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Salmon immunological defense and interplay with the modulatory capabilities of its ectoparasite *Lepeophtheirus salmonis*

Running title: *Immunomodulation by the salmon louse*

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Abstract

The salmon louse *Lepeophtheirus salmonis* (*Lsal*) is an ectoparasitic copepod that exerts immunomodulatory and physiological effects on its host Atlantic salmon. Over 30 years of research on louse biology, control, host responses and the host-parasite relationship has provided a plethora of information on the intricacies of host resistance and parasite adaptation. Atlantic salmon exhibit temporal and spatial impairment of the immune system and wound healing ability during infection. This immunosuppression may render Atlantic salmon less tolerant to stress and other confounders associated with current management strategies. Contrasting susceptibility of salmonid hosts exists and early pro-inflammatory Th1 type responses are associated with resistance. Rapid cellular responses to larvae appear to tip the balance of the host-parasite relationship in favour of the host, preventing severe immune-physiological impacts of the more invasive adults. Immunological, transcriptomic, genomic and proteomic evidence suggests pathological impacts occur in susceptible hosts through modulation of host immunity and physiology via pharmacologically active molecules. Co-evolutionary and farming selection pressures may have incurred preference of Atlantic salmon as a host for *Lsal* reflected in their interactome. Here we review host-parasite interactions at the primary attachment/feeding site, and the complex life-stage dependent molecular mechanisms employed to subvert host physiology and immune responses.

Keywords: Salmon lice, immunomodulation, virulence, aquaculture, Atlantic salmon, host-parasite relationship

Introduction

Commercial production of Atlantic salmon continues to expand in order to supply increasing consumer demands in emerging markets of the global aquaculture industry. Growth is estimated to exceed 8% per annum (1). However, intensive farming is associated with challenges, including bacterial, viral, fungal and parasitic diseases. Of these, ectoparasitic salmon lice *Lepeophtheirus salmonis* (*Lsal*) are the chief disease constraint to Atlantic salmon aquaculture sustainability in the Northern hemisphere, costing the industry an estimated >$874M US (£700M) per annum to control (2). Full costs to the industry in terms of productivity are considerably higher due to impaired growth, increased feed input, and downgrading of harvested fish (3). Furthermore, indirect costs such as coinfections with viral and bacterial pathogens and environmental impact of lice and treatments contribute signficantly to negative consumer perception of the industry (4).

It is well reported from both field and lab-based studies that salmon lice prevalence and intensity vary significantly between salmonid species. Atlantic salmon (*Salmo salar* L.) exhibits a highly susceptible phenotype (5,6). The mechanisms governing these differences and the adverse impacts incurred to susceptible hosts such as *S. salar* has received increasing attention over the last 30 years. Histopathological comparisons of localised infections in salmonids (7–9) inferred weaker inflammation and tissue proliferation were characteristics of Atlantic salmon susceptibility. Contrasting data on salmonid species-specific immunological and mucosal responsiveness to *Lsal* (10–14) suggested some association of these responses with louse-induced modulation. The evolution of molecular 'omics' analytical tools has enabled further in-depth understanding of the immunological (reviewed in (15)) and physiological changes previously described (reviewed in (16)) that occur during the course of infection. Filling knowledge gaps on parasitic mechanisms impairing the host health is important for understanding the impacts of implementing appropriate integrated pest management control strategies to reduce *Lsal* infections whilst maintaining host welfare.

Thus, the purpose of the present review was to summarize the current body of literature pertaining to the host-parasite relationship of *Lsal* and Atlantic salmon, taking note of potential complications of lice management, with a particular focus on the molecular interactions involved in successful parasitism by subverting host immunity and physiology. The co-evolutionary pressure for *Lsal* infection on *S. salar* is discussed in this context.

Infection and Host Response

Lepeophtheirus salmonis (Krøyer, 1837) is an ectoparastic copepod of the family Caligidae that infects the skin of salmonid fish (Figure 1A). The direct life cycle of *Lsal* includes five larval stages: two planktonic naupliar stages, one copepodite stage, and two chalimus stages (17,18). The infectious free-swimming copepodite uses mechanical, chemical, and visual cues for attaching to a host (19,20). Ionotropic receptors on the first antenna enables settlement on a suitable salmonid host facilitated by chemotaxis to host semiochemical cues (21–24). There are three post-larval stages: two sexed pre-adult stages and one sexed male and female adult stage (Figure 1). The primary attachment of the copepodite to the salmon is achieved by embedding modified secondary antennae into the skin epithelium. Secondary attachment follows by the formation of a frontal filament and a moult to the first chalimus stage (Chalimus I) (25,26). The filament is used to anchor the louse to the host; however, despite the penetration in host epidermis there is rarely evidence of a host response at this primary attachment site (8,26,27)(Figure 1B), with inflammation and epithelial hyperplasia restricted to the periphery of the attachment/feeding site in Atlantic salmon (7,8,15). For a broad description of the biology and lifecycle of *Lsal* the reader is referred to several comprehensive reviews (16,28,29).

During the permanently attached chalimus stages (Chalimus I and II) the parasite grows between moults (18), but only feeds within the vicinity of the attachment site prior to moulting into a mobile pre-adult. Thus, erosion inflicted by the sessile chalimus stages to the fish epidermis is far more limited (sometimes characterised by localised melanised spots (5)) than later mobile stages, and juvenile parasites are less commonly found to have blood in their gut contents, feeding predominantly on mucus and skin epithelium (30,31). In contrast, mobile pre-adult and adult stages have much greater haemotophageous feeding activity whereby the gut is often filled with blood (Figure 1A) and this stage of parasitism is associated with significant mechanical induced epithelial damage associated with grazing and intense inflammation. High pre-adult and adult lice burdens often result in degradation of the skin to underlying tissues resulting in bleeding wounds (3,9). The greater physical impact associated with these much larger aggressive feeding stages results in physiological disruption of Atlantic salmon through increased plasma cortisol, elevated plasma chloride, increased haematocrit and electrolyte levels and altered osmoregulatory capacity (5,6,32).

Behavioural responses exhibited by Atlantic salmon during experimental infection include significant flashing and jumping immediately following exposure to larval *Lsal* (5). An initial inflammatory response occurs upon attachment of copepodite/first chalimus (e.g. within 3 dpi) characterised by transcriptional pro-inflammatory mediator activity (e.g., *interleukin (il)1ß, il1R, il12, tumour necrosis factor α, prostaglandin* E_2) and T cell activity (*cd4/cd8*) (33,34). A biphasic immune response is associated with the moult from copepodite to developing chalimus whereby many immune genes are downregulated from 1-5 days post-attachment (infection; (dpi)) then subsequently upregulated to near control levels by day 10 dpi (35,36). The transition to chalimus is accompanied by transcriptomic changes in the host response (15,33–35). However, this response is followed by the down-regulation of genes throughout chalimus development, the degree of which is influenced by infection intensity (37), before a second innate inflammatory spike occurs (33,34). Although susceptible fish are relatively unresponsive histologically to attached chalimus stages (i.e. at the attachment point and feeding site by the mouth tube (Figure 1B)), epithelial cell migration, fibrosis and macrophage infiltration has been reported in Atlantic salmon associated with remnants of a frontal filament in the absence of a live chalimus (7).

It has been suggested that T_h1 and T_h17 type responses (e.g. IFN, CD8α, IL-17) could play a role in resistance of salmonids during these early (chalimus) infection stages as the expression of transcripts associated with these pathways have been shown in multiple studies to negatively correlate with larval louse burden in Atlantic salmon (36,37). An era of genomic and transcriptomic research on selective breeding for more resistant Atlantic salmon families has provided vital information on potentially protective immune responses to juvenile sea lice stages using transcriptomic profiling (36–40). For example, differences in susceptibility have been reported between strains and families of Atlantic salmon, including between wild and farmed populations (41), initiating selective breeding programs for these resistant traits. To date, only moderate genetic variation has been detected in resistance to *Lsal* in Atlantic salmon (42,43). However, by analysing quantitative trait loci (QTL) in Atlantic salmon 'less susceptible' to another sea louse species, *Caligus rogercresseyi* (*Crog*), 7 and 13% heritability was accounted for by three QTLs (39). One of the genes *transducer of erbB-2 1* (*tob1*) located on chromosome 3 of Atlantic salmon is a transcription factor that negatively regulates cell proliferation, specifically T lymphocytes (44), and exhibits significantly lower expression in skin

with attached chalimus (39). Differential expression of many T lymphocyte-associated genes during *Lsal* infection supports the role of these cells in the host response, as reported in a number of studies (34,35,37,45–47). Interestingly, overexpression of *tob1* has been described during the response of very small juvenile pink salmon prior to achieving natural resistance (0.3 g; ((48)), further supporting the role of T lymphocytes in the host response to *Lsal*.

The moult to pre-adult is accompanied by decreased systematic monocyte/macrophage activity, such as reduced respiratory burst and phagocytosis (49,50). Coincidentally, there is significant overexpression of localized innate inflammatory mediators (e.g., *il1β, il8, tnfα*; (46,51)), prostaglandin synthetases, and metalloproteinases (*mmp9* and *mmp13*), resulting in chronic wounds linked with minimal transcript activity associated with cellular proliferation (34,46,52). Expression of matrix metalloproteinases are controlled by specific signaling pathways including pro-inflammatory cytokines and are largely responsible for the degradation and remodelling of extracellular matrix (ECM) components (53), and thus play a vital role in wound healing (54). However, dysregulation of MMPs contributes to chronic wounds, a hallmark feature of susceptibility to *Lsal* (12,33,45,46,51). Regulatory T_h2 type responses could potentially be associated with protection *vis a vis* increased production of immunoglobulins and TGF-β-mediated tissue repair (36,46). However, these responses (i.e. *arginase-1*, *il10*, *tgfβ,* and alternative macrophage activation) appear delayed in Atlantic salmon (46), and are not associated with reduced lice numbers (34,52). Although antigen presentation does not appear highly activated (i.e., MH class II^+ cells) in response to *Lsal* (36,46), immunoglobulin transcripts (*igm* and *igt*) are significantly upregulated in both skin and spleen of early stage infected Atlantic salmon (34,35). Furthermore, greater levels of immunoglobulin mRNA have been demonstrated by at least one study to be associated with typical areas of skin at salmon lice attachment sites (36). Serum antibodies (IgM) from *Lsal*-infected salmon recognise *Lsal* antigen, but their protective capacity has not been demonstrated as evidence of adaptive immunity to *Lsal* is lacking (15,33,55,56).

Management and Intervention

An inability to make substantive gains in host resistance to lice through genetic selection, coupled with minor advances in ectoparasite vaccinology (42,43,57–60), and significantly lower relative

protection afforded by anti-louse functional feeds (61), has left the salmon farming industry with chemotherapeutic intervention as the main option for louse control. Decreases in lice abundance in the mid-90's were attributed to the use of azamethiphos in Canada (62), whereas use of organophosphates in Norway, dating back to the late 1970's, was associated with reduced efficacy leading to its termination by 1999. In 2000, emamectin benzoate sold under the trade name SLICE® as an in-feed treatment became available to farmers and was used extensively in the North Atlantic. Lees *et al.* (63) was the first to report reduced efficacy of SLICE® treatments in Scotland, showing longer times to achieve efficacy and post-treatment counts increasing from 2003 to 2006. Treatment failures were observed in Atlantic Canada and Norway in 2008 (64). Despite increasing reports of resistance of lice to current chemotherapeutants (64), the use of medicinal treatments has increased in every country with the exception of Norway (4,65).

With the development of resistance to available chemotherapeutants, an integrated pest management strategy has been introduced that involves a number of pharmaceutical, mechanical and biological control methods (reviewed in (2,66)). For example, prevention strategies focus on keeping the infective copepodites away from host fish (67), either by attracting the fish to depths using lights or underwater feeding (68), by physical obstruction at the top part of the cage (e.g., snorkel cages; (69)) or by keeping copepodites out of the cage using plankton skirts (70). Removal of existing lice infections can be accomplished by the use of cleaner fish (lumpfish and wrasse) that feed mainly on the larger stages of lice from the surface of the fish (71). The use of these methods is relatively recent and under continuous development to improve efficacy and reduce negative impacts on the host (72). For example, cohabitating fish inevitably enhances sources of viral and bacterial disease transmission, some of which may be notifiable (73), potentially threatening already immunocompromised hosts (74). Furthermore, treatment-induced stress may result in an enhanced susceptibility to *Lsal* infections, which further impairs immune-responsiveness to the parasite (33,52). Prolonged exposures to such stressors may result in a prolonged state of allostasis (i.e. physiological adaptation to stress leading to the release of catecholamines and glucocorticoids in an attempt to restore homeostasis), which in *S. salar* impairs leukocyte activity to bacterial antigen (16). This might ultimately render treated salmon more susceptible to microbial infections and less responsive to vaccination (75). Finally, there is a concern that the development of resistance to medicinal treatments will negatively

impact both farmed and wild fish stocks (64). This has become apparent even for environmentally sound treatments with reduced effectiveness of H_2O_2 (76), high tolerance of sea lice to freshwater treatment (77,78), and selecting for increased virulence through management practices (79).

Host Specificity

The salmon louse principally infects anadromous salmonids of the genera *Salmo*, *Oncorhynchus*, and *Salvelinus* (8,29,80); however, among juvenile members of these genera, there is a spectrum of susceptibility whereby certain species (e.g., Atlantic salmon) are more prone to the pathological effects of infestation such as epithelial degradation, tissue necrosis, altered mucosal biochemistry, enhanced susceptibility to secondary infections, anaemia, elevated plasma cortisol, osmoregulatory failure, and sometimes death ((5,16,49,81,82); Figure 2**)**. In contrast, juveniles of other species such as pink salmon (*O. gorbuscha*) are protected from these negative outcomes due to an enhanced ability to reject the parasite. For example, laboratory experiments exposing pink salmon to *Lsal* has shown that at approximately 0.7 g, juveniles develop the ability to rapidly reject the parasite, despite infection pressures exceeding 75 copepodites per fish (48,83), and this natural resistance occurs despite inadequate nutrition (84). Similarly, juvenile coho salmon (*O. kisutch*) are able to reduce parasite load from ~ 40 to < 1 lice per fish after only 18 dpi (L. Braden, *pers obs*; (27)). Inflammatory cell infiltration and hyperplastic tissue encapsulation of the copepodite has been described during the process of parasite elimination (8,86). In common garden experimental challenges, Sutherland *et al.* observed preferential infection of chum and Atlantic salmon over pink salmon (45). In contrast, experimental challenges comparing host responses in sockeye salmon (*O. nerka*) and Atlantic salmon indicate that sockeye are much more susceptible to infection and pathology associated with infection of *Lsal* (85). Chalimus survival after experimental exposure was higher on juvenile chum salmon (*O. keta*) compared to pink salmon (86), while comparative studies between Atlantic salmon and sea trout (*Salmo trutta*) indicate that the latter was more susceptible (87). Moreover, although Atlantic salmon are highly susceptible to infection, intraspecific heterogeneity in susceptibility occurs among distinct spawning stocks (41), and within full-sib families (59,88). Interestingly, fitness of the parasite appears to negatively correlate with resistance status, as development time from copepodite to pre-adult is shorter while parasitizing Atlantic salmon compared to rainbow trout or coho salmon (49). Although

these variable susceptibilities are observed experimentally among juvenile salmonids (i.e., single infection), it is important to consider that these might not fully encompass infection dynamics in the field (i.e., continuous exposure) or life history and energetic demands of the host. For example, migrating pink salmon are observed to harbour large numbers of parasites in the open ocean (89) and laboratory experiments have revealed a divergent immune response to *Lsal* in sexually maturing adults compared to juveniles with several biomarkers of resistance (e.g., the number of MHIIβ$^+$ cells) downregulated in adults (90). In contrast, coho salmon routinely exhibit the lowest prevalence of *Lsal* in field assessments (91) suggesting that resistance is maintained throughout development. Notwithstanding, the lack of an inflammatory response to anchored *Lsal* chalimii in certain salmonids is strongly correlated with susceptibility (8), and this has been shown to be a product of louse-induced immunosuppression (12).

Host Defense Mechanisms

Enhanced resistance to infection has been correlated with rapid and robust inflammatory and acute phase immune responses in skin that are paired with a regulatory T_h2-type response, infiltration of antigen presentation cells (MHIIβ$^+$ cells) and wound repair, whereas these tend to be delayed or weakened in more susceptible species (45,46,48,51,83,86,92). Iron metabolic pathways are also affected by *Lsal* infection in a species-dependent manner along with blood haematocrit and anaemia. For example, transcriptomic data on pink salmon indicates that an element of nutritional immunity (i.e. sequestration of nutrients from pathogens during infection) over chum salmon and Atlantic salmon may be associated with greater resistance of the former (45). Experiments with primary cell preparations has demonstrated a general immunomodulatory effect of *Lsal* excretory/secretory (ES) products and this appears to correlate with resistance status. For example, Lewis *et al.* exposed primary cells from salmon anterior kidneys to concentrated *Lsal* ES proteins and found that macrophages isolated from pink salmon possessed the highest phagocytic and respiratory burst activities towards *Aeromonas salmonicida* spp. *salmonicida* cells compared to either chum (*O. keta*) or Atlantic salmon (93). In contrast, macrophages isolated from *Lsal*-infected rainbow trout (*O. mykiss*) and Atlantic salmon have displayed reduced phagocytic ability and respiratory burst (49,50). Furthermore, Fast *et al.* measured a demonstrable decrease in expression of proinflammatory cytokine

il1β in Atlantic salmon head-kidney macrophages and SHK-1 cells after exposure to fractionated ES products (12).

Research indicates that the weakened host defenses described in susceptible species may be a response to parasite secretions produced during feeding. For example, skin mucus from coho salmon does not stimulate the same magnitude of ES product release from *L. salmonis* as compared to Atlantic salmon (94). This observation was corroborated more recently with ES proteins recovered from sockeye- and Atlantic-fed *Lsal* appearing at higher concentrations than either coho- or pink-fed *Lsal* (L. Braden, *pers obs*). The apparent species-specific response by the parasite was quantified after microarray hybridization, demonstrating that *Lsal* responds more aggressively to susceptible Atlantic salmon compared to either coho or sockeye salmon (95). Interestingly, in this study there was overexpression of several genes corresponding to proteins described in the secretome (96,97), including putative virulence factors that likely play prominent roles in host immunosuppression and disruption of wound healing (98). Thus, the variable host response to *Lsal* is multifactorial and involves the interaction between the parasite feeding/attack and host defense responses.

Immunomodulation

The likelihood of successful parasitism is increased by reducing host awareness of the parasite at the attachment site, which is achieved through immunomodulation (99–101). Ectoparasites secrete a cocktail of highly-evolved pharmacologically active factors in their saliva that manipulate both hemostatic and immune systems (reviewed by (102)), acting to inhibit cutaneous irritation, suppress cellular immunity, prevent blood clotting and interfere with wound healing (103). These molecules have been found to share ancestral homology with host genes or have developed independently and share no identifiable homology to the host (reviewed by (104)). Extensive characterization of these molecules in terrestrial blood-feeding arthropods (e.g., ticks, mites, lice) over the last 100 years indicates functional commonalities that represent convergent evolution across several phylogenetic lineages (105–107)).

The salmon louse has at least four different types of glands that appear to have specialized functions (108). Exocrine glands involved in the production of host-interacting proteins include type 3 tegumental glands and labial glands: the former emptying contents directly onto the host from pores in the marginal membrane, while the latter containing secretory units that empty into storage

reservoirs that are suggested to be involved in mouth tube movement (108). Due to their intimate association with the host it is likely that the contents of both these reservoirs are involved in the host-parasite relationship. Experimental characterization of the proteins excreted and/or secreted (ES proteins) by *Lsal* has generated a list of potential interacting virulence factors that share significant homology with those described for other ectoparasites (11,12,96,97). Using sequence and domain homology, these can be broadly generalized into five categories of proteins: 1.) Inhibitors of coagulation, 2.) Hemoglobinolytic enzymes, 3.) Anti-immunity, 4.) Anti-microbial, and 5.) Anti-wound healing, with several proteins not falling into any category and thus having unknown function (Figure 1C&D, Table 1).

For parasites that rely on blood as a source of energy, including *Lsal*, a critical adaptation has been the evolution of strategies to inhibit the host clotting cascade and enhance digestion of clots via fibrinolysis. In other parasites the presence of proteins involved in these processes is well documented (109–117). Similarly, the *Lsal* secretome is populated with anti-clotting proteins including alpha-2-macroglobulin, serine protease inhibitors, carboxypeptidase B, and coagulation factor IX (96,97). The extent of louse-associated interference of host coagulation and fibrinolysis was recently demonstrated in the putative louse-salmon interactome (Figure 3), where key virulence factors in the secretome were shown by an interolog-approach (a conserved interaction between a pair of proteins which have interacting homologs in another organism; (118)) to interact with critical components of the salmon host clotting and coagulation cascade such as kininogenin and plasminogen (96,97).

The degradative pathway of hemoglobin has been extensively characterized in hematophagous parasites with perhaps the most comprehensive description in that of *Ixodes ricinus* (119); however, there is remarkable similarity in the pathway among lineages, with digestion based on cooperating acidic aspartic and cysteine peptidases. Hematophagous arthropods either rely on alkaline proteolysis performed by serine peptidases (e.g., fleas), cysteine peptidases (e.g., ticks), or both serine and cysteine peptidases (e.g., triatomid bugs) for hemoglobin digestion (reviewed by (120)). The pathway of blood digestion in *Lsal* has not been elucidated; however, there is an abundance of serine-type peptidases characterized in the ES proteome which might indicate a similar digestive strategy to schistosomes ((121), Table 1). Changes in the proportion of serine endopeptidases and cysteine

proteases in the ES of *Lsal* between pre-adult and adult life stages suggests varying immune evasion and digestion activity is life stage dependent, even when lice are mobile (96).

While feeding for extended periods of time, ectoparasites compromise the epithelium which in an aqueous environment, permits colonization by opportunistic pathogens. This has led to the notion that *Lsal* may facilitate infections with bacteria or virus (122). Despite this, when examined histologically, epithelial wounds caused by *Lsal* are rarely associated with bacterial colonization, and when they have been observed it has been on the fins and gills > 150 degree-days (123,124). Even in cases where salmon have been co-exposed with *Moritella viscosa*, the bacteria was rarely isolated from *Lsal* attachment sites (< 5%), despite common clinical signs of 'winter ulcer disease' and bacterial isolation from other skin sites away from louse attachment (125). Interestingly, the feeding response of *Lsal* is associated with significant production of vitellogenin-like proteins. Primarily associated with egg-yolk production, vitellogenins have also been implicated to act in non-reproductive roles including immunity and protection against oxidants (126). In honeybees *Apis mellifera* vitellogenins in the venom are associated with antimicrobial activities (127), and vitellogenins are a major salivary antigen of ectoparasitic sheep scab mites *Psoroptes ovis* (128). The most abundant proteins in the *Lsal* secretome are vitellogenin-like proteins (96,97), suggesting they play an important non-reproductive role in the host-parasite interaction. However, this hypothesis requires experimental validation.

As the fundamental barrier in an aqueous environment, teleost skin is in a constant state of flux and is therefore extremely proficient at repairing damage within short periods of time as well as preventing pathogen colonization (96,97). Wound healing includes the highly regulated and overlapping processes of hemostasis, inflammation, proliferation, formation of granular tissue, re-epithelialization, matrix formation, and tissue remodelling (129,130), with genetic and mechanistic conservation between mammals and teleosts (131). However, in teleost fishes, the process of re-epithelization is faster, with full-thickness wounds approaching 100% closure within 12 hrs of injury and independent of coagulation or inflammation (132). Thus, for the ectoparasitic salmon louse, inhibition of host wound healing would likely be of considerable importance. It appears that a major function of *Lsal* virulence is interference with wound healing by chemically disrupting fibrinolysis. For example, the parasite secretes several proteins including collagenase and serine proteases that are predicted to interact with fibronectin, a critical component of wound healing and tissue remodeling

(133). The large number of ES proteins targeting extracellular matrix regeneration and collagen deposition in the host emphasizes the importance of this pathway during the louse-salmon interaction (98). Interestingly, expression of louse proteins that target wound healing and tissue remodeling is enhanced while parasitizing the more susceptible species (95), the phenotype of which is characterized by a poor wound healing response.

Blood-feeding arthropods must contend with their host's blood coagulation cascade, the toxic components of iron and heme, and also must circumvent host immunity. This is perhaps even more critical in parasites that feed on their hosts for an extended period of time, such as with salmon lice, in contrast to mosquitos (*Aedes aegypti*) which only have a short period of blood feeding before leaving the host. Thus, it is not surprising that among the pharmacologically active molecules present in the ES proteome of *Lsal* are several anti-immune molecules. For example, *hypodermin B* (*hypB*) has been described as a key feeding-associated gene in *Lsal*, with enhanced expression while feeding on susceptible hosts compared to resistant hosts (95). Furthermore, hypodermin B is routinely identified in the dopamine-elicited ES products of both Pacific and Atlantic subspecies of *Lsal* (95). Originally described in the cattle grub *Hypoderma lineatum* this enzyme inhibits the complement cascade by degradation of C3 as well as assisting parasite migration through degradation of protein in host tissue (96,97). Complement evasion is a common strategy among all classes of parasites (134), and downregulation of genes in both classical and lectin pathways (e.g., *c1q, mbl, clec2*) appears to be a feature of the host response to *Lsal* and *Crog* lice infections (34,35,39). This likely inhibits host production of reactive oxygen species (e.g., H_2O_2) thus effector macrophage activity, which can damage louse cuticle, and host dendritic cell maturation impacting T-cell responsiveness.

There appears to be several cases of divergent evolution in the feeding transcriptome and proteome of *Lsal*. For example, genes annotated as β- and γ-crystallin in the louse transcriptome are part of the co-regulated suite of virulence-associated transcripts involved in the feeding response on susceptible Atlantic salmon (95). Furthermore, β/γ-crystallins have been identified in the proteome, supporting their role in the host-parasite interaction (97). Crystallins are a diverse group of water soluble, multifunctional proteins that are related to stress or metabolic-associated proteins (135). In higher vertebrates, they appear to only function in the eye lens, but the presence of several crystallin-like proteins with non-optical roles in invertebrates supports mutational diversification throughout

evolution (135). For example, Piatigorsky *et al.* (136) showed a jellyfish crystallin-like protein shared between 25-50% similarity to saposin-containing protein NK-lysin, a saposin-containing protein (SAPLIP). Interestingly, SAPLIPs are prominently featured in the feeding transcriptome of *Lsal*, and are significantly induced while feeding on susceptible Atlantic salmon (95). These are a diverse family of lipid-interacting proteins that are conserved phylogenetically and have high sequence and function variability (137). SAPLIPs from liver flukes *Fasciola hepatica* and *Clonorchis sinensis*, the protistan *Entamoeba histolytica*, and nematodes *Necator americanus* play key roles in the host-parasite interaction (138–140). In the ES proteome of *Lsal*, proteins with saposin-associated domains have been identified, including antimicrobial peptide NK-lysin and saposin-type protein A (96,97). Interestingly, sequence analysis of *Lsal* NK-lysin indicates this protein is > 35% similar to amoebapore B, a major virulence factor of *Entamoeba* spp (139).

To avoid deleterious host immune responses, ectoparasites deploy several classes of proteases that can be characterized by the chemical composition of their active site: cysteine, serine, aspartic, metallo- and threonine. The feeding response of *Lsal* prominently features expression of serine (e.g., trypsins, chymotrypsins) (Figure 1C&D), cysteine (e.g., cathepsins), and metalloproteases (e.g., astacins) (96,97). The high number of proteases in the ES proteome indicates the importance of these enzymes during the host-parasite interaction. Indeed, network analysis of the predicted louse-salmon interactome revealed that several of these proteases interact with salmon host proteins involved in inflammatory, extracellular matrix and tissue remodelling processes (98). Thus, the salmon louse may be achieving immunosuppressive effects through the actions of secreted proteases as what is described for many other parasites. For example cysteine proteases from the liver fluke *Fasciola hepatica* downregulates inflammation by degradation of TLR3, and modulates cellular effectors by cleaving host-derived immunoglobulin (141). Ectoparasite mites and ticks also rely on cysteine proteases as major virulence factors, and the abundance of these proteins in the secretomes of the parasites highlights the importance of these proteases in the host-parasite interaction (142–144). Interestingly, the greatest cathepsin L activity from *Lsal* has been reported at the sessile chalimus stage, when prevention of immune recognition would be of outmost importance to prevent host rejection (145). Inhibition of host recognition during attached chalimus stages may also be accomplished through production of prostaglandins. For example, Fast *et al* (146) identified the

arachidonic acid metabolite PGE_2 in the ES products of *Lsal* and this was negatively correlated with expression of pro-inflammatory mediators. Secretion of PGE_2 is effective in modulating responses of dendritic cells (DCs) in nematodes (147) and ticks (101,148); however, the effect of *Lsal*-PGE_2 on salmonid DCs has not been quantified and the overall contribution of PGE_2 to *Lsal* virulence remains to be elucidated.

Co-evolutionary Impacts & Future Perspectives

Our current understanding of louse immunomodulation of the host is a snapshot of a dynamic relationship after millions of years of co-evolution. Evolutionary theory predicts that the host and parasite could currently be a stable strategy between the host and parasite, could involve static within-population dimorphism or polymorphism; experiencing arms race dynamics, in which the host and parasite are escalating their immune and modulatory responses, respectively, to gain advantage over the other; or finally, there could be fluctuating selection dynamics, due to oscillatory feedbacks from the host and parasite, and these can be enhanced or dampened by environmental and life history inputs (149). As some fitness costs to the host are expected to occur with increased host resistance and parasite virulence (i.e., parasite-derived factors contributing to parasite fitness), arms race dynamics are not expected to continue indefinitely, eventually leading to either a stable strategy between the host and parasite or fluctuating selection dynamics (149,150). Over the last 20-30 years of intensive salmon aquaculture, these dynamics may have been significantly altered, whereby the costs of enhanced louse virulence are reduced with the constant supply of new hosts to the marine environment on a 20-24 month cycle. Recent publications have suggested strong potential for microevolution of virulence in lice and other pathogen populations that can be selected for under intensive aquaculture conditions (79,149,151). Anti-louse medicinal intervention and removal of host populations at harvest may disentangle the natural selection towards host adaptation to combat louse virulence (i.e. reduce pathology) in the cage system and the positive traits normally associated with it such as increased survival and fecundity. While this theory may expect little to no changes in adaptation of the host to the pathogen, Masri *et al.* (152) has shown that in some cases the presence of co-evolution with the host leads to selective advantage of virulence fixation (i.e., no change in virulence) in the pathogen population, compared to one-sided adaptation. Whether intensive

aquaculture is enhancing immunomodulatory mechanisms, and thereby virulence in the louse, effectively tipping fluctuating selection dynamics in the parasite's favour over the short-term, or this is a longer-term trend, perhaps our greatest knowledge gap is whether/how this co-evolutionary trajectory might be altered. For example, expression of virulence factors in *Lsal* are overexpressed while feeding on non-native host populations (e.g., *L. salmonis oncorhynchii* on Atlantic salmon), which may be disrupting natural evolutionary processes (95). In another parasitic copepod-host system (*Mytilicola* spp. infecting blue mussels), Feis *et al.* (153) describe separate invasion fronts lead to different co-evolutionary trajectories (i.e. host resistance, tolerance, susceptibility), and *Lsal*, among other Caligid copepods, have already demonstrated this within the salmonid host lineage described earlier. However, shifts in these trajectories once established has not been shown. Theoretical exploration of perturbations to the natural system (i.e. host-parasite co-evolutionary forces) from aquaculture, using different modelling approaches would be an interesting new area of investigation in the future.

Another major knowledge gap we have is the extent of co-evolution of the louse with other potential pathogens of the salmonid host. Overgaard *et al.* (154), through knock-down studies, observed significant impacts of *Lsal* rhabdoviruses on subsequent salmon inflammatory/immune responses, potential benefiting the copepod during infection. Co-infection studies also show the synergism between *Lsal* and another rhabdovirus infection (infectious hematopoetic necrosis virus; IHNV) as well as the orthomyxovirus infectious salmon anemia virus (ISAv), in the salmonid host (74). These synergies are linked to the immunomodulatory capabilities of *Lsal* dampening inflammation and anti-viral responses in the host. Co-evolutionary selection dynamics on the louse-virus relationship are unknown and how they may enhance or dampen the impacts on the hosts is an important question for both salmonid culture and fisheries ecology.

The development of novel anti-louse treatments relies on a holistic understanding of the host-parasite relationship between *Lsal* and its various salmonid hosts. Advances in genomics along with the sequencing and annotation of the salmon louse (155), Atlantic salmon (156,157) and other salmonid species genomes (e.g., (158)) has certainly perpetrated a more thorough understanding of this relationship. However, there are many gaps that need to be addressed, including improved methods to identify secretory products released specifically during the copepodite-chalimus transition

stages and functional characterization of the various virulence factors and their effects on host biology.

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Figures

Figure 1. Larval (chalimus) and adult stages of *Lepeophtheirus salmonis* and aspects of host-parasite interaction. (A) Dorsal skin of Atlantic salmon commonly infected by mobile stages of *Lsal.* Gravid Adult females attached posterior to the dorsal fin causing extensive erosion to the skin (annotated parasite inset). When actively feeding the gut is often filled with blood; (B) Following initial attachment on Atlantic salmon the copepodite moults into a sessile chalimus which feeds within the vicinity of the anchored filament. Host response is limited at the feeding site of the mouth tube due to louse immunomodulation. Some melanisation can be observed. The chalimus can attach to scales feeding on host mucus and epithelium (inset left) and commonly fins (micrograph) where erosion of epidermis can extend to the basal membrane but with no / limited inflammatory responses occurring around the periphery of the feeding site (micrograph - H&E) (C-D); Secreted proteins can inhibit coagulation, prevent wound healing, facilitate digestion and immune evasion/suppression, and may be anti-microbial. For example trypsins are highly expressed in the larger more aggressive feeding adult stages (159,160). (Fluorescent micrographs of *in situ* hybridisation (ISH)-labelled *L sal* trypsin. Enhanced red = Trypsin expression. Enhanced autofluorescence emission was used for distinguishing anatomical structures). AF = Adult female, CHAL = Chalimus, AM = Adult male, dor fin = dorsal fin, mt = mouth tube, ff = frontal filament, ceph = cephalothorax, ant = antennae, ova = ovaries, gs = genital segment. Photo micrographs and ISH figures courtesy of James Bron and Jacquie Ireland, Institute of Aquaculture, University of Stirling.

Figure 2. **Spectrum of susceptibility of salmonids to the salmon louse.** Among species of salmonids there is a variable host response such that some species are resistant (e.g., coho, pink salmon), some are very susceptible (e.g., sockeye salmon, Atlantic salmon), while others (e.g., chinook salmon) are of medium susceptibility. This susceptibility has been demonstrated in the laboratory to be related to life history and involve a weakened inflammatory, cellular, and tissue regenerative response at the site of attachment which is strongly linked to immunomodulatory effects of the salmon louse. Associated relevant literature is shown in superscript. Figure is depicting relative susceptibility.

Figure 3. The host-parasite interaction between *Lepeophtheirus salmonis* and Atlantic salmon. Proteomic characterization of the *Lsal* secretome has identified at least 5 classes of proteins that facilitate the parasite evading the host immune system during feeding and attachment. Several proteins that are prominent in the secretome do not share sequence homology with known parasite virulence factors and appear to be novel to *Lsal* and are thus classified with "unknown" function.

Table 1. Protein families identified in the ES proteome of *Lsal* showing the associated orthologous proteins orthogous to "Acari" (when available), the putative role in the host-parasite relationship, and references supporting this functional role. Protein families are grouped based on Pfam conserved domains, and the number of proteins in each family are indicated in brackets.

			Putative role in HP interaction[c]						
Protein families	**Example protein[a]**	**Orthologous protein[b]**	**Anticoagulation/ Fibrinolytic**	**Hemoglobinolytic**	**Anti-immunity/ Antioxidant**	**Antimicrobial**	**Wound healing**	**Unknown**	**References[d]**
A2M (1)	Alpha-2-macroglobulin	Alpha-macroglobulin, [*Ixodes scapularis*]	•						(102,117)
Actin (2)	Actin	Actin [*Rhipicephalus microplus*]	•					•	(105)
Alk_phosphatase (1)	Intestinal alkaline phosphatase	Alkaline phosphatase-like protein 2 [*Leptotrombidium deliense*]			•				(161)
ATP-gua_Ptrans (1)	Arginine kinase	Arginine kinase [*Haemonchus contortus*]			•				(162)
Peptidase_A1 (1)	Cathepsin D	Cathepsin D2 [*Ixodes ricinus*]		•					(119,163)
Astacin (3)	Zinc metalloproteinase nas-4	Astacin-like metalloprotease toxin 5 [*Galendromus occidentalis*]			•		•		(119,163)
Carb_anhydrase (1)	Carbonic anhydrase 2	Carbonic anhydrase [*Ixodes scapularis*]						•	(164)
CD36 (1)	Scavenger receptor class B	Lysosome membrane protein 2-like protein [*Dinothrombium tinctorium*]			•			•	(165,166)

Cellulase (1)	Endoglycoceramidase	Pikachurin [*Tetranychus urticae*]						•	n/a
CN_hydrolase (1)	Vanin-like inflammatory protein 1	Carbon-nitrogen hydrolase [*Ixodes scapularis*]						•	n/a/
Cofilin_ADF (1)	Cofilin/actin-depolymerizing factor homolog	Actin depolymerizing factor [*Ixodes scapularis*]	•				•		(167)
COIL (1)	Glutamine rich 2	n/a						•	n/a
Crystallin (3)	Gamma crystallin A	n/a						•	n/a
DUF227 (1)	Juvenile hormone-inducible protein	n/a						•	n/a
EB (1)	Chitin deacetylase	Polysaccharide deacetylase-like [*Sarcoptes scabiei*]						•	n/a
Enolase_C (1)	Enolase	Enolase [*Ornithodoros moubata*]	•				•		(168)
Fasciclin (1)	Embryo cathepsin L-associated protein	Fasciclin domain-containing protein [*Ixodes scapularis*]					•		(169)
Ferritin (1)	Ferritin heavy chain	Ferritin [*Ornithodoros moubata*]			•	•			(170)
GSHPx (1)	Glutathione peroxidase	Phospholipid-hydroperoxide glutathione peroxidase [*Rhipicephalus microplus*]			•				(171–173)
GTP_EFTU_D3 (1)	Elongation factor 1-alpha-like	Elongation factor 1-alpha [*Varroa destructor*]	•						(105)
Histone (2)	Histone H2B	Histone H2B [*Varroa jacobsoni*]						•	(105)
Kuniz_BPTI (1)	Tissue factor pathway inhibitor	Papilin-like [*Dermatophagoides pteronyssinus*]	•		•				(112)
Lectin_C (1)	Mannose receptor, type C	n/a						•	n/a
Lipocalin (1)	Fatty acid binding protein 4	Fatty acid-binding protein [*Tetranychus urticae*]			•				(174)

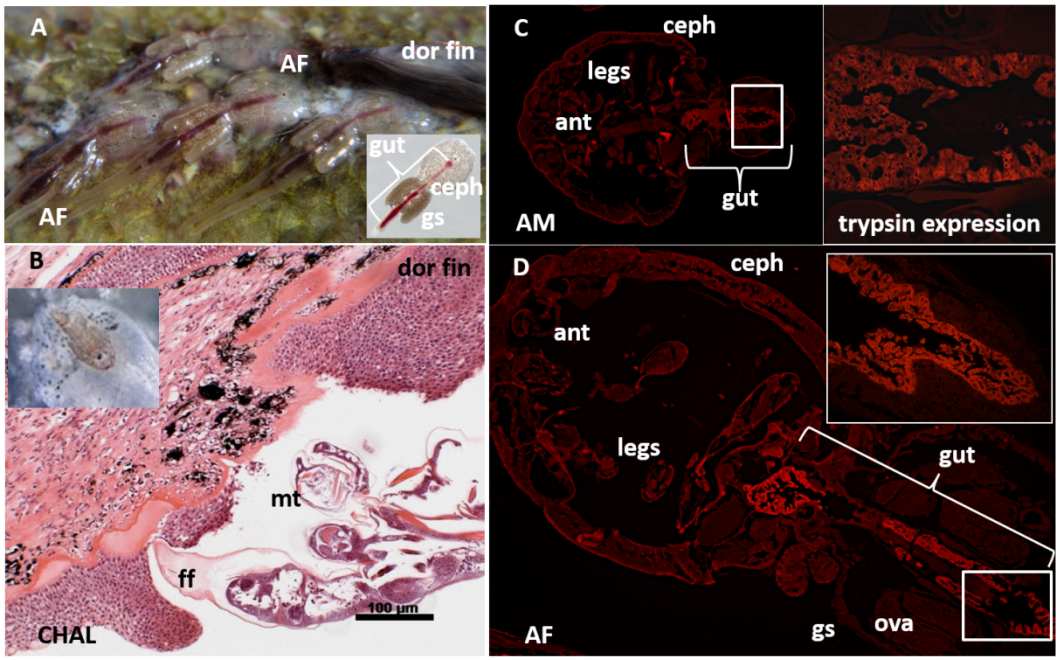
Peptidase_C1 (3)	Cathepsin L	Cathepsin L1-like [*Varroa jacobsoni*]		•	•		•		(117,119,141–143,163)
Peptidase_M13 (2)	Neprilysin-2	Neprilysin-2 [*Galendromus occidentalis*]	•		•				(163,175,176)
Peptidase_M14 (1)	Carboxypeptidase B	Carboxypeptidase B-like [*Leptotrombidium deliense*]	•	•					(119,177–179)
Peptidase_M17 (1)	Leucyl aminopeptidase	Aminopeptidase-like protein 6 [*Sarcoptes scabiei*]		•					(119,163)
Redoxin (1)	Redoxin	Peroxiredoxin [Psoroptes ovis]			•			•	(180)
Ribosomal_L40 (1)	Ribosomal protein L40	Ribosomal protein L40 [*Ixodes scapularis*]						•	(170,181)
SapB_2 (3)	Antimicrobial NK-lysin like	Ameobapore B precursor [*Entamoeba invadens*]		•	•		•		(137,139,140)
E1_DerP2_DerF2 (1)	Ganglioside GM2 activator-like	Ganglioside GM2 activator-like protein [*Sarcoptes scabiei*]			•				(182)
Thioredoxin (2)	Thioredoxin	Thioredoxin [*Amblyomma variegatum*]			•				(181,183–185)
TIM (1)	Triosephosphate isomerase	Sar s 25 allergen (triosephosphate isomerase-like) [*Sarcoptes scabiei*]	•		•				(105,186)
Trypsin (17)	Trypsin 1	Serine protease [*Ixodes scapularis*]	•	•	•		•		(117,119,121,163, 176,187)
Vitellogenin_N (3)	Vitellogenin 1	Vitellogenin-2 [*Haemaphysalis longicornis*]			•	•			(125,126,188)
	Prostaglandin E2	Prostaglandin E2 [*Ixodes scapularis]*		•	•				(147,189)

[a]An example protein is given when there are multiple proteins present within the same family

[b]Orthologous proteins in other ectoparasites were determined using sequence homology with an e-value cutoff of 10^{-5}

[c]The role of *L. salmonis* proteins in the host-parasite interaction were inferred by homology to orthologues in other parasite systems

[d]References pertaining to the inferred function are given



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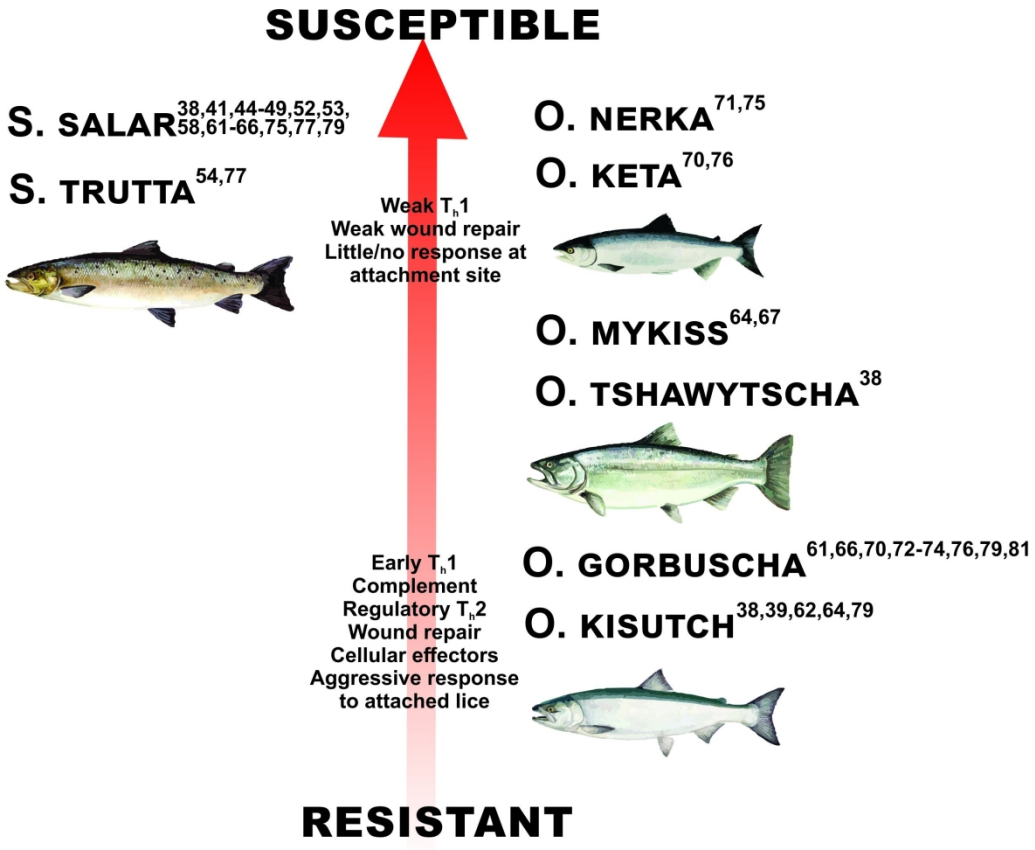

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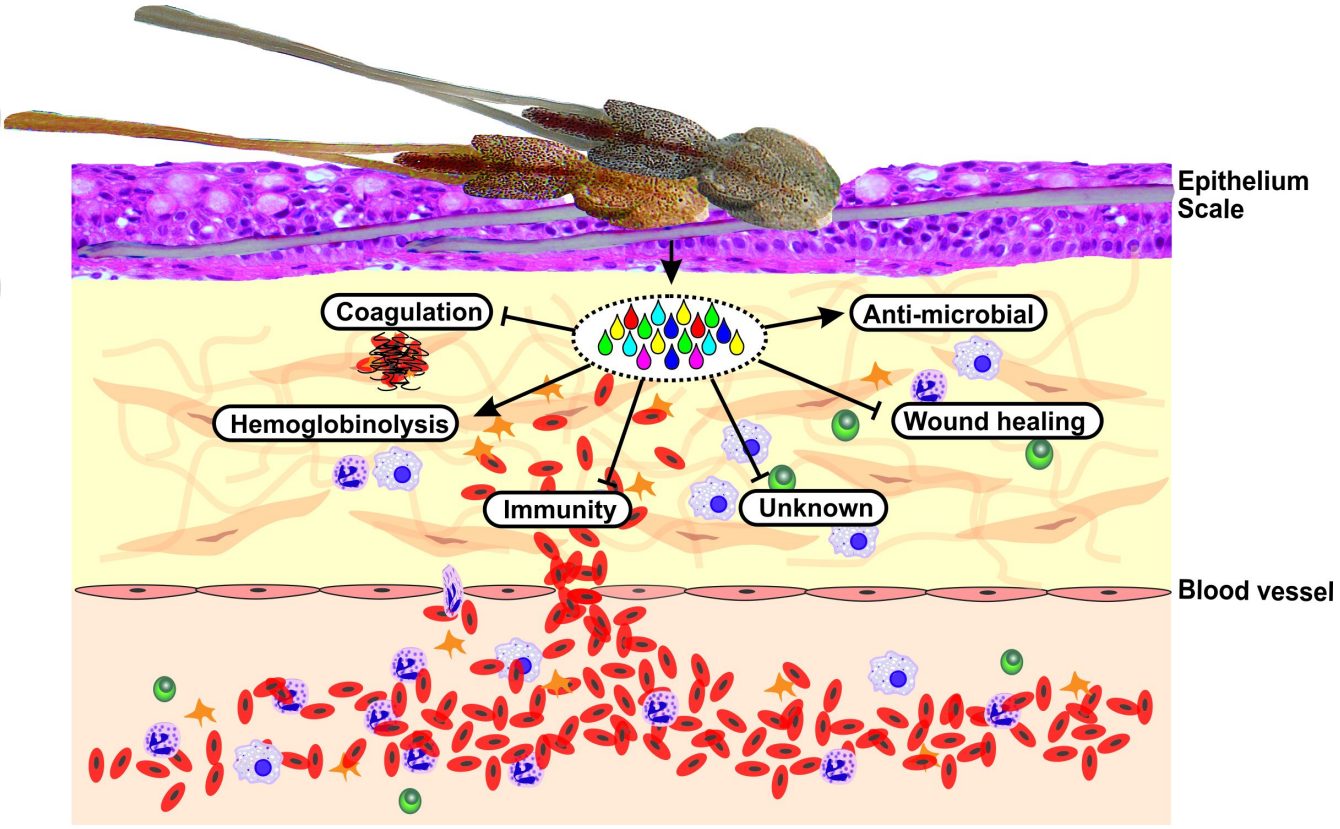

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