARB 3′ RACE	GTCAAGAATTTTTCTCATGCGCCAA	3′ RACE
LsHSCARB fwd	AGCGGATAAACTCGATGGCT	ISH/RNAi
LsHSCARB T7 fwd	TAATACGACTCACTATAGGGAGAAGCGGATAAACTCGATGGCT	ISH/RNAi
LsHSCARB rev	TTTGCTTGGCGCATGAGAAA	ISH/RNAi
LsHSCARB T7 rev	TAATACGACTCACTATAGGGAGATTTGCTTGGCGCATGAGAAA	ISH/RNAi
Cod_CPY185 fwd	TAATACGACTCACTATAGGGATAGGGCGAATTGGGTACCG	RNAi
Cod_CPY185 rev	TAATACGACTCACTATAGGGAAAGGGAACAAAAGCTGGAGC	RNAi
SYBR *LsEF1α* fwd	GGTCGACAGACGTACTGGTAAATCC	qPCR
SYBR *LsEF1α* rev	TGCGGCCTTGGTGGTGGTTC	qPCR
SYBR *LsHSCARB* fwd	TCCGCTTGATCCCCATGTTC	qPCR
SYBR *LsHSCARB* rev	GCCAACGACATAGCCAAGAGC	qPCR

Table 1. Primers used for RACE, *in situ* hybridization (ISH), RNA interference (RNAi) and qPCR. T7 promoter extension is underlined.

populations, and as resistances to various treatments have occurred recently, effective methods for pest control are in high demand[11]. The life cycle of this parasite is divided into eight stages separated by molts. These are in chronological order: nauplius I and II, copepodid, chalimus I and II, preadult I and II, and adult lice[12,13]. The developmental pace of *L. salmonis* correlates for the most part with temperature. At 10 °C, development from fertilization to mature adult lice is completed in approximately 40 (♂) to 52 (♀) days[14]. From hatching and until it reaches the infectious copepodid stage, *L. salmonis* is planktonic and survives on energy reserves from the yolk sac. When these eventually wear down, the copepodid has to infect a salmonid host in order to complete its life cycle. Once attached to a suitable host, *L. salmonis* feeds off the host's skin and blood[15]. By hematophagy, the parasite is exposed to significant amounts of hemoproteins and other nutrients.

The salmon louse is likely dependent on its vertebrate host for heme supply; consequentially there needs to exist a way of absorbing heme from ingested blood within the digestive tract of the parasite. However, heme transport through the cell membrane as well as intra- and intercellular heme trafficking are generally poorly understood. An organism lacking endogenous heme provides the opportunity to study trafficking of the cofactor without further confounding effects by endogenous cellular synthesis. In the heme auxotroph *C. elegans*, heme transporter HRG-1 transmembrane proteins mediate heme uptake and homeostasis. Whereas HRG-1 primarily localizes to endosomal and lysosomal organelles, its paralogue HRG-4 localizes to the plasma membrane and is expressed in the intestine; therefore HRG-1 and HRG-4 might act in a concerted fashion to take up environmental heme[16]. However, the *hrg-4* gene is nematode-specific implying that different mechanisms of heme uptake exist in other animal species. The mammalian proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) was also initially proposed as an intestinal heme transporter[17], but its function has since been debated, and later research characterized PCFT/HCP1 as a folate transporter with at best minor affinity to heme[18].

Limited success in identifying an evident candidate for the function of intestinal heme absorption has led to a shift in the traditional thinking as to which characteristics a heme receptor or transporter should fulfill. As the porphyrine ring is an amphipathic molecule with both polar and nonpolar properties, it has been suggested that it may be trafficked, alike lipids, via membrane-tethered complexes, lipid transfer proteins, vesicular trafficking, or lipid transporters[19]. A gene found to be highly expressed in the salmon louse intestine encodes a CD36-like protein. By sequence similarity, it belongs to the scavenger receptor class B (SCARB) family. Proteins of the SCARB family consist of two transmembrane domains, an extracellular ligand-binding domain, and short intracellular N- and C-terminal tails. Mammalian homologous proteins have been reported to scavenge a large variety of ligands (albeit not including heme), e.g. various lipoproteins such as oxidized and non-oxidized LDL[20], beta-carotene[21], and very long chain fatty acids[22].

In this study, we characterize the SCARB-like (*LsHSCARB*) gene and protein found in *L. salmonis* and hypothesize that LsHSCARB facilitates heme uptake across the intestinal membrane. Localization of the gene and protein, structural analysis, functional knockdown studies and a recombinant binding assay support our hypothesis. Our data support the existence of a novel pathway of heme scavenging from the arthropod intestine, and yield a potential new drug target for sea lice control.

Results

Sequence analysis. The full salmon louse heme scavenger (*LsHSCARB*) cDNA sequence (Ensembl metazoa stable ID: EMLSAG00000005382, GenBank accession: CDW29028.1) was verified by 5′ and 3′ Rapid amplification of cDNA ends (RACE) PCR using sequence specific primers (Table 1). The gene consists of a 220 bp 5′-untranslated region (UTR), an ORF of 1677 bp and a 288 bp 3′-UTR. The ORF translates into a 559 amino acid (aa) long protein with two predicted transmembrane domains in the N- and C-terminal (from aa 7 → 28 and from aa 528 → 549) with the terminals located intracellularly (Fig. 1c), and a signal anchor sequence from aa 1 → 23. The protein has a predicted N-linked glycosylation site at aa 260 and three predicted O-linked glycosylation sites at aa 302, 303 and 376. The sequence of the ORF was analyzed in InterproScan, and was characterized as belonging to the cluster of differentiation 36 (CD36) family (PF01130). A protein BLAST search of the *L. salmonis* full length protein showed 31% identity with a scavenger receptor class B1 in the kuruma prawn, *Marsupenaeus*

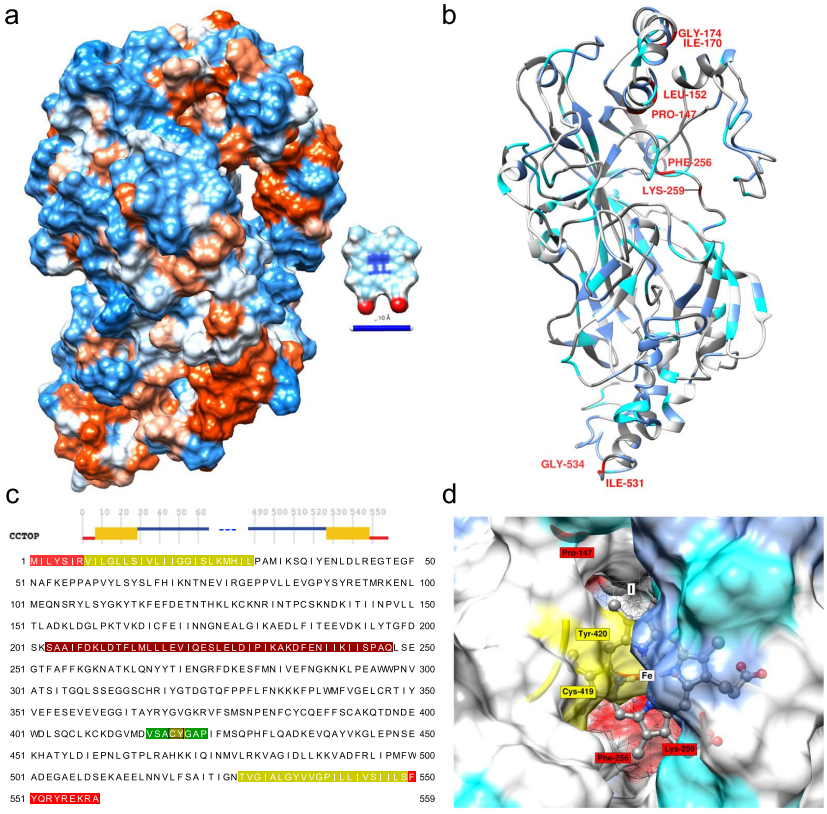

Figure 1. Structural *de novo* prediction for the protein sequence of LsHSCARB. Best scoring tertiary structure model generated from i-TASSER depicted as (**a**) hydrophobic surface (red: hydrophobic, blue: hydrophilic) The ligand protoporphyrin IX (C34H32N4O4, ZINC26671872) is depicted for comparison, scale bar = 10 Å. (**b**) ribbon representation with predicted secondary structure, and annotated residues with potential implication in heme binding (dark gray: aromatic, blue: aliphatic, cyan: potential axial ligand (C, M, Y, K, or H), red: residues predicted by HemeBIND). (**c**) Consensus membrane topology prediction by CCTOP over residues and sequence, red: intracellular, orange-green: trans-membrane, blue: extracellular domain, dark-red: lateral hydrophobic surface of the hydrophobic pocket, green: interior surface of predicted binding pocket. (**d**) Results from Receptor-Ligand docking by AutoDock Vina with the best-scoring (I) docking position. The highest scoring viable docking position is located inside the hydrophobic pocket, Cys-419 and Tyr-420 (yellow) are proximal to the hypothetical iron center of a heme ligand (Fe), residues are in same color code as in (b).

japonicus (GenBank accession: AKO62849), and 29% identity with the freshwater shrimp, *Macrobrachium nipponense* scavenger receptor B1 (GenBank accession: ALK82306).

Homology modelling and heme binding. A three-dimensional model of LsHSCARB was predicted using i-TASSER (Fig. 1a,b). The highest scoring model I (S-score = −1.33) presents with a lateral cavity in the extra-cytoplasmic domain that is enriched with hydrophobic residues. By sequence alignment with the highest ranked threading template (4F7B, Lysosomal Integral Membrane Protein, LIMP-2, RMSD = 0.56, Supplementary Fig. S1) the presence of a hydrophobic loop (residues 202–236: SAAIFDKLDTFLMLLLEVIQESLELDIPIKAKDF, Fig. 1a–c) is detected within an insertion in LsHSCARB (Supplementary Fig. S2). The opposite surface of the cavity consists of a loop region (residues 416–423: VSACYGAP, Fig. 1c) with central Cys-419 Tyr-420 (CY) residues (Fig. 1d).

Using HemeBIND as a specialized predictor of heme-binding residues to integrate sequence and structural information following residues were predicted as potential heme interacting: Pro-147, Leu-152, Ile-170, Gly-174, Phe-256, Lys-259, Ile-531, and Gly-534 (Fig. 1b). While Ile-531 and Gly-534 are located in the intracellular part of the receptor, all other residues belong to the predicted extracellular domain.

We then performed docking simulations of the generated model I using protoporphyrin IX (PPOIX) as the ligand in AutoDock Vina. The best viable docking pose is predicted inside the hydrophobic pocket with

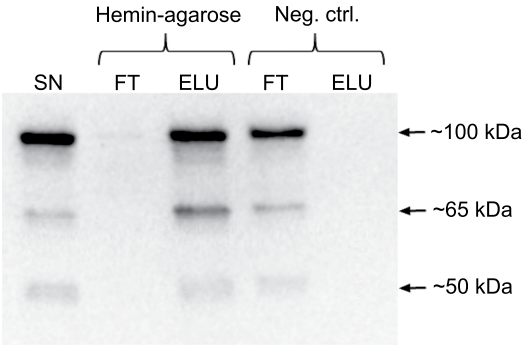

Figure 2. Hemin-agarose pull-down assay. Cell lysates of *E. coli* Bl21 cells expressing LsHSCARB (residues 31–523) were mixed with hemin-agarose (Sigma-Aldrich), and samples analysed by western blot using an anti-LsHSCARB antibody (Genscript). LsHSCARB was detected in the supernatant (SN), the elution (ELU) of hemin-agarose, and the flow through (FT) of the negative control. Exposure time = 29.5 sec. The experiment was conducted three independent times with similar results.

the CY residues extending over the iron-center of heme (Vina score: −9, Fig. 1d, Supplementary Data S1–4). To assess whether the observed scores are comparable to docking results with known hemoproteins and other heme-binding proteins, we analyzed the best docking poses of ten experimentally obtained structures with heme ligand after removing the heme moiety and re-docking with PPOIX (Supplementary Table S1). Nine out of ten docking attempts placed the ligand at a distance of <2 Å between centroids, and six out of ten attempts resulted in an RMSD <1 Å between the docking pose and the experimental structure. The only failed docking attempt (3GNF, Cytochrome c-553 from *Bacillus pasteurii*) also presented with the worst Vina score of all experiments. All successful re-docking attempts to heme-proteins presented with a Vina score ≤−9. In addition, we tried docking PPOIX with two alternative PDB structures of LIMP-2, one of which was used as a threading template by i-TASSER to generate the LsHSCARB homology model. To the best of our knowledge, LIMP-2 has not been previously reported as a heme-interacting protein. These resulted in very similar optimal VINA scores of −7.6 and −7.7 respectively (Supplementary Table S1).

When comparing *in silico* docking results and HemeBIND predictions, three out of six predicted extracellular residues are within a distance of <2 Å from the electrostatic surface of the docked ligand, Lys-259 interacts directly with the ligand surface, whereas Pro-147 and Phe-256 are proximal to the docking site (Fig. 1d)

In order to investigate whether LsHSCARB was able to bind heme directly, we expressed the extracellular part of LsHSCARB (residues 31–523) in *E. coli* Bl21 De3 cells and investigated its ability to bind hemin-conjugated agarose resin. Cells were lysed and the supernatant containing the recombinant protein was incubated with either hemin-conjugated agarose resin or un-conjugated agarose resin as negative control. As can be seen in Fig. 2, little or no LsHSCARB was detected in the hemin-agarose flow through, whereas an evident band was visible in the negative control flow through. Upon washing of the resin, any protein bound to the agarose was eluted by heat denaturation and sampled for detection by western blot (elution). The most evident band on the membrane is at 100 kDa, which corresponds with the predicted molecular weight of the recombinant protein (100.6 kDa), but there are also fainter bands seen at approximately 65 and 50 kDa. While LsHSCARB clearly could be detected in the hemin-agarose elution, no band was visible in the negative control (Fig. 2), strongly indicating that LsHSCARB is a heme-binding protein. Full length image of the western blot with the protein standard can be seen in Supplementary Fig. S3.

***In situ* hybridization.** To initially characterize the gene, we determined the localization of its transcript by *in situ* hybridization. In the adult female louse, the transcript of *LsHSCARB* was solely detected in cells lining the intestine (Fig. 3c).

Immunohistochemistry. Immunohistochemistry was used to detect the localization of the LsHSCARB protein in the adult female louse by polyclonal anti-LsHSCARB antibody. The protein was detected in the intestinal border facing the intestinal lumen identical to the location of *LsHSCARB* mRNA (Fig. 3d).

RNAi mediated ablation of *LsHSCARB*. In order to assess the effect of RNA interference-mediated ablation of *LsHSCARB*, two experiments were implemented. The first was terminated upon extrusion of the second pair of egg string (38 days). At this point, no visible phenotypic alteration was observed. Knockdown efficacies, as well as heme and protein measurements are documented in Supplementary Figs S4–S7. Knockdown efficacy was on average 99% and the heme concentration in knockdown lice was significantly ($p = 0.02$) reduced by on average 58% compared to control lice. As no visible phenotype alteration was observed despite lower levels of heme, the second experiment was prolonged to observe the effect of an extended period of ablation. Observed values for effect size and standard error from experiment 1 were used as input to conduct power analysis; thus, a minimum $n = 8$ samples per group was required to achieve adequate power ($1\text{-}\beta > 0.80$) to detect the observed change in

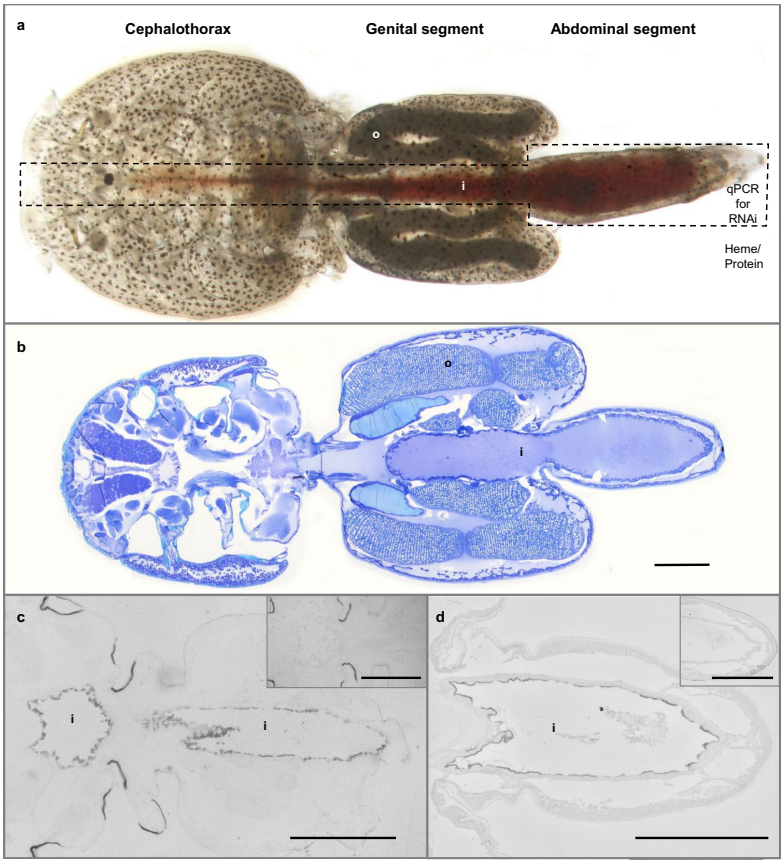

Figure 3. Anatomy of a salmon louse. Photograph of an untreated adult female louse. The blood-filled intestine in the boxed area was dissected out after RNAi to assess knockdown success by qPCR, and the rest of the body was used for heme/protein analyses (**a**). Section of an untreated adult female louse toluidine blue dyed is shown with various structures named. Scale bar: 1 mm (**b**). *In situ* hybridization shows that the transcript of *LsHSCARB* is localized in cells lining the intestinal tract of the adult female salmon louse. The negative sense control in the upper right corner showed no signal. Scale bar: 1 mm (**c**). Immunohistochemical detection of LsHSCARB using anti-LsHSCARB antibody in an adult female louse. Protein is located in the intestine, and the negative control in the upper right corner showed no signal. Scale bar: 500 µm (**d**). Abbreviations: o = oocyte, i = intestine.

heme concentration, while n = 10 allows for a vast increase in statistical power ($1\text{-}\beta > 0.90$). The following results are from experiment two.

Knockdown efficacy after injection of double-stranded RNA was assessed in the dissected intestine of each louse (Fig. 3a) by qPCR. At the time point after the extrusion of the fifth egg string pair (observed in the control lice), *LsHSCARB* mRNA was significantly reduced in intestinal tissues of the treated group (Fig. 4). Seven out of ten animals in the treated group had between 95 and 99% reduction in mRNA; the other three animals in the treated group had a reduction of 60, 32 and 32%. All animals were included in the statistical analyses (n = 5 (control) and 10 (treated), p = 0.005).

Histology and phenotype. Upon termination of the second experiment, RNAi knockdown animals appeared anatomically normal by visual inspection, although they had shorter egg strings than control animals (Fig. 5a,c). Histological sections of control and dsRNA-treated lice revealed no morphological change other than in the oocytes (Fig. 5b,d). Oocytes from the *LsHSCARB* dsRNA-treated group appear larger and chorions more swollen than in the control group, even though lice from both groups were at the same stage of oogenesis (i.e. egg strings hatched on the same day after terminating the experiment). An annotated histological section of an untreated adult female louse colored with toluidine blue is depicted in Fig. 3b.

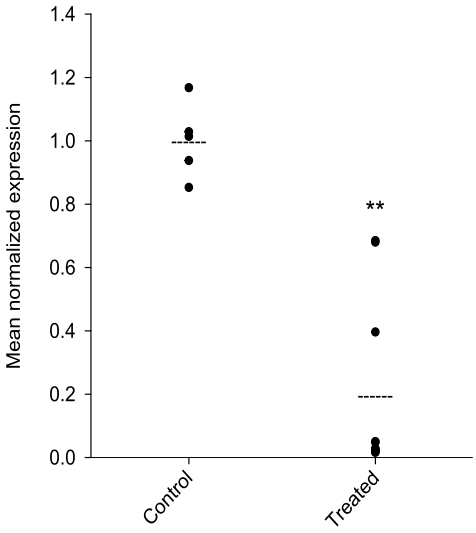

Figure 4. Gene expression analysis by qPCR reveals that *LsHSCARB* is down-regulated 69 days after induced RNAi. The stapled lines indicate the mean value of each group, and the dots represents expression values in individual lice. Asterisks indicate a significant difference between control and treated group. **: significant at $p \leq 0.01$. $n = 5$ (control) and 10 (treated).

Egg string lengths and offspring development. 67% of the control lice were recovered upon termination of the second *LsHSCARB* RNAi experiment. Here, 19 out of 20 lice carried egg strings, with a mean length of 21 mm (Fig. 6a). 50% of the treated lice were recovered at the end of the experiment, and of these, 12 out of 15 lice presented with egg strings with a mean length of 16.5 mm whereas 3 had none (Fig. 6a) ($n = 19$ (control) and 12 (treated), $p = 0.001$). Hatching and molting success of the egg strings were monitored. Emerging copepodids were counted, and the number of live animals in relation to their respective egg string length is shown in Fig. 6b ($n = 19$ (control) and 12 (treated), $p = 0.0002$). One egg string pair from the control group did not hatch, and three pairs of egg strings did not hatch in the treated group. These are denoted at 0 copepodids per mm egg string, and are included in the calculations (Fig. 6b).

Heme and protein levels. To measure the effect of RNA interference-mediated ablation of *LsHSCARB* on heme absorption, total heme levels were analyzed from body tissues excluding the intestine that is filled with salmon blood (Fig. 3a). Two animals in the *LsHSCARB* dsRNA-treated group displayed body heme levels below the LOD, and their heme levels were set to the threshold value. Heme levels are significantly lower in the treated animals (Fig. 6c). Total protein levels from the same lysates were slightly, but not significantly ($p = 0.089$) lower (Fig. 6d). In addition, the adjusted heme levels per protein unit were significantly lower ($n = 10$, $p = 0.011$) in the knockdown group compared to the control group (Fig. 6e).

Starvation. To investigate whether the mRNA expression of *LsHSCARB* in the adult female salmon louse is affected by the presence of host blood in the louse intestine, adult female lice were collected from fish and thereby separated from their food source and starved for zero (sampled immediately), one, two, four and eight days. The expression profile shows a down-regulation in mRNA levels of *LsHSCARB* (Fig. 7) using lice fixed immediately after sampling as calibrator. Down-regulation is significant from day two (day 1: $p = 0.124$, day 2: $p = 0.03$, day 4: $p = 0.007$, day 8: $p = 0.003$). Compared to lice sampled immediately after being removed from fish, lice starved for eight days have on average an 85% down-regulation of transcript levels of *LsHSCARB*.

Discussion

As a hematophagous parasite without the option of synthesizing heme on its own, the salmon louse is dependent on acquiring heme from the blood of its host to sustain essential cellular processes. In the present study, we propose a likely mechanism by which the salmon louse takes up heme from its blood-filled intestine. Although the hypothesis about the existence of an intestinal heme receptor has been framed already in 1979[23], few studies have since identified proteins involved in heme trafficking over intestinal membranes, one of the hindrances potentially being that many animal models have endogenous heme. Using the natural heme auxotrophic parasite *L. salmonis* as a model organism provides a great opportunity to study heme trafficking.

In this study, we have characterized a gene encoding a scavenger receptor class B-like protein that may facilitate heme absorption over the intestinal membrane of the salmon louse. While CD36 is abundant in epithelial cells of the mammalian small intestine and might play a role in gut homeostasis[23], to the best of our knowledge, homologous proteins have not been previously implicated in heme internalization. Nevertheless, CD36-like

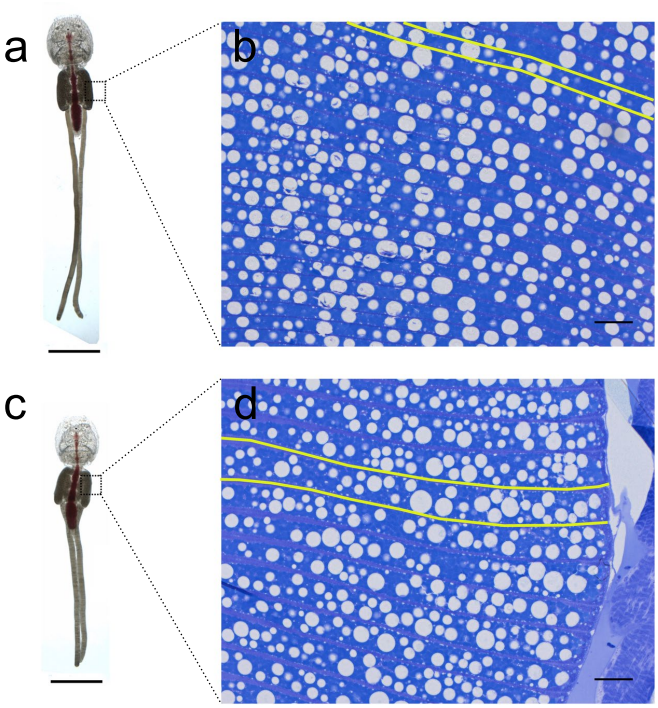

Figure 5. Photographs of representative adult female lice from control (**a**) and *LsHSCARB* knockdown group (**c**). Toluidine blue colored histological sections of oocytes found in indicated areas in genital segments of these lice are shown in right panel for control (**b**) and knockdown (**d**) lice. Yellow lines are drawn along two chorions in each group (**b**,**d**) to indicate the boundaries of one oocyte. Lipid droplets are observed throughout the oocytes. Scale bars: (**a**,**c** = 5 mm, **b**,**d** = 50 µm).

receptors bind a multitude of ligands, among them thrombospondin-1, oxidized low-density lipoprotein, beta carotene, long-chain fatty acids, and pathogens[20–22,24]. Other scavenger receptors comprise an even wider family of integral membrane proteins with variety of domain architectures and a broad range of ligands, among them hemoproteins. As an example, Scavenger receptor cysteine-rich type 1 protein M130 (CD163) is involved in removal of the hemoglobin/haptoglobin complex by macrophages[25,26]. Compared to LsHSCARB, CD163 has only a single transmembrane domain and low overall sequence similarity to the receptor characterized here. Membrane topology prediction on the amino-acid sequence of LsHSCARB convincingly yields two transmembrane helices (residues 7 → 28 and 528 → 549), a feature distinguishing class B from all other characterized scavenger receptors. Hence, by domain architecture and by sequence and structural similarity we sustain the hypothesis that LsHSCARB is homologous to class B scavenger receptors.

The ability of recombinant LsHSCARB to bind heme was investigated by a hemin-agarose binding assay, as has also been used for other heme-binding proteins[27,28]. Three bands appear upon detection with anti-LsHSCARB, at approximately 100, 65 and 50 kDa. The band at 100 kDa is the strongest, and corresponds with the predicted molecular weight of the recombinant LsHSCARB with an MBP fusion tag (100.6 kDa). The other two bands are less evident, and could be degradation products, or alternative isoforms of the protein. Our results clearly show that recombinant LsHSCARB is able to bind hemin-agarose resin *in vitro*, but not agarose resin without conjugated hemin, further supporting our hypothesis that it is a heme-binding protein.

3D protein model and the result of *in silico* docking with protoporphyrin IX were also in accordance with our initial hypothesis. We tested the ability of AutoDock Vina to predict binding sites of a variety of known heme-binding proteins for calibration. For the majority of cases, the best binding pose was predicted with high accuracy with respect to RMSD and distance between centroids, giving further confidence that heme ligands could fit into the proposed docking pose geometrically. Of note, for two very similar experimental structures of a homologous receptor to LsHSCARB, the lysosomal integral membrane protein (LIMP), which has not been described as having heme affinity, docking yielded much worse but near identical scores, giving us further confidence in the robustness of the approach. It is further worth noticing that our *in silico* model, unlike crystal structures, does not contain any side-chain information to guide the docking process. While we did not find a known heme-binding motif (e.g. cysteine-proline residues or CXXCH motif), the predicted binding pocket is enriched for non-polar residues (mostly isoleucine and leucine) and contains cysteine-tyrosine (CY) residues on the

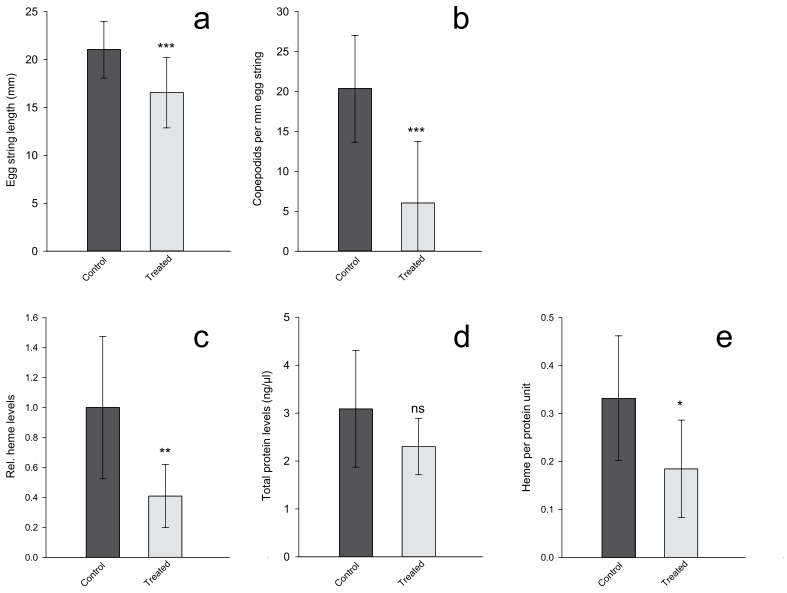

Figure 6. Effect of *LsHSCARB* knockdown. Top: mean egg string lengths (±SD) (**a**) and mean number of copepodids per mm egg string (±SD) (**b**) in control (dark grey) and *LsHSCARB* dsRNA treated (light grey) group ($n = 19$ for control and 12 for treated). Bottom: mean relative heme levels (±SD) (**c**). Mean total protein levels (±SD) (**d**) and adjusted heme levels to the amount of protein (±SD) (**e**) of lice from the control group (dark grey bars) compared to the *LsHSCARB* knockdown group (light grey bars) ($n = 10$). Asterisks indicate a significant difference between control and treated group. Ns: not significant, *: significant at $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

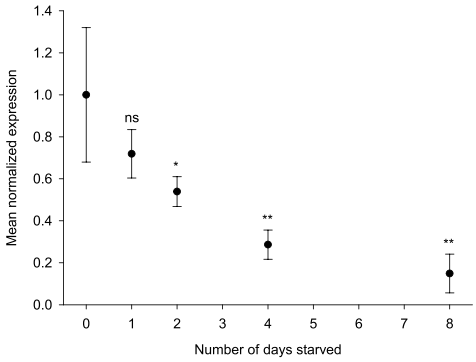

Figure 7. Relative expression of *LsHSCARB* in relation to starvation of adult female lice for 0, 1, 2, 4 and 8 days. Lice taken at 0 days of starvation were used as a calibrator. Asterisks indicate significant difference from day 0. ns: not significant, *: significant at $p \leq 0.05$, **: ≤ 0.01. $n = 5$ per sampling day.

opposite interior surface, potentially forming the axial ligand to the iron atom of heme. In a comprehensive structural analysis of 125 non-redundant heme-binding protein chains, Li *et al.* (2011) reported that heme-binding pockets were enriched with non-polar residues that create a hydrophobic environment for heme binding, with leucine, isoleucine, and valine slightly more abundant[29]. Besides, the same study also reported enrichment of aromatic residues, whereas we did not observe aromatic residues within the predicted pocket except for the tyrosine residue. Of note, the extracellular domain is strongly enriched for three out of the five common axial ligands of heme compared to LIMP[29]. The structural characteristics of a heme receptor could however be evolutionarily adapted to support a rather transient binding mode compared to other heme-proteins, and this might explain

some of the differences observed. Transient binding would further explain slightly lower Vina scores for our receptor, in contrast to proteins with catalytic heme groups, such as nitric oxide synthase, cytochrome P450's or peroxidases. We further note the presence of multiple residues on the protein surface that have been identified as potentially heme-binding either by prediction or as commonly known axial ligands (Fig. 1b)[29,30]. The existence of multiple unmapped binding modes could contribute to increased efficiency of heme absorptions by binding multiple ligands. More in-depth structural analyses of the receptor in complex with heme are nevertheless required to precisely determine binding modes.

Both transcript and protein of the putative heme scavenger were exclusively expressed in epithelial cells lining the midgut of the salmon louse, further supporting our hypothesis that this protein is important for nutrient absorption from the blood-filled intestine of the salmon louse. Because of the localization of the transcript, we dissected out the intestine to assess knockdown success by qPCR (Fig. 3a). Consequently, the rest of the body could be used for heme and protein measurements. This way, fish-blood in the intestine is excluded and only absorbed heme is measured. As fish blood is highly enriched in heme as seen by the red pigmentation due to heme, measuring heme levels in the blood-filled intestine would otherwise lead to a wrongful estimate of absorbed heme levels.

In the first RNA-interference knockdown experiment (terminated after 38 days) there was no visible phenotypic alteration in the knockdown animals. However, the heme levels of the knockdown animals are significantly lowered. This drove us to prolong the second experiment to investigate how a longer period in the absence of *LsHSCARB* would affect the parasite. The next RNAi mediated knockdown (terminated after 69 days) of *LsHSCARB* also caused a significant reduction in the amount of heme in the tissues of the louse. Moreover, ablation caused lice to have 21% shorter egg strings, and 70% lower hatching success. The two animals with less effective knockdown and egg strings present (32% down-regulation for both) had a 74% greater hatching success than the animals with 95–99% knockdown. We conclude that detrimental effects of nutrient deprivation accumulate over the prolonged knockdown period and manifest in vastly reduced fecundity.

The necessity of heme for development is widely recognized, and the reduction of viable offspring due to heme depletion has been reported on several occasions. The blood fluke, *Schistosoma mansoni*, requires exogenous heme for the production of eggs as incubation with cyclosporin A inhibited heme uptake, and thus reduced fecundity[31]. The requirement of heme for growth is also reported for the hemoparasite *Leishmania tarentolae*[32]. Perner *et al*. (2016) found that serum fed ticks (*Ixodes ricinus*) did not have embryonal development in eggs, whereas adding 10% hemoglobin to the serum rescued embryogenesis[33]. Furthermore, the silencing of a maternal heme-binding protein in the blood sucking insect *Rhodnius proxilus* impaired embryogenesis[34]. The heme measurements in this study indicate that the knockdown animals contain on average 60% less heme than the control animals. The remaining heme could be due to redundant uptake systems within the intestine that should be further investigated. However, since RNA-interference is not able to mediate a complete knockout, remaining transcripts of *LsHSCARB* could lead to residual production of LsHSCARB protein in addition to remaining LsHSCARB protein translated before dsRNA injection. These effects could contribute to continuous, albeit reduced, heme uptake. A passive diffusion of heme across the intestinal border membrane seems unlikely, as free heme is lipophilic and could lead to peroxidation of membrane lipids[5].

Even though scavenger receptors have not been implicated in intestinal heme absorption before, ablation of similar genes has been done in other blood-feeders. A CD36-like scavenger receptor was knocked down by RNAi in the hard tick *Haemaphysalis longicornis*[35]. The experiment showed impact on blood-feeding behavior, egg production and the hatching rate. It is however unknown whether this is related to heme absorption, as the level of heme was not analyzed. Later, the authors presented that the receptor was involved in granulocyte-mediated microbial phagocytosis in ticks as well as mediating systemic RNAi in ticks[36,37]. We suggest investigating the role of this receptor in the absorption of heme in the mid-gut of the tick further.

Although the ablation of *LsHSCARB* led to shorter egg strings and lower hatching success, histological analyses did not reveal any drastic morphological alteration that could explain these observations. The only difference observed was in the oocytes. In treated animals, the oocytes appear larger and swollen, and also less structured than in the controls, despite the fact that all egg strings hatched on the same day, indicating that oocytes were at a similar stage of maturation. These observations lead to the conclusion that *LsHSCARB* is essential for normal oocyte development in *L. salmonis*.

We further observe that starving animals causes a decrease in the transcript level of *LsHSCARB*. This suggests a positive regulatory feedback system where a nutrient-rich blood-filled intestine leads to induction of gene expression. Down-regulation due to starvation is in contrast to reports from the free-living heme-auxotroph *C. elegans*, where the expression of HRG-1 is up-regulated as a response to decreasing levels of heme[16]. However, the lifestyle of the hematophagous parasite *L. salmonis* differs strongly from that of *C. elegans*. The salmon louse is, unlike *C. elegans*, either on or off its host. Thus, it is either able to feed or not, and will thus not experience a true gradient in heme concentration. Expression of a gene that is solely required for nutrient uptake is energy consuming and thus potentially wasteful under nutrient deprivation. This argument could contribute to explain increased down-regulation under prolonged starvation. In accordance with our findings, a nutrient induced positive feedback mechanism was observed in the crustacean *Macrobrachium nipponense*, where a scavenger receptor was up-regulated by various dietary lipid sources[38]. Further, Staron *et al*. (2017) reported induction of several heme associated proteins under dietary hemoglobin rescue[39].

In conclusion, the results from functional studies, both *in vitro* and *in vivo,* as well as *in silico* data indicate that *LsHSCARB* is likely encoding a receptor of heme in the salmon louse intestine. The investigation of the CD36-like protein in relation to heme transport shown in this study may further elucidate the trafficking of heme in other species, which as of today is a process that remains enigmatic. Because ablation of the receptor mRNA gives rise to significant reduction of the fecundity of the parasite, the receptor could find its application as a new drug target in pest control of blood feeding parasites. More generally, we have demonstrated the possibility that class B

scavenger receptors, among a wide range of other known ligands, could also mediate heme absorption. It remains an open question if this ability is a singular evolutionary event, or if some of the many orthologous sequences in other animals have a similar function. Either way, given that heme is the most bioavailable iron source for humans, medical applications could arise in the future, prospectively for disorders related to iron uptake or the heme biosynthesis pathway.

Material and Methods

Sequence analysis, in silico modelling and docking. Initial data for searching transcript and protein sequences and primer design were extracted from the *L. salmonis* genome annotation in Ensembl Metazoa (https://metazoa.ensembl.org/Lepeophtheirus_salmonis). Gene expression data were retrieved from LiceBase (https://licebase.org). Pathway reconstruction was performed using the KEGG Automatic Annotation Server on all predicted protein sequences[40]. All following analyses are based on the GenBank sequence CDW29028.1. Glycosylation prediction was performed using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and the NetOGlyc 4.0 Server[41]. Protein BLAST searches were conducted against the GenBank and SwissProt databases[42]. Conserved domain search was conducted using InterProScan[43] and consensus membrane topology prediction was performed using CCTOP[44]. Prediction of protein 3D-structure was done in i-TASSER[45], and docking of the best scoring i-TASSER model (model I) with protoporphyrin IX (PPOIX, ZINC26671872) from the ZINC database[46] was performed using AutoDock Vina with default parameters[47], using model I as receptor and PPOIX as ligand. Docking was evaluated using experimental heme-containing protein structures and other proteins not known for heme affinity (Supplementary Table S1) after manually removing the heme moiety and non-standard residues, if present. Visualization of structures and docking was done in UCSF Chimera[48]. RMSD and distances between centroids were calculated for all C, N, and O atoms shared between HEM residues in PDB structures and docking poses using UCSF Chimera and the R-package Rpdb (https://cran.r-project.org/package=Rpdb). Enriched residues that could serve as axial ligands to heme (C, M, Y, K, H)[29] were evaluated in a conserved region only (Supplementary Fig. S1), after pruning potential N- and C-terminal His-tags and terminal gap regions: conserved region was extracted from structural alignment of model I, and PDB ids 4F7B and 4Q4B after re-aligning by MUSCLE[49]. The HemeBIND web-server was used to predict heme-binding residues in model I[50].

Plasmid construction and recombinant LsHSCARB protein expression. ORFs coding for the extracellular part of LsHSCARB (residues 31–523), synthesized and subcloned into a pETM41/His-MBP plasmid by GenScript, were transformed into *E. coli* Bl21 De3 cells for protein expression. Cells were grown at 28 °C until reaching an OD600 of 0.6, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM. Proteins were expressed o/n at 18 °C and harvested by centrifugation. Cell pellets were lysed in a buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, cOmplete EDTA-free protease inhibitor (Roche), 1 mg/ml lysozyme and either 0.1% FOS16 or 2% DDM), incubated on ice for 30 min and sonicated for 1.5 min with 1 second impulses at 50 amp. The cell sonicate was centrifuged at 15,000 g for 30 min at 4 °C and the supernatant collected. The protein concentration of the supernatant was determined by Direct Detect spectrometer and the supernatant was further used in downstream experiments.

LsHSCARB binding to hemin-agarose. 100 µl of hemin-agarose beads (Sigma, H6390) were washed four times with 50 mM Tris-HCl pH 7.4, 150 mM NaCl prior to use. The beads were then resuspended in 100 µl buffer and added to an Eppendorf tube with 350 µl of the supernatant containing 2.5 µg/µl protein. Incubation was performed on a rotating wheel for one hour at room temperature. After incubation, the flow through was sampled and mixed with SDS-PAGE sample buffer to assess the unbound fraction. The beads were washed four times with wash buffer and finally the beads were incubated in denaturing buffer containing 2% (wt/vol) SDS, 1% (v/v) β-mercaptoethanol, and 500 mM Tris-HCl pH 6.6 for two min at room temperature followed by five min at 99 °C. The beads were centrifuged, and the supernatant was collected for analysis by western blot. Agarose beads with a glutathione ligand (sepharose 4b) were used as negative control and treated in the same manner as the hemin-agarose. Samples were separated on a SDS-PAGE and detected by anti-LsHSCARB on western blot. Initially, the protein was expressed with an N-terminal His-tag. In order to verify that the observed binding to hemin-agarose was not due to His-tag affinity for iron, the His-tag was removed by Q5 site-directed mutagenesis (NEB). The protein without His-tag was used in one of three binding experiments.

Animals. A laboratory strain of *L. salmonis* was raised on Atlantic salmon (*Salmo salar*) in tanks with seawater (salinity 34.5‰ and temperature 10 °C) as earlier described[51]. Fish were daily handfed a commercial diet and maintained according to Norwegian animal welfare regulations. Experiments were approved by the governmental Norwegian Animal Research Authority (ID8589). Fish carrying lice from RNA interference (RNAi) experiments were kept in single-fish tanks[52]. Egg string pairs of salmon lice were incubated and hatched in single wells in a flow through system[51] until being used for experiments.

RNA isolation, cDNA synthesis, and qRT-PCR. All tissues destined for RNA extraction were stored in RNAlater (Ambion) first at 4 °C overnight, and then at −20 °C until usage. Total RNA was isolated from adult female louse intestines or whole animals using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. Samples were homogenized using 5 mm stainless steel beads and a TissueLyser II (Qiagen) for 1 min at maximum frequency. Genomic DNA was digested from the samples by DNase treatment using DNase I (Invitrogen). RNA was quantified and its purity checked by NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). Isolated RNA was stored at −80 °C until further use. RNA (300 ng) was reverse transcribed to complementary DNA (cDNA) using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene), and diluted tenfold with RNAse-free water before being stored at −20 °C. *LsHSCARB* knockdown efficiency and the effect of starvation were validated by assessing the transcript levels by quantitative PCR (qPCR) using the previously validated

reference gene *LsEF1α*[53]. qPCR was run on a QuantStudio 3 qPCR machine using PowerUp SYBR Green Master Mix (Applied Biosystems) on duplicate samples under standard 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, followed by a melt curve analysis at 60–95 °C). Duplicate samples had a difference in Ct-values < 0.35. A six-point standard curve of twofold dilutions was prepared to assess the PCR efficiency of the assay: $E\% = (10^{1/slope} - 1) \times 100$[54], which was 94% for *LseEF1α* and 100% for *LsHSCARB*. Relative differences (ΔΔCt) in threshold were calculated and transformed by the formula $2^{-\Delta\Delta Ct}$[55].

***LsHSCARB* knockdown by RNA interference.** Double stranded (ds) RNA fragments for *LsHSCARB* were generated using the Megascript® RNAi kit (Ambion) with cDNA from an adult female louse as template. A fragment for trypsin (CPY185) from Atlantic cod (*Gadus morhua*) was used as negative control as this has no significant sequence similarity to transcripts of the salmon louse[56]. Primers used for fragment synthesis are listed in Table 1. Fragments were diluted to 600 ng/µl and bromophenol blue was added to visualize successful injections[56]. Knockdown was investigated in female lice only. On the startup day of the experiment, preadult II female lice were removed from reservoir fish by forceps and dsRNA solutions were injected using borosilicate glass capillaries and pressure from a mouth tube. Lice were placed in seawater to recover for four hours before being put on fish held in individual tanks. Ten females and seven males were placed on each fish. For each fragment, three fish in individual tanks were used. The first experiment was terminated after the extrusion of the second egg string pair of mature adult female lice 38 days after injection. The second experiment was terminated upon the extrusion of the fifth egg string pair of mature adult female lice at 69 days after injection. Upon termination, all lice were removed from the fish and photographed under a binocular. Two control lice and three treated female lice were stored in Karnovsky's fixative, whereas the remaining lice were dissected as shown in Fig. 3a and stored in RNAlater (intestine) and homogenized in lysis buffer (rest of the body). Egg strings, if present on the adult female lice, were incubated in seawater in flow through hatching wells. Egg string hatching time point and the development of the larvae were monitored.

***In situ* hybridization.** The localization of *LsHSCARB* mRNA was detected in the adult female salmon louse by using *in situ* hybridization (ISH) as earlier described[57,58]. An RNA antisense (AS) probe of 731 bp was made from a target specific cDNA template (see Table 1 for primer sequences). A sense (S) probe acted as negative control for the transcript localization, whereas hybridization with a known set of probes detecting *LsTryp1* acted as positive control[59]. The labelled probes were visualized by using anti-digoxigenin (DIG) alkaline phosphatase fragment antigen binding (FAB) fragment (Roche) and a chromogen substrate containing levamisole (Sigma), nitroblue tetrazolium (NTB; Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche). Microscopy images were captured by an Axio Scope A1 light microscope connected to Axiocam 105 (Zeiss).

Heme and protein extraction. Whole body tissues of adult female lice excluding intestine (Fig. 3a) were homogenized in 700 µl lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% v/v Triton-X-100 (Sigma-Aldrich), 1 × cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche)) using a mortar and pestle, followed by using a syringe and needle. Homogenates were centrifuged at 12,000 rpm at 4 °C for 15 min. Supernatants were either used directly or stored at −80 °C until use.

Fluorescent heme quantification assay. The heme quantification method by Morrison (1965)[60] was adapted to *L. salmonis* and performed as following. Supernatants from heme/protein extractions were diluted 1:20 in 150 µl of 2 M oxalic acid containing 1% w/v iron oxalate (both from Sigma-Aldrich). The solution was divided into two aliquots, one boiled at 99 °C for 30 min and one left at room temperature. By boiling, non-fluorescing heme is reduced to fluorescent protoporphyrin IX[60–62]. The aliquot kept at room temperature was used to control that the tissues did not contain porphyrins naturally. Four parallels of lysis buffer diluted 1:20 and boiled in the oxalic acid solution were used as the blank and to calculate the limit of detection (LOD = 3× standard deviation (SD) of the blank). A positive control of 400 ng/ml hemin (Sigma-Aldrich) (dissolved in DMSO and diluted 1:20 in 2 M oxalic acid containing 1% w/v iron oxalate) was boiled for 30 min and included in three parallels to ensure the samples being within the linear range of the assay (serial dilution from 0–400 ng/ml, Supplementary Fig. S8). A high precision cell made of Quartz SUPRASIL® (Hellma Analytics) held the samples of about 50 µl as they were excited at 406 nm, and emission was read between 600–605 nm using a LS-50B fluorescent spectrometer (Perkin-Elmer). The reading speed was set to 50 nm/min. The cell was rinsed thoroughly with Milli-Q H_2O between each sample. All samples were read at 25 °C.

Protein quantification. Total protein levels were quantified using a bicinchoninic acid (BCA) assay with a Bovine serum albumin (BSA) standard (both from Sigma-Aldrich). Two dilutions of each lysate (1:10 and 1:20) were prepared and further diluted in BCA working reagent (1:20) and incubated at 37 °C in a PCR machine for 30 min. Two microliters of the sample were mounted to the instrument and absorbance was read at 562 nm using NanoDrop-1000 spectrophotometer. Negative samples were measured frequently to ensure no protein residues interfered with the experimental sample readings. Protein amounts were used to calibrate heme levels.

Histology. Animals destined for histological analyses were fixed in Karnovsky's fixative for a minimum of 24 h at 4 °C. Fixed animals were then rinsed in 1xPBS and dehydrated in 70% EtOH (15 min), 96% EtOH (2 × 15 min), 1:1 absolute EtOH:infiltration solution (1% w/v Hardener I (benzoyl peroxide) in Technovit 7100 resin (Nerliens Meszansky A.S)) (2 h), and incubated overnight in 100% infiltration solution on a shaker. Plastic embedding was done in 15:1 infiltration solution:Hardener II. Two micrometer thick sections were obtained using a microtome (Leica RM 2165) and placed on microscope slides (VWR International). Sections were stained in filtered toluidine blue for 30 s and rinsed thoroughly in H_2O to remove background stain. Dry slides were mounted with DPX mounting solution (Sigma-Aldrich) and covered with glass cover slips. Microscopy images were captured as

described under the *in situ* hybridization section. Images of whole animals were processed and stitched together using an ImageJ plugin as described by Preibisch *et al.*[63].

Starvation experiment. Adult female lice were removed from the fish and placed apart from their food source in individual incubators in running sea water, and left there starving for 0 (sampled immediately), 1, 2, 4 and 8 days. Whole animals were sampled in RNAlater according to Trösse *et al.* (2015)[64]. qPCR was conducted as described above.

Immunohistochemistry. Polyclonal anti-LsHSCARB antibodies (0.8 mg/ml) were produced in rabbits by the company GenScript. The whole extracellular part of the protein was used for the antibody production (PAMIKSQIYENLDLREGTEGFNAFKEPPAPVYLSYSLFHIKNTNEVIRGEPPVLLEVGPYSYRETMRKENLMEQNSRYLSYGKYTKFEFDETNTHKLKCKNRINTPCSKNDKITIINPVLLTLADKLDGLPKTVKDICFEIINNGNEALGIKAEDLFITEEVDKILYTGFDSKSAAIFDKLDTFLMLLLEVIQESLELDIPIKAKDFENIIKIISPAQLSEGTFAFFKGKNATKLQNYYTIENGRFDKESFMNIVEFNGKNKLPEAWWPNVATSITGQLSSEGGSCHRIYGTDGTQFPPFLFNKKKFPLWMFVGELCRTIYVEFESEVEVEGGITAYRYGVGKRVFSMSNPENFCYCQEFFSCAKQTDNDEWDLSQCLKCKDGVMDVSACYGAPIFMSQPHFLQADKEVQAYVKGLEPNSEKHATYLDIEPNLGTPLRAHKKIQINMVLRKVAGIDLLKKVADFRLIPMFWADEGAELDSEKAEELNNVLFSAITIGNT). Paraffin embedded sections (3.0 µm) of an adult female louse were incubated at 60 °C for 30 min and treated with Histo-Clear II (National Diagnostics) for 2 × 10 min. Sections were then rehydrated in ethanol for 3 min per treatment (2 × 100%, 96%, 80%, 50%) and then rinsed in MilliQ H_2O. Following this, sections were washed 2 × 2 min with TBST (150 mM NaCl, 50 mM TRIS, 0.05% Tween, pH 7.6). Blocking was done with Superblock TBS (ThermoScientific) for 30 min at room temperature before the sections again were washed with TBST for 2 × 2 min. The primary antibody was diluted 1:8000 in TBST and left on the sections for 1 hour at room temperature. The primary antibody was washed off with TBST (2 × 2 min) before incubating with a 1:100 dilution of the secondary antibody (goat anti-rabbit IgG, Sigma-Aldrich) for 30 min at room temperature, and then washed off with TBST (2 × 2 min). Sections were flushed with processing buffer (100 mM Tris-NaCl, 50 mM $MgCl_2$, pH 9.5), and then incubated with processing buffer for 10 min. Staining was done with BCIP/NBT Liquid Substrate System (Sigma-Aldrich) for 3 min until a visible color appeared, upon which the reaction was stopped in MilliQ H_2O. The negative control was incubated with secondary antibody only. Sections were mounted with ImmunoHistomount (Sigma-Aldrich), and images were captured and processed as described under the *in situ* hybridization section. Two individuals were investigated.

Statistics. All statistical analyses were conducted using R and IBM SPSS Statistics 23 for Windows. The data sets' parametric requirements were checked by Shapiro-Wilk's test of normality. Levene's test was then used to check for equality of variances. Independent two-sample *t*-tests were conducted to evaluate the difference in means between control and experimental groups. For data sets not fulfilling the parametric requirements a Mann-Whitney U test was performed (knockdown effect and number of copepodids after hatching). Power analysis was performed in R using the observed effect size and standard error in experiment 1, assuming a two-sided alternative hypothesis. A p-value ≤ 0.05 was considered statistically significant. Data are presented as mean values ± standard deviations (SD). Graphs were prepared in SigmaPlot 13.0 and processed in Inkscape.

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Author Contributions

E.I.H. performed all laboratory experiments and drafted the manuscript. M.D. performed *in silico* work, framed the functional hypothesis and proposed the candidate. F.N. and C.E. supervised the project. S.I.S. and A.M. helped design and perform the work with the recombinant protein and heme-binding assay. All authors discussed the results and contributed to the final manuscript.

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III

Host gill attachment enables blood-feeding by the salmon louse (*Lepeophtheirus salmonis*) chalimus larvae, and alters parasite development and transcriptome

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KEYWORDS

Salmon louse; ectoparasite; blood-feeding; gills; transcriptome

ABSTRACT

Blood-feeding is a common strategy among parasitizing arthropods, including the ectoparasitic salmon louse (*Lepeophtheirus salmonis*), feeding off its salmon host's skin and blood. Blood is rich in nutrients, among these iron and heme. These are essential molecules for the louse, yet their oxidative properties render them toxic to cells if not handled properly. Blood-feeding might therefore alter parasite gene expression. We infected Atlantic salmon with salmon louse copepodids and sampled the lice in four different experiments at day 10 and 18 post infestation. Parasite development and presence of host blood in their intestines were determined. We find that lice start feeding on blood when becoming mobile preadults if sitting on the fish body, however they may initiate in blood-feeding at the chalimus I stage if attached to gills. Lice attached to gills develop at a slower rate. Lice of similar instar age from gills versus lice from skin epidermis were sampled in three different experiments, and gene expression was analyzed by RNA-sequencing. By differential expression analysis, we found 355 transcripts elevated in lice sampled from gills and 202 transcripts elevated in lice sampled from skin consistent in all experiments. Genes annotated with "peptidase activity" are among the ones elevated in lice sampled from gills, while in the other group genes annotated with "phosphorylation" and "phosphatase" are most predominant. Transcripts elevated in lice sampled from gills are often genes relatively highly expressed in the louse intestine compared with other tissues, while this was not the case for transcripts found elevated in lice sampled from skin. In both groups, more than half the transcripts were also higher expressed after attachment. In conclusion, blood-feeding results in an alteration in gene expression, and a premature onset of blood-feeding likely causes the parasite to develop at a slower pace.

INTRODUCTION

The Atlantic salmon louse, *Lepeophtheirus salmonis salmonis* (Krøyer, 1837) (Crustacea: Caligidae) (Skern-Mauritzen et al., 2014), is an obligate ectoparasite of salmonid fish, such as the Atlantic salmon (*Salmo salar*). The parasite is of major concern for the aquaculture sector in the Northern Hemisphere, as it causes challenges for the industry with its high fecundity and resistance towards several chemotherapeutants (Aaen et al., 2015). The parasite life cycle consists of both planktonic and parasitic stages (Hamre et al., 2013; Johnson and Albright, 1991a). Upon hatching from a fertilized egg, the parasite is in the nauplius I stage. Thereafter, the salmon louse molts into the nauplius II stage, and further to the infective copepodid stage. Successive molting occurs on the host, first to the parasitic chalimus I and II. These stages are attached to the host by their elongated frontal filament (Bron et al., 1991; Gonzalez-Alanis et al., 2001), and are therefore immobile. Another molting renders the parasite mobile, as it is no longer secured by the frontal filament, but holds itself by using its cephalothorax as a suction cup. These stages are the preadult I and II and adult lice. Now, the parasite grazes on larger parts of its host, selecting its preferred feeding site and is causing greater damage to the fish (Bjørn and Finstad, 1998; Grimnes and Jakobsen, 1996). Progression of the salmon louse life cycle is temperature dependent, and at 10 °C, the time from fertilization to mature adult is approximately 40 (male) to 52 (female) days (Johnson and Albright, 1991b), or 38 (male) to 44 (female) days for the fastest developers (Hamre et al., 2019).

The alimentary canal of the salmon louse develops during the copepodid stage (Bron et al., 1993). The alimentary canal is composed of a mouthpart, an esophagus, a midgut, and a hindgut ending in a short rectum (Bron et al., 1993; Nylund et al., 1992). Vertebrate blood is a highly nutritious tissue fluid that is constantly renewed. Hematophagy (blood-feeding habit) is therefore a common strategy among parasitizing arthropods. The salmon louse diet is considered to consist of the skin and blood of its host (Brandal et al., 1976), and the blood-filled

intestine is visible as a red line throughout the salmon louse body. Upon ingestion of blood, hematophagous parasites need to express genes encoding proteins that can manage the blood components. Blood is particularly enriched in proteins that contain the pro-oxidant molecules heme and iron. These are essential cofactors for the salmon louse, yet also highly toxic if not bound and detoxified by chaperones. Therefore, the alimentary canal needs to withstand, digest and absorb components of the food bolus. Trypsin-like enzymes (Johnson et al., 2002; Kvamme et al., 2004), a lipid transfer protein (Khan et al., 2017), a putative heme scavenger receptor (Heggland et al., 2019a) and the iron storage units of ferritin (Heggland et al., 2019b) are all expressed in the salmon louse midgut.

The distribution of copepodids on wild and farmed hosts shows that the preferred settlement site is on the fins and scaled body of the host (Bron et al., 1991). Some groups have reported the settlement of lice on gills as well, however this is considered rather uncommon (reviewed by Treasurer and Wadsworth (2004)). In laboratory trials, on the other hand, lice are often found on gills, although there still seems to be a higher preference for the fins and body (Bjørn and Finstad, 1998; Treasurer and Wadsworth, 2004). Copepodid gill settlement is therefore often considered an experimental artefact due to an altered host behavior during laboratory infestations (Treasurer and Wadsworth, 2004). Gill tissue in teleost fish is highly vascular, whereas skin epidermis is not. The chalimus frontal filament, appendages and mouth tube have been shown to not breach the basement membrane within the salmon skin (Jones et al., 1990), thus not reaching the dermal vascular layer. Salmon lice settling on gills might therefore be more prone to ingest a blood meal than those lice elsewhere on the host during early stages of attachment.

The genome of the Atlantic salmon louse is fully sequenced and high-throughput transcriptomics studies have been conducted under various experimental conditions using microarrays as well as sequencing. Examples of such experimental settings include host-

parasite interactions on different hosts (Braden et al., 2017), hosts fed different diets (Sutherland et al., 2017), response to drugs (Sutherland et al., 2014), larval stress response (Sutherland et al., 2012), parasite sex differences (Poley et al., 2016), and development (Eichner et al., 2008). Recently, we have used RNA-sequencing (RNA-seq) to investigate patterns of gene expression during molting in parasitic larval stages of *L. salmonis* (Eichner et al., 2018). Transcriptome plasticity in response to hematophagy has been investigated in various arthropods for which controlled blood-feeding is possible. Arthropod species subjected to such controlled feeding trials include mosquitos (*Aedes* species (Bonizzoni et al., 2011; Bottino-Rojas et al., 2015; Huang et al., 2015), *Anopheles gambiae* (Marinotti et al., 2005)), the biting midge *Culicoides sonorensis* (Nayduch et al., 2014), and ticks (*Ixodes* species (Kotsyfakis et al., 2015; Perner et al., 2016). However, investigating transcriptional changes induced by a blood meal within the salmon louse is challenging, as no protocol for feeding lice *in vitro* exists. To overcome this limitation, equally developed lice of the same batch, infecting the same fish, were sampled from host body attachment sites with predicted differing access to blood.

In this study, we infected Atlantic salmon with salmon louse copepodids and sampled the lice on the 10th and 18th day post infestation (dpi), when the lice were in the chalimus I and chalimus II stage respectively, or had recently molted to the preadult I stage. Parasite settlement site and visible presence of host blood in louse intestines were determined. Transcriptomes of equally developed lice sampled from different locations (gills and the body/fins), representing lice with access to blood versus lice without access at 10 and 18 dpi, were examined by RNA-sequencing. Specific aims of this study were to investigate i) visible blood ingestion from various sampling locations, ii) development of lice from locations differing in blood access, and iii) differences in gene expression of immobile lice from locations with unequal access to blood.

MATERIAL AND METHODS

Animals

Atlantic salmon lice (*L. salmonis salmonis*) (Skern-Mauritzen et al., 2014) were raised on Atlantic salmon in tanks with seawater (salinity 34.5‰ and temperature 10 °C) (Hamre et al., 2009). A laboratory strain of *L. salmonis* called LsGulen (Hamre et al., 2009) was used. Fish were daily handfed commercial dry pellets and maintained according to Norwegian animal welfare regulations. Experiments conducted herein were approved by the governmental Norwegian Animal Research Authority (ID7704, no 2010/245410). Fish were anesthetized by a mixture of methomidate (5 mg/l) and benzocaine (60 mg/l) prior to handling. For sampling of early developmental stages of lice, fish were killed by a swift blow to the head. Salmon louse egg string pairs were incubated and hatched in incubators in a seawater flow through system (Hamre et al., 2009). Emerging copepodids were used to infect fish in 500-liter tanks. Copepodids between 4 and 14 days post hatching were used. A total of 34 or 37 fish in the two experiments respectively were infected with about 70 copepodids per fish. The amount of copepodids used was estimated as described by Hamre et al. (2009). Prior to infestation, the tank water was lowered and copepodids spread on the surface.

Experiment termination and tissue sampling

At 10 and 18 dpi, fish were sacrificed and lice were removed with forceps and photographed. The gills were cut out and observed under a microscope. Any lice present were sampled, photographed and placed on RNAlater in individual tubes. Measurements of lice were done on photographs. Salmon louse developmental stages were determined by measuring the total length (TL) and cephalothorax length (CL) as earlier described (Eichner et al., 2018, 2015). For RNA isolation prior to RNA-seq, lice were sorted into groups of equal developmental status within each group as described by Eichner et al. (2018), and between groups from different

sampling locations (gills, skin). Five chalimus I or four chalimus II lice respectively were pooled together in one sample. Eight (Ex1, 10 dpi), six (Ex2, 10 dpi) or five (18 dpi) replicates were sampled per group (lice sampled from gills, lice sampled from skin). After RNA isolation, samples from both 10 dpi experiments and from one of the 18 dpi experiments were analyzed by RNA-seq.

RNA isolation and sequencing

RNA was isolated as described before (Eichner et al., 2014). In brief, pools of four or five chalimus larvae were homogenized in TRI reagent and mixed with chloroform (both Merck). The water phase was withdrawn and further used with the RNeasy micro kit (Qiagen) for RNA isolation according to the manufacturer's instructions. RNA was stored at -80 °C until being used. Library preparation and RNA-sequencing were conducted by the Norwegian Sequencing Centre, Oslo as previously described (Eichner et al., 2018). Briefly, sequencing libraries were prepared from 0.5 µg total RNA using the TruSeq stranded mRNA reagents (Illumina). Indexed libraries were blended into a single pool and sequenced during three runs of a NextSeq 500 instrument (Illumina) using 76 base pair (bp) single end reads. Image analysis and base calling were performed using Illumina's RTA software version 2.4.11, and data were converted to fastq format using bcl2fastq version 2.17.1.14. Raw sequencing data has been deposited in the NCBI database under BioProject ID PRJNA577842.

Data processing of RNA-seq

Obtained sequences were quality controlled by FastQC v0.11.5 (Andrews, 2016). Reports were summarized using MultiQC 1.0 (Ewels et al., 2016). As reference genomes, we used a combination of the Ensembl Metazoa reference assembly of the nuclear genome (LSalAtl2s, http://metazoa.ensembl.org/Lepeophtheirus_salmonis) and the mitochondrial genome RefSeq sequence NC_007215.1 (Tjensvoll et al., 2005). The gene models found in Ensembl Metazoa

were further augmented with gene models derived from full-length sequences of LsFer1 and LsFer4 obtained by rapid amplification of cDNA ends (RACE) (Heggland et al., 2019b), by aligning the RACE consensus sequences against the nuclear assembly with GMAP (Wu and Watanabe, 2005). RNA-seq reads were aligned against the reference using the STAR aligner (Dobin et al., 2013). Then, alignments were sorted and indexed using SAM-tools (Li et al., 2009) and saved in BAM format. Technical replicates were merged prior to counting using the merge function in SAM-tools. RNA-seq reads and their overlap with annotated nuclear and mitochondrial transcripts were counted using featureCounts (Liao et al., 2014), using settings for strand-specific and reverse stranded libraries.

Differential expression (DE) analysis was done with DESeq2 (Love et al., 2014) on raw counts using Galaxy (Giardine et al., 2005) under the Norwegian e-Infrastructure for Life Sciences (NeLS) platform (Tekle et al., 2018). Prior to DE analysis, all transcripts with less than four counts were removed. Venn diagrams were prepared using the BioVenn platform (http://www.biovenn.nl/) (Hulsen et al., 2008). Hierarchical clustering as well as GO annotation enrichment were performed in J-Express (Dysvik and Jonassen, 2001; Stavrum et al., 2008). GO terms were summarized in REVIGO (Supek et al., 2011).

Transcript annotation

Protein-coding transcripts were annotated by running NCBI-Blast+, BlastP version 2.6.0+ (Altschul et al., 1990; Camacho et al., 2009) of their corresponding predicted Ensembl protein sequences against the GenBank (NR) (https://www.ncbi.nlm.nih.gov/) and SwissProt (Bairoch and Apweiler, 2000) databases. GO terms (full terms and GOslim annotation) and protein families (Pfam) were automatically assigned by InterProScan 5 (Jones et al., 2014).

RESULTS

Distribution and characteristics of lice

Figure 1 depicts the distribution of different developmental stages and instar ages of lice on the host at 10 and 18 dpi. At 10 dpi, most lice are attached to the fins, but there is also a high proportion of lice on the body and gills. At 18 dpi, however, the highest proportion of lice is found on the body of the salmon. Different stages are distributed differently between sites. At 10 dpi, there are chalimus II larvae (mainly males) on the body and fins, but none on gills. Here, we rather find chalimus I (higher proportion of middle than old) larvae. At 18 dpi, there are preadult I females on the body and fins, but not on the gills. On gills, we rather find a higher proportion of old chalimus II females at 18 dpi. There is a higher proportion of preadult I male lice found on the body compared with the fins. On the fins, most of the preadult I lice are young females.

Upon termination of the two experiments at 18 dpi, all lice were removed from the salmon, and their settlement site, developmental stage, the presence of a frontal filament, and the visibility of an intestine filled with blood were assessed (Table 1). On the body and fins, most lice were at the preadult I stage, while on the gills, most lice were in the chalimus II stage. The majority of the preadult I lice were located on the host body, and the minority was located on the gills. The preadult I lice on the gills, however, were more often secured by their frontal filament. Of the preadult I lice still attached by the filament, only the ones on the gills had a blood-filled intestine (Fig. 2c). None of the lice on the fins had a blood-filled intestine, and on the host body, only the mobile lice had apparently fed on blood. Additionally, chalimus I (Fig. 2a) and chalimus II (Fig. 2b) larvae attached to the gills had fed on blood, whereas lice of the same ages on the fins and body had no visible blood in the intestine.

Effect of gill settlement on gene expression in chalimus larvae

In order to determine the effect of gill settlement on the gene expression in chalimus larvae, RNA-sequencing of pooled individuals was performed. All counts per million (CPM) values can be found in Supplementary Table S1. The overall gene expression of the individual samples in comparison with chalimus I and chalimus II larvae of different instar age (data taken from Eichner et al. 2018) is shown in a correspondence analysis (CA) plot (Fig. 4).

All lice from this study sampled at 10 dpi are clustering together with chalimus I larvae sampled directly before molting as well as molting ones (old, molt) from Eichner et al. (2018) and all lice sampled at 18 dpi from this study are clustering with chalimus II lice sampled directly before molting (Eichner et al., 2018). Lice sampled from gills and lice sampled from skin differed also slightly in their overall gene expression. Lice from Ex1 at 10 dpi cluster together with lice from the respective group (from gills or from skin) of Ex2, showing that the two experiments are composed of comparable batches of lice. DE analyses were performed for each experiment separately. MA plots as well as a principle component analysis (PCA) for each experiment are shown in Figure 5. A list of all genes with log2 fold change and false discovery rate (FDR) adjusted p-values (padj) for each experiment can be found in Supplementary Table S2. 5878 genes were differentially expressed in at least one of the experiments (Supplementary Table S3).

Most DE genes were found in Experiment 1, 10 dpi (2188 or 2015 for gill and skins samples respectively). In Experiment 2, 10 dpi only, 1112 or 1081 transcripts respectively were found, of which 79% or 68% respectively are overlapping with the ones found in Ex1. DE genes found at 18 dpi are less overlapping with DE genes found in Ex1 at 10 dpi. Only 43% or 32% respectively of the genes found here are overlapping with genes from the respective groups in Ex1, 10 dpi and 35% or 28% respectively are overlapping with Ex2, 10 dpi (Fig. 3). 616 genes are DE in all three experiments, of which 355 are elevated in lice samples from gills and 202

are elevated in lice samples from skin (Supplementary Table S6 and S7), while 59 are significant different, but regulation directions are differing between experiments (31 elevated in lice from gills at 10 dpi but lower at 18 dpi, 24 the other way around and 4 are differing between the two experiments sampled at day 10) (Supplementary Table S8). Transcripts solely regulated at either 10 dpi or 18 dpi are listed in Supplementary Table S4 and S5, respectively.

We found a high number (49) of genes annotated with Pfam domain PF00040: "Fibronectin type II domain" among the DE genes. Mostly, these are elevated in lice sampled from gills at 10 dpi. However, a smaller number of PF00040 is within under the DE genes which are elevated in lice sampled from skin than from gills. Additionally, the Pfam domain PF00089: "Trypsin", are mostly found in the group of lice sampled from gills.

The sizes and overlaps of gene sets that were DE in each experiment separated by expression pattern (elevated in lice sampled from gills or elevated in lice sampled from skin respectively) are depicted together with most representative GO terms in Figure 3 Venn diagrams. All significantly enriched GO terms are listed in Supplementary Table S9 where summarized GO annotations belonging to biological process are visualized in a TreeMap (REVIGO). "Peptidase activity" is an enriched GO term in lice sampled from gills across all groups (except the ones exclusively found in Ex2, 10 dpi), and in particular "serine type endopeptidase activity", whereas "serine-type endopeptidase inhibitor activity" is enriched in lice sampled from skin. Notably, "glycolysis" as well as "oxidoreductase activity" are GO terms highly enriched in genes elevated in lice sampled from gills. GO terms containing "phosphorylation" as well as "phosphatase" are found enriched in nearly all groups in genes elevated in lice sampled from skin (also here, the exception is the ones exclusively found in Ex2, 10 dpi).

Equal gene expression changes throughout all three experiments

To determine which genes may be important in relation to the blood meal in general, independent of the stage of the lice in the different experiments, we investigated the transcripts which were either significantly elevated in lice sampled from gills in all three experiments, or significantly elevated in lice sampled from skin. We found 355 transcripts elevated in all three experiments in lice from gills, and 202 from skin. Of the 355 genes elevated in lice sampled from gills, 60% had predicted Pfam domains, and of the 202 elevated in lice sampled from skin, 82% had predicted Pfam domains. A highly prevalent Pfam domain in the DE genes found in all 3 experiments elevated in lice sampled from gills is PF00089: Trypsin; 108-279. Other more frequent found domains are PF01400: Astacin (Peptidase family M12A), PF02469: Fasciclin domain, PF05649: Peptidase family M13, PF00171: Aldehyde dehydrogenase family, as well as different Zinc finger domains. In the group of DE genes which were elevated in lice sampled from skin, prevalent domains are PF00040: Fibronectin type II domain; PF00069: Protein kinase domain, PF00096: Zinc finger, PF00135: Carboxylesterase family, PF01391: Collagen triple helix repeat. A full list can be found in Supplementary Table S6 and S7. GOslim was used to minimize GO categories. DE genes in lice sampled from gills fall under fewer GOslim categories than DE genes elevated in lice from skin, even though there are more genes in former DE group (Fig. 6). Often, genes in the group found elevated in lice sampled from gills fall under the enriched GO group “catalytic activity” (all are shown in Fig. 6a). Remarkably strong enriched (factor 30) DE genes elevated in lice from skin, are genes belonging to “extracellular matrix”. However, only five genes are in that group. More than 30 genes were found in GO categories “catalytic activity”, “hydrolase activity”, “binding” and “ion binding” (Fig. 6b). All enriched GOslim terms, numbers of genes found in each category, and enrichment factor for the two groups are shown in Figure 6.

42% of the 355 genes elevated in lice sampled from gills have a fold-change more than two compared to skin. When also taking the p-value into account we find 21%, which are strongly regulated (average fold-change over two and average padj-value ≤ 0.005). In this group, the strongest elevated transcript is most similar to a nematode astacin (EMLSAT00000010457). Among the other strong ones regulated are three transcripts with FNII domains, another transcript with an astacin domain, as well as nine transcripts with trypsin domains, as well as a chemosensory protein (EMLSAT00000005105), yet also many transcripts with no annotation or known protein domains. There are several transcripts with similarity to various proteases that are up-regulated in lice from gills. All genes are listed in Supplementary Table S6.

In the group of genes elevated in lice sampled from skin, we find fewer genes highly differentially expressed than in lice sampled from gills. Only 16% have a fold-change of two or higher compared to samples from gills. Most up-regulated in this group is a transcript with no predicted annotation or Pfam domains (EMLSAT00000009920). Among the strongest elevated genes in all three experiments (average fold-change over two and p-value ≤ 0.005) in lice sampled from skin are several transcripts with predicted FNII domains. Moreover, we find several genes with no annotation or known protein domains. All genes are listed in Supplementary Table S7.

We further looked at the expression profile of these transcripts during the course of development, as well as in various tissues (Edvardsen et al., 2014; Eichner et al., 2018; http://licebase.org) . We were particularly interested in determining if these transcripts were also elevated in the louse intestine compared with other tissues, or if these transcripts are up- or down-regulated after attachment or after molting to preadult, the expected time point for accessing host blood. Of the 202 genes elevated in lice from the skin, 124 were also investigated in an oligo microarray regarding to expression in different tissues (gut adult female, gut adult

297 male, ovaries, testis, subcuticular tissue and brain) and from the 355 genes elevated in lice
298 sampled from gills 209 were represented in that study (Edvardsen et al., 2014).

299 Among transcripts elevated in lice sampled from gills, 94 (26%) are higher expressed in the
300 intestine (only 5% of transcripts were lowest in intestine compared with other tissues
301 investigated) (LiceBase). Moreover, 39 of these were very highly expressed in the intestine,
302 compared to other tissues (more than 100 times) (LiceBase). 77 of these elevated in intestine
303 were also analyzed in the microarray study investigating different tissues of *L. salmonis* and 52
304 were also found highest expressed in the intestine there (Edvardsen et al., 2014). 66% of the
305 transcripts are elevated after attachment and 55% are higher expressed in preadult lice than in
306 chalimus II when comparing to the time series data (Eichner et al., 2018).

307 Only 17 (8%) of the transcripts in the DE gene group elevated in lice sampled from skin are
308 also higher expressed in the intestine than in other investigated tissues (12% lowest of all tissues
309 investigated) (LiceBase). Only two were much higher (more than 100 times) expressed in
310 intestine than other tissues (LiceBase). However, the ten of these also found on the oligo
311 microarray were not highest expressed in the intestine, except one (EMLSAT00000008355)
312 (Edvardsen et al., 2014). Here two others were found highest expressed compared with the other
313 tissues analyzed. 57% of the transcripts are elevated after attachment. Nearly all of the strongest
314 regulated transcripts are elevated after attachment when comparing with LiceBase data. 53%
315 are higher expressed in preadult lice than chalimus II (Eichner et al., 2018).

316 ## DISCUSSION

317 In this study, we have investigated the biology of blood-feeding in the marine ectoparasitic
318 salmon louse with a special focus on gene expression of immobile lice situated on host gills.
319 We chose immobile lice, because this allowed us to focus on those individuals that had stayed
320 at one location at least since extruding the frontal filament in the late copepodid stage. Being

attached to the gills allowed the lice to initiate blood-feeding prior to becoming mobile. Two parallel experiments terminated at 10 days post infestation, and one experiment terminated at 18 days post infestation were included in the RNA-seq and subsequent gene expression analyses.

At 10 dpi, the amount of lice is rather evenly distributed between the investigated body parts (32 or 29% on body, 43 or 39% on fins and 24 or 30% on gills in Ex1 and Ex2 respectively). The favored site at 18 dpi is the body with 66 or 61% in Ex1 or Ex2 respectively (15 or 14% on fins and 17 or 23% on gills). Lice at day 10 are in chalimus I or chalimus II stage and attached by the frontal filament. At day 18, we found 75 or 74% preadult lice in Ex1 or Ex2 respectively. Of these, 10 or 21% respectively are attached with a filament, while the others are mobile and can freely move on the fish. The finding that lice are differently distributed when being mobile rather than attached, and mainly found on the body of the fish, suggests that the mobile preadult lice choose the general body surface as a preferred feeding site and migrate there from host fins and gills when becoming mobile. The majority (80-90%) of the preadult I lice on fins are females. Female lice are known to develop slower than males (Hamre et al., 2019, 2013), and this as well indicates that the lice tend to leave the fins for other host feeding areas when becoming mobile. A preadult I louse that is still attached to its host by its frontal filament has recently molted from the chalimus II stage, and has stayed at that feeding site since attachment. There are no preadult I lice with a visible blood-filled intestine on the fins, whereas we find this on the gills and the body. Interestingly, of the lice still on their filament, only those on gills have apparently fed on blood. Moreover, already in the chalimus I stage, we find lice with blood-filled guts on the gills, but not on any other feeding site. As the preadult I lice on the body with a blood-filled intestine are mobile, these lice have either started with blood-feeding in the mobile preadult I stage or are preadult lice migrated from the gills, meaning that blood-

feeding is initiated from the mobile preadult I stage and onwards in the development of the salmon louse occurring under natural conditions.

Development of lice on the gills was delayed, compared to development of lice on body or fins. At 10 dpi, no chalimus II lice were found and a higher proportion of chalimus I lice was of less developed instar age on gills. Comparing only the attached chalimus at 18 dpi (25% of all lice), 48 or 59% in Ex1 or 2 respectively are found on gills. In addition, on gills, there is a higher proportion of male chalimus II lice, which develop faster than females. Developing on host gills caused the salmon lice to develop slower than those developing on other locations. There have been contradicting results about this in the past (Johnson, 1993; Johnson and Albright, 1992), however here we have determined instar ages, and not only the developmental stages, which adds more confidence to our results.

We conclude that during the normal development on the body or the fins, the salmon louse does not start to feed on blood until reaching the mobile preadult I stage. By that reasoning, we wanted to compare gene expression of chalimus larvae located on the vascular gills with access to blood with that of chalimus larvae of equal development from the rest of the body. The salmon louse has approximately 13,000 protein encoding genes (http://metazoa.ensembl.org/Lepeophtheirus_salmonis), and we find in our RNA-seq analyses that over 5800 genes had an altered expression in at least one of our experiments. As expected, we found a higher amount of overlapping DE genes in the two experiments sampled at 10 dpi. These are chalimus I larvae, which are soon molting to chalimus II, while lice sampled at 18 dpi are chalimus II larvae, shortly prior to molting to preadult I lice. As such, all lice are sampled at a similar instar age. However, phenotype and lifestyle differ in preadult lice and one can expect expression of genes in preparation for this stage in the lice sampled at 18 dpi. The high amount of DE genes exclusively found in Ex1, 10 dpi could be caused by batch differences between Ex1 and Ex2, or could be as a result of more powerful statistics due to a higher amount

of parallel samples (8 vs 6 biological parallels of each group in Ex1 and Ex2, respectively). However, we know also that minor differences in development have a high impact on gene expression (Eichner et al., 2018), and individual differences occurring within groups, with possible consequences between groups, could bias the results.

To investigate gene expression caused by nutrition differences, we mainly concentrated on the DE genes found in all 3 experiments. Transcripts over-expressed in lice sampled from gills could be important for hematophagy. However, many (70 of 74) of the strongest DE genes in this group are not highest expressed in the intestine, but rather other tissues, suggesting these contribute to other functions in the louse that may be modified by hematophagy. Genes elevated in lice from gills show a more homogenous GO annotation (fewer GOslim categories) than the ones elevated in lice from skin, suggesting that several DE genes are involved in the same processes. There are also more genes with a greater fold change within the group of DE genes elevated in lice sampled from gills (42% over 2-fold change, whereas only 16% in lice sampled from skin), pointing towards a high demand of these gene products when feeding on blood. However, as GO terms can be unspecific or general, the following discussion deals with selected groups of transcripts.

Iron and heme

Among the regulated transcripts, the iron storage units of ferritin (*LsFer1* (RACE sequence) and *LsFer2*: EMLSAT00000006305) are both elevated in chalimus larvae sampled from gills compared with other settlement sites. We have previously established that these genes are important for the adult female salmon louse blood-feeding and reproductive success, as the parasite had a clear gut and failed to produce viable eggs upon silencing the two genes (Heggland et al., 2019b). Blood contains several iron-proteins, and when initiating blood-feeding, the salmon louse needs to obtain a way of storing and detoxifying iron absorbed from the blood. Up-regulating ferritin when ingesting a blood meal is therefore an important defense

mechanism for a blood-feeding parasite. The putative heme scavenger receptor, *LsHSCARB* (EMLSAT00000005382), is elevated in lice on gills at 18 dpi compared to lice on skin. We recently found that upon silencing *LsHSCARB* by RNA interference, adult female lice had absorbed less heme and produced fewer viable eggs and less offspring (Heggland et al., 2019a). Lacking early (10 dpi) transcriptional elevation of *LsHSCARB* could indicated alternative mechanisms of absorption during the earlier developmental stages, or the existence of a post-transcriptional mode of regulating the LsHSCARB protein. Alternatively, the lack of early regulation might serve to maintain homeostasis of heme levels when feeding on the vascular gills.

Detoxification

A Glutathione S-transferase (GST) (PF02798) transcript (EMLSAT00000009830) was elevated in lice on gills in all experiments. GSTs are major detoxification enzymes. A GST in the hard tick *Ixodes ricinus* (IrGST1) (GenBank ID: MF984398) was also found to be elevated in the midgut of blood-fed ticks compared with serum-fed ticks (Perner et al., 2016). Further characterization of IrGST1 showed that it was heme-inducible and the recombinant protein was able to bind heme *in vitro* (Perner et al., 2018). The authors speculated that IrGST1 is important for detoxifying excess heme to avoid cytotoxicity in the tick (Perner et al., 2018). Recombinant GSTX2 (GenBank ID: AAK64286.1) of *A. aegypti* also binds heme (Lumjuan et al., 2007), and was elevated in a heme-incubated *A. aegypti* Aag2 cell line (Bottino-Rojas et al., 2015). Of the six different predicted salmon louse proteins with the GST domain (PF02798), EMLSAP00000009830 is the most similar to both IrGST1 and *A. aegypti* GSTX2. The connection of GST and blood-feeding in the salmon louse is an interesting topic for future studies, as we per today do not know what mechanisms the salmon louse depends on to detoxify heme.

Digestion

Food protein hydrolysis is a fundamental step of digestion, and is mediated by peptidases that enzymatically cleave peptide bonds. Blood is highly enriched in protein, and one of the most abundant ones is the gas transporter hemoglobin. Investigating changes in the salmon louse transcriptome upon initiating blood-feeding could thus give clues as to which enzymes are essential for breakdown of blood components. Trypsin is a digestive enzyme belonging to the S1A subfamily of serine endopeptidases, and five main trypsin-encoding transcripts in the salmon louse intestine have previously been characterized (Johnson et al., 2002; Kvamme et al., 2004). Trypsins and other proteins involved in protein degradation were found elevated e.g. in blood-fed mosquito *A. aegypti* (Bonizzoni et al., 2011). Twenty-eight transcripts with trypsin as the only predicted protein domain (PFAM: PF00089) (29 in total with trypsin + other domains) were found to be elevated in lice on host gills at day 10 (Ex1 and 2) and 18 dpi. Of these, 11 are predicted to be highest expressed in the intestine compared with other tissues investigated in the salmon louse (LiceBase; Edvardsen et al., 2014; Supplementary Table S6). A heat map showing the expression patterns for all transcripts with trypsin domains found DE in all 3 experiments taken from LiceBase and from the time-series study are shown in a hierarchical cluster in Figure 7. *LsTryp1* (GenBank ID: AY294257, best blast hit: EMLSAT00000004828) is elevated in all three experiments in lice on gills. One transcript with a trypsin domain only (EMLSAT00000004988) is found to be elevated in lice on host skin at both 10 dpi (Ex1 and Ex2) and at 18 dpi. RNA-seq data in LiceBase however show that this transcript has a low expression in the louse intestine and is rather expressed in antenna and feet. It might therefore be of importance for other purposes than blood meal digestion.

Peptidases other than trypsins are also regulated in lice on host gills. There are 17 transcripts with Pfam domains “peptidase” other than trypsins elevated in lice on gills in all experiments. Among these are four transcripts with Astacin-domains (Peptidase family M12A) and five are

M13 peptidases (Fig. 7). Both groups are metallopeptidases, and are enriched in arthropods. Astacin-like metallopeptidases are implicated in digestive processes, but are also reported to have anticoagulative effects, as they are found to have fibrinogenolytic activity in spider venoms (Trevisan-Silva et al., 2010). M13 metallopeptidases are widely distributed in animals, and e.g. make up the major group of the hematophagous tick degradome (Mulenga and Erikson, 2011). Furthermore, we also find many of the same types of peptidases elevated rather in lice from skin (one with Astacin domain, two with Peptidase family M13 domain). This could indicate different modes of digesting a blood meal versus digesting components of ingested salmon skin. Further investigation into the elevated trypsins and other peptidases expressed in the salmon louse gut should be conducted.

Putative anti-coagulation

Blood coagulation is a key mechanism in maintaining homeostasis in vertebrates if a blood vessel were to rupture. A parasite feeding on vertebrate blood would therefore require mechanisms in order to counteract this to maintain its feeding habit. Anti-coagulation factors targeting host proteins could thus be vital for the successful blood-feeding in the parasitizing arthropod. A thrombin (coagulation factor) inhibitor, hemalin, was found to be important to avoid clotting of the blood meal in the bush tick *Haemaphysalis longicornis* (Liao et al., 2009). A salmon louse transcript (EMLSAT00000003009) encoding two Kunitz/Bovine pancreatic trypsin inhibitor domains (PF00014), as also found in the tick hemalin, was elevated here in all experiments in lice on gills. However, four other transcripts with the same domain were elevated in lice from skin in all three experiments (EMLSAT00000000152, EMLSAT00000007907, EMLSAT00000008877 and EMLSAT00000009255).

We also find serine protease inhibitors (serpins, PF00079) regulated. From the 15 predicted serpin transcripts in the louse, four were DE in all three experiments. Two were elevated in lice on gills (EMLSAT00000010931 and EMLSAT00000001743), one elevated in lice on skin

(EMLSAT00000011353), while the last (EMLSAT00000005224) was expressed lower at 10 dpi, but elevated at 18 dpi in the lice sampled from gills. One transcript (EMLSAT00000000552) was elevated in lice on gills at 10 dpi (Ex1 and Ex2). Anti-coagulation factors could be targets for pest control as they are likely secreted and in contact with the host, and thus probably vital for the host-parasite interaction.

Fibronectin type II

The approximately 60 amino acid long fibronectin type II (FNII) domain (PF00040) is a protein domain found within the glycoprotein fibronectin. It contains four conserved cysteine residues that form disulfide bridges. These residues are important for e.g. fibronectin's collagen binding properties (Guidry et al., 1990). The FNII domain is also found within the vertebrate blood coagulation protein Factor XII (McMullen and Fujikawa, 1985). The FNII domain is the most expanded protein domain of the salmon louse with over 200 copies within over 80 genes identified so far (http://metazoa.ensembl.org/Lepeophtheirus_salmonis). Some of these genes (*LsFNII1*, *2* and *3*) have been characterized, and are expressed in tegumental type 1 (teg1) glands of the salmon louse (Harasimczuk et al., 2018; Øvergård et al., 2016). Teg1 glands are exocrine and their secretory ducts are extending to the dorsal and ventral side of the salmon louse (Øvergård et al., 2016). The functions of FNII-containing proteins have not been determined in the salmon louse, however it has been suggested that proteins with the domain may be of importance for lubricating the integument and functioning as an anti-fouling agent, or as part of the salmon louse fuzzy coat (acid mucopolysaccharide layer (Bron et al., 2000)) (Harasimczuk et al., 2018). Genes with FNII domains expressed in teg1 glands have also been suggested to be of importance for host immune modulation by the parasite (Øvergård et al., 2016).

We find that several FNII-containing genes are significantly regulated in chalimus larvae in our dataset. Five transcripts with predicted FNII domains are elevated in all three experiments in

lice on gills. Of these, all but one (EMLSAT00000011958) are predicted to be up-regulated after louse attachment (http://licebase.org; Supplementary Table S6) (Fig. 7). Seven transcripts with FNII are rather found elevated in lice on skin in all three experiments. Here as well, all but one transcript (EMLSAT00000006178) are up-regulated after louse attachment (http://licebase.org; Supplementary Table S7). The FNII encoding genes characterized by Øvergård et al. (2016) and Harasimczuk et al. (2018) are not among the transcripts regulated in all experiments here (*LsFNII1*: EMLSAT00000012082, *LsFNII2*: EMLSAT00000007294, *LsFNII3*: EMLSAT00000009744), however LsFNII1 was elevated in both experiments at 10 dpi, and LsFNII3 at 18 dpi as well as in Ex1 10 dpi. The transcripts elevated in lice on gills should be further characterized, in order to elucidate a possible role of FNII in blood-feeding. Given the earlier reports that FNII domains in vertebrates may be important for blood clotting, one hypothesis is that proteins with FNII domains only could have an anti-coagulant effect.

CONCLUSIONS

Blood is a major dietary component for the ectoparasitic salmon louse, which the parasite has access to when attached to a salmonid host. We find that the salmon louse initiates blood-feeding during the mobile preadult I stage. However, if the parasite is attached to host gills, it may start feeding on blood already at the chalimus I stage. The premature onset of blood-feeding caused lice on gills to develop at a slower pace than lice that were attached to host fins and general body surfaces. Chalimus lice of equivalent age on gills versus other attachment sites were therefore analyzed for gene expression comparisons. Several genes are elevated in lice attached to the gills, and among these, we find e.g. genes of importance for the absorption, storage and/or transportation of the pro-oxidative molecules iron and heme, digestive and detoxification enzymes, genes that could be important for anti-clotting of host blood, and

several genes with FNII domains. The results of this study raise a number of new gene targets to investigate further in order to elucidate the blood-feeding habit of the infamous salmon louse.

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AVAILABILITY OF DATA

The datasets supporting the conclusions of this article are included within the article and its additional files. Raw RNA-sequencing data files have been deposited to NCBI BioProject under the accession number PRJNA577842. A preprint of this manuscript and its supplementary files have been made publicly available on bioRxiv: https://doi.org/10.1101/815316.

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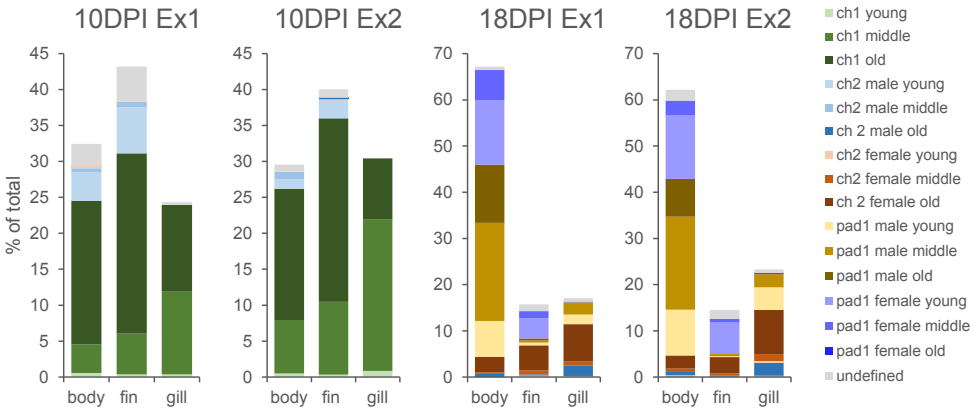
714 FIGURES AND TABLES

715 **Table 1.** Number and characteristic of preadult I lice sampled from different parts of the fish at
716 18 days post infestation. Ex1: 34 fish (average 43 lice per fish), Ex2: 37 fish (average 24 lice
717 per fish). Pad1 = preadult I, ch2 = chalimus II.

	Experiment 1			Experiment 2		
	body	fin	gills	body	fin	gills
% pad1 of total (pad1 + ch2)	93	50	29	92	62	35
% female of pad1	33	80	5	31	90	1
# pad1 found	228	40	27	206	55	62
# pad1 on filament	12	10	7	12	21	36
# pad1 with visible blood	30	0	18	27	0	53
# pad1 with visible blood on filament	0	0	2	0	0	30
% pad1 on filament	5	25	74	6	38	58
% pad1 with visible blood	13	0	67	13	0	85
% pad1 with visible blood on filament	0	0	11	0	0	57

718



719

720 **Figure 1.** Distribution of different stages and instar ages of lice on fish at 10 days post
721 infestation and at 18 days post infestation sampled from the fish body, fins and gills
722 respectively. Lice instar ages were defined on photographs as described in the main text.

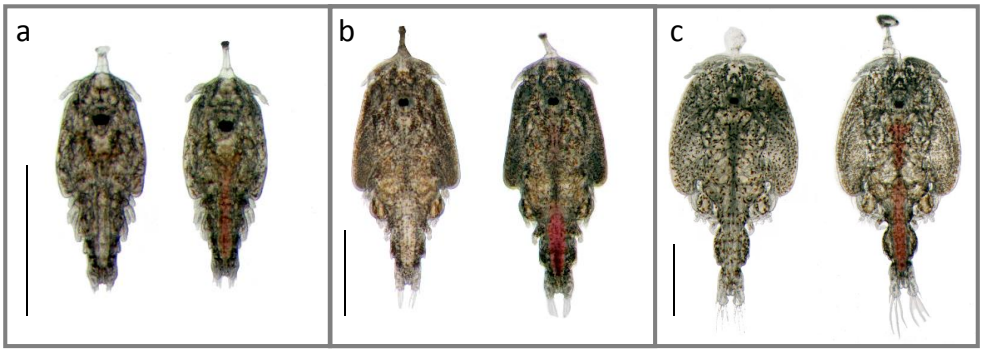
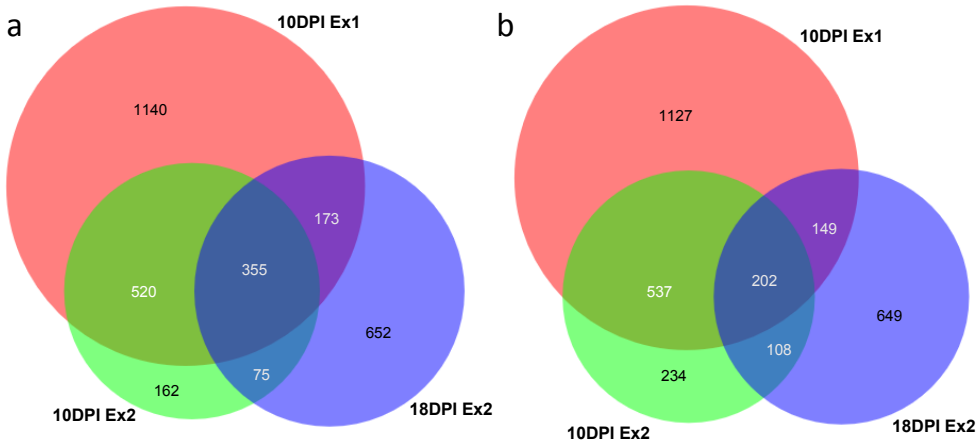
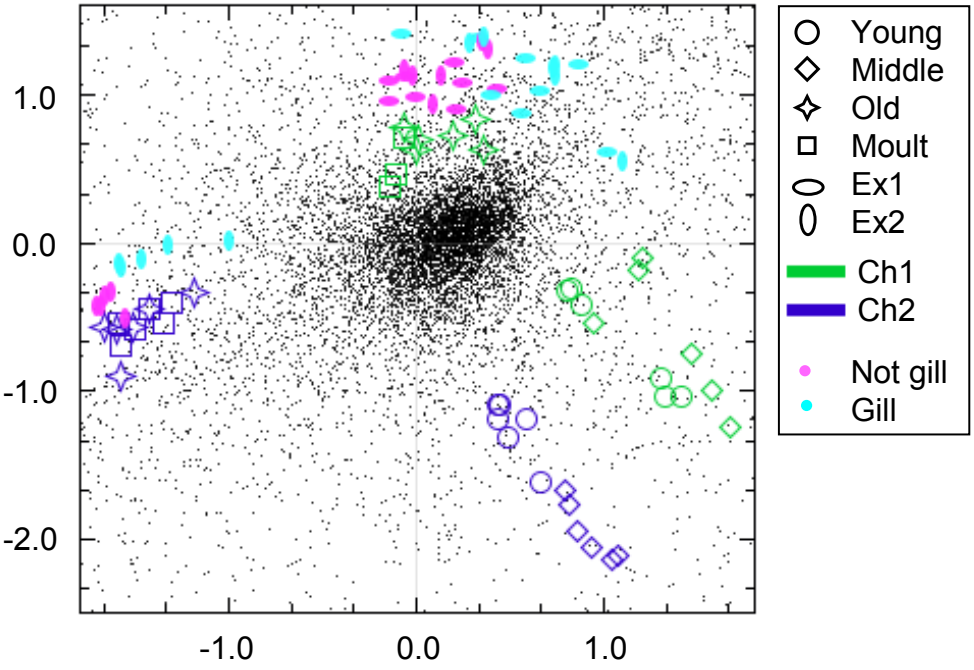


Figure 2. Photographs of salmon lice with (right) and without (left) a blood-filled intestine. Chalimus I larvae sampled 10 days post infestation (a). Chalimus II larvae sampled 18 days post infestation (b). Preadult I lice on frontal filament sampled 18 days post infestation (c). The lice with blood-filled guts were sampled from the gills, and the others were sampled from the fins of their host. Scale bars = 1 mm.



Experiment	Higher in lice sampled from gills	Higher in lice sampled from skin
all 3 Ex	protein metabolism glycolytic process	protein phosphorylation
10DPI Ex1 and 10DPI Ex2	protein metabolism carbohydrate metabolism	phosphorus metabolic process
10DPI Ex1 and 18DPI Ex2	protein metabolism carbohydrate metabolism	cellular response to chemical stimulus
10DPI Ex2 and 18DPI Ex2	protein metabolism carbohydrate metabolism	protein phosphorylation response to stress
10DPI ex1 only	translation chromosome organization	transport regulation of intracellular signal transduction
10DPI ex2 only	second-messenger-mediated signaling	chitin metabolism regulation of cellular component organization
18DPI ex2 only	transport carbohydrate metabolism protein metabolism localization response to stress	transport dephosphorylation regulation of cellular process biological regulation

Figure 3. Venn diagram showing the number of differentially expressed genes between chalimus larvae sampled from gills versus those sampled from skin, found in three different experiments, at 10 or 18 days post infestation. Representative GO terms for each group are given in the table in the bottom panel. The numbers of genes elevated in chalimus larvae sampled from gills are shown in (a) and the ones elevated in chalimus larvae sampled from fish skin are shown in (b). A full list of enriched GO terms are shown in Supplementary Table S9.

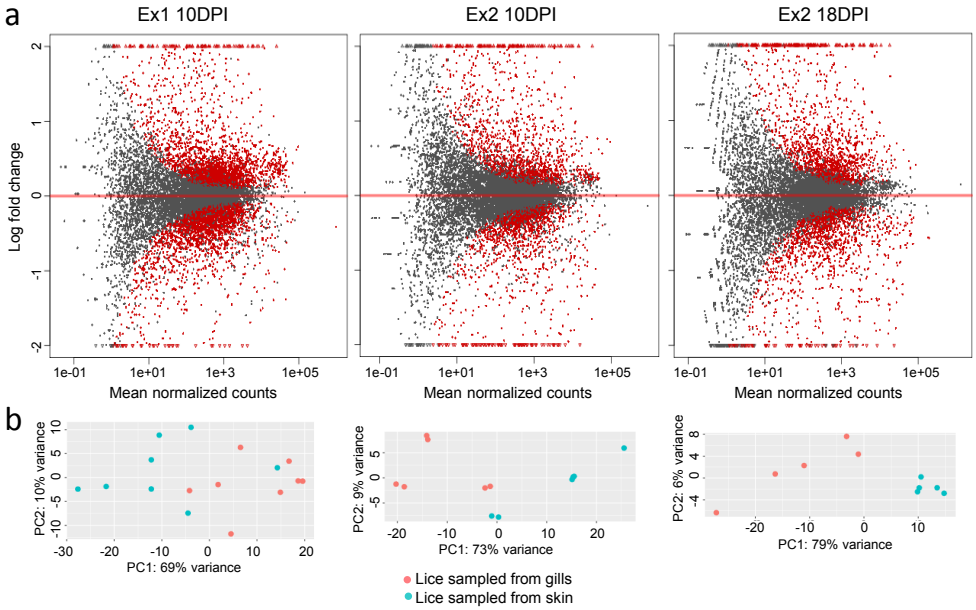


736

737 **Figure 4.** Correspondence analysis (CA) plot of the samples analyzed (pink and turquoise dots)

738 in comparison with other chalimus larvae divided into various instar ages taken from Eichner

739 et al. (2018). Ch1 = chalimus I, Ch2 = chalimus II, Ex = experiment.



740

741 **Figure 5** a) MA plot comparing the different conditions (lice sampled from gills vs lice sampled
742 from skin at 10 days post infestation (DPI) and 18 DPI respectively) in the three experiments:
743 Experiment (Ex) 1, sampled at 10DPI, Ex2 sampled at 10DPI and Ex2 sampled at 18DPI. The
744 average log intensity for a dot in the plot (A) is shown on the x-axis and the binary logarithm
745 of the intensity ratio (M) is shown on the y-axis. b) Principle component analysis (PCA) plots
746 of the same data.

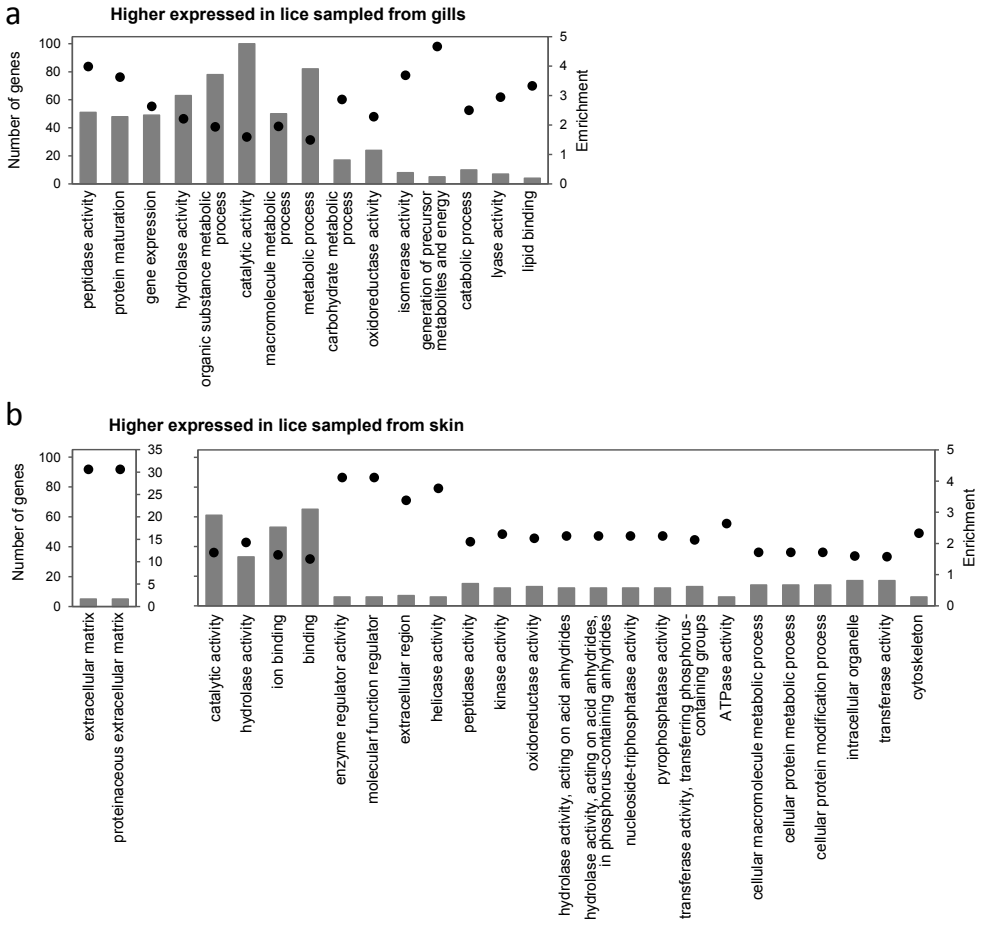
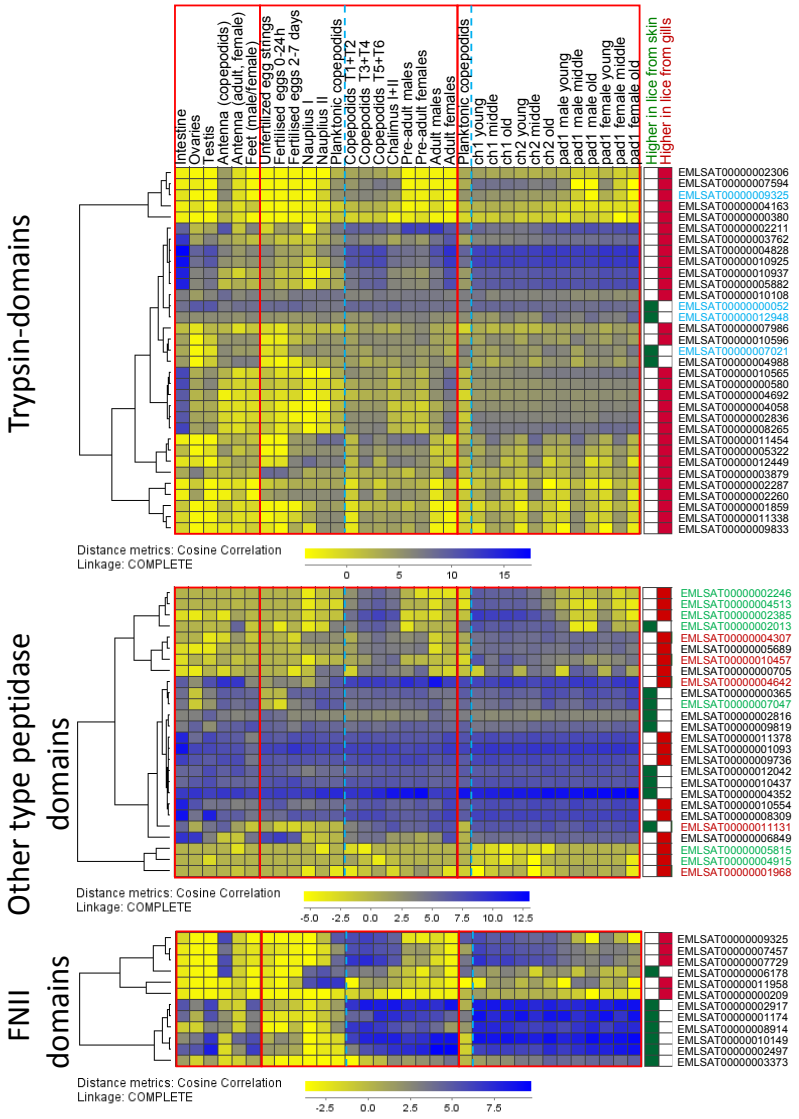


Figure 6. GOslim annotations of genes elevated in lice sampled from gills (a) and genes elevated in lice sampled from skin (b) in all three experiments. Columns indicate the number of genes in each GOslim category, while black dots show the enrichment of genes in that category in relation to number of genes of the specific category in the whole dataset.



752

753 **Figure 7.** Hierarchical clustering of the expression profiles of genes found DE in all three
754 experiments with trypsin, other peptidase and FNII domains after expression profiles from
755 LiceBase (various tissues and stages) and from the time series study (average values of
756 biological parallels) by Eichner et al. (2018). A blue stippled line is separating planktonic and
757 parasitic stages. Stable IDs with font in blue = domains other than trypsin predicted as well,
758 green = predicted M13 peptidases, red = predicted astacin peptidases.

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