INTERACTIONS BETWEEN SALMON MACROPHAGES AND PATHOGENIC BACTERIA IN THE PRESENCE OF SECRETIONS ISOLATED FROM *LEPEOPHTHEIRUS SALMONIS*

by

Danielle Lee Lewis

B.Sc. Vancouver Island University, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Animal Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April, 2013

Abstract

In response to stimuli (i.e., salmon mucus) the sea louse, *Lepeophtheirus salmonis*, produces pharmacologically active substances (prostaglandin E_2, trypsin-like proteases and cathepsin). Lice-derived secretions impair the genetic expression of pro-inflammatory mediators in the commercial salmon-head kidney (SHK-1) cell line and head kidney macrophages isolated from Atlantic salmon (*Salmo salar*); however, effects on the functionality of these cells has not been explored. Related to the development of an inflammatory response, salmon species (*Oncorhynchus* spp. and *Salmo* spp.) exhibit differences in infection rates and threshold tolerances to *L. salmonis*. The objective of this study was to determine if the presence of *L. salmonis* secretory and excretory products (SEPs) alters the innate immune response of salmon. More specifically, the present study examined if the presence of SEPs altered phagocytic activity and respiratory burst response of salmon macrophages. Phagocytosis assays were performed using SHK-1 cells and the bacterial pathogen, *Aeromonas salmonicida*, in the presence/absence of SEPs. To address species-specific differences, phagocytosis and respiratory burst assays were completed using macrophages isolated from pink (*Oncorhynchus gorbuscha*), chum (*O. keta*), and Atlantic (*S. salar*) salmon in the presence/absence of SEPs. SHK-1 cells incubated with SEPs plus *A. salmonicida* had a significantly higher phagocytic index (223.2 %) than cells incubated with *A. salmonicida* alone (136.5 %). Macrophages isolated from pink salmon had a pronounced production of superoxide (O_2^-) in the presence of SEPs that was not observed in chum or Atlantic salmon macrophages. Interestingly, pink salmon macrophages had a lower phagocytic index (15.8 %) than the more *L. salmonis*-susceptible species, chum (55.1 %) and Atlantic (26.4 %) salmon. Furthermore, the presence of PGE_2, proteins and other

undetermined molecules in SEPs appear to have a biologically relevant concentration at which they no longer exert an effect on phagocytosis in SHK-1 cells. This study provides the first evidence of altered macrophage function in response to *L. salmonis* secretions and provides insight into the complex interactions that occur at the parasite-host interface (i.e., the skin).

Preface

Parts of Chapter 2 will be submitted for publication in a scientific journal with the help of co-authors, Duane Barker and Scott McKinley. I, Danielle Lewis, was the primary author and will submit the manuscript. I performed all the research, wrote the manuscript, and included comments made by D.B and S.M. All research in Chapter 2 was conducted at Vancouver Island University (VIU) and fish were maintained under VIU Animal Care protocol 2010-05TR. Lice and secretions were collected in collaboration with Laura Braden (University of Victoria Ph.D. candidate), at the Pacific Biological Station (PBS), and fish used for primary culture were maintained at PBS by Colin Novak (University of British Columbia M.Sc. candidate) and Laura Braden. Funding for the preparation of this manuscript was provided by an NSERC Strategic Projects Grant (STPGP 372605-08) awarded to Duane Barker, Kyle Garver and Simon Jones.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Full name
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
A. sal	*Aeromonas salmonicida*
AAMs	alternatively activated macrophages
ANOVA	analysis of variance
APCs	antigen presenting cells
APPs	acute phase proteins
APR	acute phase response
ASW	autoclaved saltwater
β2-m	Beta 2- microglobulin
CAMs	classically activated macrophages
C/EBP	CCAAT- enhancer binding proteins
CCAAT	cytidine- cytidine-adenosine- thymidine
CD4+	helper T cell
CD8+	cytotoxic T cell
COX	cyclooxygenase
CRP	C- reactive protein
D.O.	dissolved oxygen
DA	dopamine
DAMPs	damage associated molecular patterns
^{o}C	degrees Celsius
DMSO	dimethyl sulfoxide
dpi	days post-infection
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
g	grams
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICE	interleukin-1 converting enzyme
IFN	interferon
Ig	immunoglobulin
IHN	infectious haematopoeitic necrosis
IkB	nuclear factor of Kappa light polypeptide gene enhancer in B-cells inhibitor

Abbreviation	Full name
IL	interleukin
iNOS	inducible nitric oxide synthase
IPN	infectious pancreatic necrosis
ISA	infectious salmon anaemia
kDa	kilodalton
KOH	potassium hydroxide
L	litre
L-15	Leibovitz-15
LMW	low molecular weight
LPS	lipopolysaccharide
M	molarity
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MH	major histocompatibility
µm	micrometer
µg	microgram
µL	microliter
mL	milliliter
mm	millimeter
MMP	matrix mellatoproteinase
MRC1	mannose receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitro-blue tetrazolium
NCCs	non-specific cytotoxic cells
NF-kB	nuclear factor of Kappa light chain enhancer of activated B cells
ng	nanogram
NK	natural killer
nm	nanometer
NO	nitric oxide
O_2	oxygen
O_2^-	superoxide
OmpA	outer membrane protein A
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PGDS	prostaglandin D synthase
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
PMNs	polymorphonuclear leukocytes

Abbreviation	Full name
PRR	pattern recognition receptors
rIL-1β	recombinant interleukin 1-beta
ROIs	reactive oxygen intermediates
SAA	serum amyloid A
SAP	serum amyloid P
SAVs	salmon alphaviruses
SEPs	secretory and excretory products (of *Lepeophtheirus salmonis*)
SHK-1	salmon head kidney cell line
spp.	species
TLRs	toll-like receptors
TMS	tricaine methanesulfonate
TNF	tumor necrosis factor
TSA	tryptic soy agar
UV	ultraviolet
VIU	Vancouver Island University

Acknowledgements

Firstly, this work would not have been possible without the funding provided by the National Sciences and Engineering Research Council (NSERC) Strategic Projects Grants (STPGP 372605-08) awarded to Duane Barker, Kyle Garver and Simon Jones.

I would like to thank the many, many people who supported and guided me throughout this process, without them this project would not have been. I would like to thank Brad Boyce and Marine Harvest Canada for their donation of Atlantic salmon and for continually allowing me to visit farm sites. A lot of trips were made to farm locations over the last couple years and, even when I showed up at inopportune times, the staff of Marine Harvest was kind, helpful and welcoming. Jon Richard at the Pacific Biological Station offered me his time and expertise in cell culture, provided me with SHK-1 cells and lent me supplies when I received the dreaded news of a "backordered product", which seemed to happen more often than not. Gord Edmondson, Dave Switzer, and Dan Fox provided technical support, especially in tank set-up and maintenance, and always put up with my continuous requests for help. Robert Wager, Barbara Campbell, Rosemarie Ganassin, John Morgan and Helen Gurney-Smith were always willing to lend me their equipment, without question. Laura Braden shared her knowledge and enthusiasm of sea lice, and was always willing to offer support and advice. Colin Novak took care of fish and was an excellent travel companion. Katie Verkaik and Brenna Collicutt were more helpful than they know, thank you for the assistance, encouragement and laughs.

A huge thank you to my supervisors, Duane Barker and Scott McKinley, and my supervisory committee, Shannon Balfry, Colin Brauner and Dan Weary. Duane encouraged

me to do my master's and then answered the endless emails and questions that accompanied. He provided me with opportunities that most students never have; the trips to Oregon, Quebec and Prince Edward Island are cherished memories. Scott was always encouraging and supportive, pushing me to succeed. Shannon helped me decipher my results and, along with Colin and Dan, made recommendations on how to strengthen my thesis. I thank each of you for all your feedback throughout this process.

Finally, I would like to thank my friends and family, this thesis is a product of their continuous support. Thank you for knowing when to be there and reminding me that there are things more important than sea lice.

Dedication

To my dad,

without you the following pages would have been blank…

1 Introduction

1.1 Innate immune system of teleosts

The innate immune system is the first line of defense for any animal, it functions to maintain homeostasis. In fish, the innate immune system has a more significant role in immunity than the specific immune system (Woo, 1992; Magnadóttir, 2006). Following stimulation by foreign molecules, the innate immune system responds in a rapid and non-specific manner (Tort *et al.*, 2003). The components of innate immunity are commonly divided into physical, cellular and humoral factors (Woo, 1992; Magnadóttir, 2006).

The innate immune system acts independently of prior exposure to an organism and utilizes germline-encoded pattern recognition receptors (PRRs) that identify and bind molecular patterns (Magnadóttir, 2006; Whyte, 2007; Magnadóttir, 2010). There are two categories of molecular patterns that initiate an immune response, pathogen associated molecular patterns (PAMPs) and molecular patterns exposed after damage to the host's tissue due to infection, necrotic changes, and cell death (damage associated molecular patterns = DAMPs) (Magnadóttir, 2006). PAMPs are molecules, such as lipopolysaaccharide (LPS), peptidoglycan, and mannose, that are absent in eukaryotic cells but are shared by major groups of pathogenic microorganisms (Magnadóttir, 2006; Whyte, 2007). Activation of the immune system occurs after recognition of PAMPs by PRRs (Tort *et al.*, 2003). Toll-like receptors (TLRs), important PRRs, are found on the membranes of dendritic cells and macrophages (Basset *et al.*, 2003; Bone and Moore, 2008; Magnadóttir, 2010). The binding of ligands to TLRs can result in phagocytosis, production of proinflammatory cytokines, upregulation of co-stimulatory molecules on antigen presenting cells (APCs) and the

maturation of naïve dendritic cells (Basset *et al.*, 2003; Alvarez-Pellitero, 2008). TLRs directly affect macrophages, stimulating them to produce antimicrobial proteins and peptides, inducible nitric oxide synthase (iNOS) and other oxidative factors (Alvarez-Pellitero, 2008). Initiation of the innate immune system stimulates molecules that are essential for the activation of the specific immune system (Watts *et al*., 2001).

1.1.1 Physical factors of innate immunity

The first line of defense for a fish against an infectious organism is the mucus and epithelium (Woo, 1992). Mucous membranes of the gills, skin, digestive system and genito-urinary tract serve as an initial source of protection from microorganisms (Bols *et al*., 2001). Mucus functions in numerous ways to prevent establishment of microbes on a host. First, it is responsible for preventing attachment of microorganisms by being continually sloughed (Woo, 1992; Bols *et al*., 2001; Watts *et al*., 2001). If attachment is successful, the mucus then acts as a barrier that the microorganism must penetrate to infect the host (Woo, 1992; Bols *et al*., 2001; Watts *et al*., 2001). And finally, the mucus of fish contains important antibacterial peptides, complement factors and immunoglobulins (Bols *et al*., 2001; Magnadóttir, 2006; Magnadóttir , 2010; Prabhackar, 2010) that aid in defense against foreign invaders.

1.1.2 Cellular components of innate immunity

Cellular immunity is the product of non-specific cytotoxic cells (NCCs), granulocytes (neutrophils) and monocytes/macrophages (Woo, 1992; Magnadóttir, 2006; Whyte, 2007). The cellular response of the innate immune system is highly non-specific allowing for large populations of cells to be mobilized rapidly upon stimulation (Whyte, 2007). The cellular response is biphasic; neutrophils are the first cells to appear at the site of infection followed

by monocytes/macrophages (Whyte, 2007). NCCs function to destroy tumour cells, virus-infected cells and protozoan parasites (Bols *et al*., 2001; Whyte, 2007). There are conflicting results as to the specific types of granulocytes present in teleost fish. Neutrophils, eosinophils and basophils have all been reported; however the presence/absence of eosinophils and basophils is variable depending on the fish species studied (Yoder, 2004). In contrast, neutrophils have been identified across multiple fish species and possess bactericidal activity (Yoder, 2004). Macrophages play an important role in the killing of a wide range of pathogens (bacteria to parasites) (Secombes, 1990). They also function as important accessory cells that initiate specific immune responses such as antigen presentation (Secombes, 1990; Yoder, 2004). Generally, the neutrophil is considered the more efficient phagocytic cell; however, when foreign particles are large or when particle load is great, macrophages are viewed as more effective (Auger and Ross, 1992). For salmonids, the majority of phagocytic uptake is performed by macrophages (Trust, 1986).

Upon recognition of an invading microorganism, macrophages extend pseudopodia over regions of the microorganism expressing opsonins (Auger and Ross, 1992). Opsonins are recognition protein molecules that bind to specific sites on the microorganism and macrophages (Auger and Ross, 1992). Macrophages can also identify microorganisms that possess surface PAMPs (e.g., mannose) and phagocytosis can be initiated without opsonisation of the microorganism (Speert, 1992). After binding of the pseudopodia has occurred, the process of internalization begins and once engulfed, the microorganism is encased and microbial destruction begins (Speert, 1992; Salyers and Whitt, 2002). A major microbicidal mechanism of macrophages is the release of reactive oxygen intermediates (ROIs) in a process known as respiratory burst. During respiratory burst, oxygen (O_2) is

reduced to superoxide (O_2^-) (Speert, 1992; Salyers and Whitt, 2002). Superoxide produced is spontaneously converted, or catalyzed by superoxide dismutase, to hydrogen peroxide (H_2O_2) which is a more potent antimicrobial (Nagelkerke *et al*., 1990; Auger and Ross, 1992).

1.1.3 Humoral components of innate immunity

The humoral component of innate immunity consists of cell associated receptors or soluble molecules including: complement, lysozymes or neutral proteases, acute phase proteins, transferrin, chemokines and cytokines (Woo, 1992; Magnadóttir, 2006; Whyte, 2007).

The complement system can become activated through three pathways: alternative, lectin and classical (Whyte, 2007; Magnadóttir , 2010). The three pathways result in the formation of a membrane attack complex (MAC) leading to cell lysis or increased phagocytosis through opsonisation of the pathogen and activation of the specific immune response (Whyte, 2007; Magnadóttir, 2010). Lysozyme is an enzyme that functions to disrupt the cell wall, resulting in lysis, by hydrolysing the β-[1,4] linked glycoside bonds of bacterial cell wall peptidoglycans (Bols *et al*., 2001; Magnadóttir, 2006). Lysozyme has a greater effect on Gram positive bacteria since it can act directly upon the cell wall but also has the capacity to lyse Gram negative bacteria once the LPS layer has been disrupted by complement and/or other enzymes (Bols *et al*., 2001). Acute phase proteins (APPs) are plasma or serum proteins that respond to tissue damage, infection or inflammation (Bols *et al*., 2001). Examples of acute phase proteins in fish include: C-reactive protein (CRP), serum amyloid P (SAP), serum amyloid A (SAA), and transferrin (Bayne and Gerwick, 2001). CRP functions to regulate phagocytosis and activate the classical complement pathway (Bols

et al., 2001). Cytokines (e.g., interferons, interleukins and tumor necrosis factor) and chemokines act as signaling molecules that control and synchronize the innate and acquired immune responses including migration of immune cells to the site of infection (Secombes *et al*., 1996; Secombes *et al*., 2001; Magnadóttir, 2006). Cytokines are produced in response to stimulation of PRRs and they initiate a cascade of signaling pathways that are responsible for proliferation, recruitment, survival and maturation of cells (Yasukawa *et al*., 2000; Aoki *et al*., 2008)

1.2 The specific immune system of teleosts

The poikilothermic nature of fish restricts the specific immune response; there are limited antibody repertoires, immunological memory is usually less pronounced and lymphocyte proliferation is relatively slow compared to homeotherms (Woo, 1992; Watts *et al*., 2001; Magnadóttir, 2006; Whyte, 2007). There is a degree of overlap between the innate and specific immune responses that covers the temporal lag (10-12 weeks) between the immediate response of the innate system and onset of the specific system (Basset *et al*., 2003; Magnadóttir, 2010). In comparison to the innate immune response, activation of the specific immune response is relatively slow but long lasting (Magnadóttir, 2010).

The specific immune response is comprised of lymphocytes, particularly the B- and T- cells (Magnadóttir, 2010), and relies on the ability of these cells to recognize and respond to antigens associated with foreign organisms (Smyth, 1994). B-cells are responsible for the production of antibodies and upon activation, will multiply and differentiate into memory cells and plasma cells which secrete the appropriate antibody (Bone and Moore, 2008; Magnadóttir, 2010). Once exposed to an antigen, memory cells will reside within an

organism and upon re-exposure to the same antigen, quickly produce more antibodies (Bone and Moore, 2008).The result of antigen-antibody binding can have two outcomes: binding itself may be sufficient to inactivate the antigen or activation of complement will occur, leading to the formation of lytic complexes and ultimately cellular destruction (Smyth, 1994). Stimulation of T-cells occurs once receptors on the cell recognize a pathogen that is associated with a major histocompatibility (MH) marker on an APC (Magnadóttir, 2010). There are two classes of MH molecules, class I and class II (Smyth, 1994). Antigen epitopes arising from within the APC (e.g., from intracellular parasites) bind to class I molecules (Smyth, 1994). In contrast, epitopes from antigens of extracellular parasites bind to class II molecules (Smyth, 1994). T-cells will differentiate into one of 2 forms, cytotoxic T-cells ($CD8^+$) (Th1 response) or helper T-cells ($CD4^+$) (Th2 response) (Wagner *et al*., 2008). Cytotoxic T-cells ($CD8^+$) will attack and destroy host cells that are infected with viruses or other intracellular pathogens (Bone and Moore, 2008). Unlike the NCC's of the innate immune system, $CD8^+$ cells require specific antigen presentation to recognize target cells (Bone and Moore, 2008). Helper T-cells ($CD4^+$) are responsible for the activation of B-cells and $CD8^+$ cells (Bone and Moore, 2008).

1.3 Factors that affect the immune response in fish

The development of both the innate and acquired immune response in fish is affected by several factors, most notably: temperature, stress and age (Watts *et al*., 2001; Uribe *et al*., 2011). In the low temperature ranges for an individual species, immunosuppressive effects have been observed (Watts *et al*., 2001). Antibody synthesis can be particularly impaired by colder thermal extremes (Watts *et al*., 2001); whereas, innate components of immunity are typically more active at colder temperatures (Magnadóttir, 2010). Virgin T-cells and B-cells

are the most affected; memory T-cells and macrophages are seemingly less affected (Watts *et al*., 2001). MacArthur *et al*. (1983) reported no difference in the clearance rate of turbot erythrocytes from circulation in plaice (*Pleuronectes platessa*) acclimated at 5^{o}, 12^{o} and 19^{o} C, suggesting that phagocytosis is temperature-independent in that species.

Chronic stress, which is typically measured by increased circulating cortisol, is generally immunosuppressive for both the innate and specific responses (Watts *et al*., 2001). Depression of phagocytic activity (i.e., mean number of bacteria phagocytized) has been observed in rainbow trout (*Oncorhynchus mykiss*) within three hours of acute stress (Narnaware *et al*., 1994).

1.4 Immune response to parasites

Successful parasitism comes from the parasite being able to (i) evade the host's immune response or (ii) suppress the host immune response (Sher *et al*., 2003). Thus, host-parasite relationships have co-evolved to include a strictly regulated immune response; uniquely characterized by the induction of a $CD4^{+}$ cell response (Sher *et al*., 2003).

Populations of helper T-cells will become differentiated into one of 2 subsets, Th1 or Th2, following presentation of an antigen to $CD4^{+}$ cells (Wagner *et al*., 2008). In mammals, T- helper cells are produced in the thymus (Manning and Nakanishi, 1996). Th1 cells produce interferon-γ (IFN-γ) and are particularly effective against intracellular organisms (Roitt, 1997). The invasion of phagocytic cells by intracellular organisms will trigger secretion of interleukin-12 (IL-12) which in turn stimulates IFN-γ production by natural killer (NK) cells (Roitt, 1997). Conversely, Th2 cells secrete the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 and are highly adapted to defense against extracellular parasites (Roitt,

1997; Sher *et al*., 2003; Diaz and Allen, 2007). The development of Th1 and Th2 responses following cytokine regulation indicates a mechanism by which innate immunity guides the effector T- cell response and coordinates the host's response to eliminate a parasite (Weaver *et al*., 2006). Development of Th1 immunity is enhanced by signals from the innate immune response; whereas, Th2 immunity could arise in response to extrinsic IL-4 or as a default pathway following inhibition of innate immune signals (Murphy and Reiner, 2002).

Parasites that fail to stimulate tumor necrosis factor-α (TNF-α) production, followed by NK cell activation, induce a Th2 response; whereas, parasites that promote IFN-γ production by stimulating interactions of infected macrophages with NK or T-cells will direct a Th1 response (Leiby *et al*., 1994). The development of a Th2 response is considered to be the immune system's adaptation to counteracting effects of parasites (Díaz and Allen, 2007). The Th2 response contains elements such as alternatively activated macrophages (AAMs) that produce proteins during injury; thereby introducing a mechanism of tissue-repair (Díaz and Allen, 2007). Many of the processes of Th2 responses promote the containment of large bodies (i.e., protozoan parasites) through granuloma formation and matrix deposition (Allen and Wynn, 2011).

Fish possess lymphocyte populations analogous to the T- and B- cells of mammals and the thymus is also believed to be the source of helper T-cells (Manning and Nakanishi, 1996). Moreover, T-cell antigen receptors that interact with MH class I and II molecules have been identified in rainbow trout (Manning and Nakanishi, 1996). Many of the cytokines responsible for the differentiation of helper T-cells and resulting Th1/Th2 responses have been described in various fish species but the occurrence of, and switching between, the two responses has not yet been fully described (Bird *et al*., 2006; Buchmann, 2012). However,

evidence for a Th2-like response in fish exists. Lack of an inflammatory response, typical of proinflammatory cytokines and a Th1 response, but upregulation of genes encoding SAA and immunoglobulin M (IgM) (similar to a mammalian Th2 response) occurs in certain strains of Atlantic salmon (*Salmo salar*) infected with *Gyrodactylus salaris* (Kania *et al*., 2010). In contrast, a more susceptible Atlantic salmon strain developed a sustained inflammatory reaction that had a negligible effect on the parasite (Kania *et al*., 2010). In carp (*Cyprinus carpio*) infected with *Trypanosoma borreli*, there is evidence of parasite-specific antibodies that mediate parasite attachment to activated macrophages and subsequent parasite lysis by nitric oxide (NO) (Wiegertjes *et al*., 2005). Similarly, carp infected with *Sanguinicola inermis* are capable of localized granuloma formation and encapsulation of parasite eggs by eosinophils, neutrophils and macrophages (Richards *et al*., 1996; Wiegertjes *et al*., 2005).

1.5 Lepeophtheirus salmonis

1.5.1 Biology of *L. salmonis*

Lepeophtheirus salmonis (Copepoda: Caligidae) is a parasitic copepod that exists naturally on salmonids (Salmonidae) (Pike and Wadsworth, 1999). *L. salmonis* has a direct life cycle, requiring only one host for completion (Hayward *et al*., 2011). The life cycle of *L. salmonis* consists of ten stages: two planktonic, free-living naupliar stages, one infective copepodid stage, four chalimus stages, two pre-adult stages, followed by the final adult stage (Pike and Wadsworth, 1999; Hayward *et al*., 2011). The chalimus stages are attached to their host by a frontal filament and are non-motile (Hayward *et al*., 2011). The pre-adult and adult stages are motile and can freely move over the surface of the fish host or swim in the water column, transferring from one fish host to another (Ritchie, 1997; Hayward *et al*., 2011).

The distribution of *L. salmonis* is circumpolar in the northern hemisphere, being widespread in both the North Atlantic and North Pacific oceans (Hayward *et al*., 2011). Recently, it has been determined that the two populations (Atlantic and Pacific Ocean) of *L. salmonis* do not interbreed and belong to different lineages (Yazawa *et al*., 2008). The Pacific form of *L. salmonis* co-evolved with Pacific salmon (*Oncorhynchus* spp.) and the Atlantic form co-evolved with Atlantic salmonids (*Salmo* spp.) independently (Yazawa *et al*., 2008; Hayward *et al*., 2011); this geographic isolation of the two populations may impact differences in susceptibility to *L. salmonis* infection observed between Pacific and Atlantic salmon (see below).

1.5.2 Species susceptibility to *L. salmonis* infection

Susceptibility to *L. salmonis* infection is influenced by numerous interacting factors including: stress and nutritional status, effectiveness of the host's immune system and genetics (reviewed in Pike & Wadsworth, 1999; Hayward *et al*., 2011).

Laboratory studies comparing susceptibility of coho (*Oncorhynchus kisutch*), chinook (*O. tshawytscha*), and Atlantic salmon (*Salmo salar*) observed differences in copepod intensity at 5, 15 and 20 days post-infection (dpi); with coho exhibiting less parasites than the other species (Johnson and Albright, 1992). Similarly, inflammation was observed in the dermis of coho salmon at 1 dpi, while little to no inflammatory response was observed in Atlantic salmon and chinook salmon up to 20 dpi (Johnson and Albright, 1992). Likewise, a suppression of macrophage respiratory burst activity and phagocytic capacity following *L. salmonis* infection has been reported for Atlantic salmon at 14 and 21 dpi (Mustafa *et al*., 2000a, Fast *et al*., 2002), while there was no inhibition of these activities observed for coho

salmon (Fast *et al*., 2002). Atlantic and chinook salmon appear to have similar susceptibilities; however, the age structure of *L. salmonis* on the salmon species differs following infection, suggesting that the copepod develops faster on Atlantic salmon (Johnson and Albright, 1992). Physiological differences between pink (*O. gorbuscha*) and chum (*O. keta*) salmon experimentally infected with *L. salmonis* copepodids have also been observed. Pink salmon had consistently fewer *L. salmonis* than sized-match chum salmon at 14 dpi (Jones *et al*., 2007). Additionally, following a high exposure to *L. salmonis* (735 copepodids $fish^{-1}$), chum salmon had a lower weight than control fish; whereas, there was no difference in weight for pink salmon (Jones *et al*., 2007). Recent studies suggest juvenile (> 0.7 g) pink salmon have a threshold tolerance of 7.5 *L. salmonis* g^{-1} (Jones and Hargreaves, 2009); whereas, Atlantic salmon smolts (~ 60 g) can experience detrimental effects at an intensity of only 0.75 *L. salmonis* g^{-1} (Finstad *et al*., 2000).

Lepeophtheirus salmonis secretes trypsin-like proteases and prostaglandin E_2 (PGE_2) during feeding (Firth *et al*., 2000; Fast *et al*., 2003; Fast *et al*., 2004). It was observed that in the presence of mucus isolated from rainbow trout and Atlantic salmon, a greater percentage of *L. salmonis* produced low molecular weight (LMW) proteases than lice exposed to mucus isolated from coho salmon, suggesting a preference for host mucus (Fast *et al*., 2003). Following exposure to PGE_2 and secretions isolated from *L. salmonis*, expression of IL-1β and MH class I decreased in head kidney macrophages isolated from Atlantic salmon (Fast *et al*., 2007). In arthropod parasites, trypsin-like proteases inhibit phagocytosis in monocytes. Such enzymes would aid *L. salmonis* by decreasing host phagocytic activity and immune responses after infection (Fast *et al*., 2003). Prostaglandins are thought to regulate vasodilation, anti-coagulation and T-lymphocyte regulation (Fast *et al*., 2007). The secretion

of proteases and PGE_2 has been hypothesized as one mechanism by which *L. salmonis* evades host immune responses (Fast *et al*., 2004; Fast *et al*., 2007)

1.5.3 Pathophysiology of *L. salmonis* infection

Parasitic copepods feed on host mucus, tissue and blood (Johnson *et al*., 2004). The attachment and feeding activities of copepodid and chalimus stages of *L. salmonis* can cause localized tissue damage and minor tissue responses on most hosts (Johnson *et al*., 2004). Later stages in the life cycle (pre-adult and adult) typically elicit only minor tissue damage except in situations of high infection intensity (Johnson *et al*., 2004). With severe *L. salmonis* infections, extensive skin erosion and hemorrhaging has been observed on the head and back and in the peri-anal region (Johnson *et al*., 2004). Depending on the severity of infection, physiological effects of *L. salmonis* infection can include: stress responses, immunological impairment and osmoregulatory dysfunction (Johnson *et al*., 2004; Jones *et al*., 2008; Hayward *et al*., 2011). In British Columbia, heavy infestations on farmed salmonids and damage due to *L. salmonis* are rare; therefore, sea lice are not considered a serious health concern (Saksida *et al*., 2011)

Pathology is typically associated with pre-adult and adult stages of the copepod (Jónsdóttir *et al*., 1992). Skin collected from Atlantic salmon following infection demonstrated a depressed oval ring corresponding to the dimensions of *L. salmonis* marginal membrane (Jónsdóttir *et al*., 1992). In naturally and experimentally infected Atlantic salmon, tissues below the cephalothorax of the parasite were severely damaged. Conversely, inflammation was more pronounced in areas surrounding the site of attachment rather than directly under the louse (Jónsdóttir *et al*., 1992). Similarly, experimentally infected coho,

Chinook and Atlantic salmon demonstrated partial to complete erosion of the epidermis on the fins at the attachment and feeding sites of *L. salmonis* copepodids (Johnson and Albright, 1992). In addition to the direct effects of *L. salmonis* on the epidermis of fish, an indirect, stress-associated response in salmon skin has been observed and includes: necrosis in pavement cells, increased apoptosis in inner cell layers and enhanced mucus production (Nolan *et al*., 1999).

Under circumstances of heavy infestation by *L. salmonis*, host death can occur (Pike and Wadsworth, 1999). Death is usually associated with the development of secondary infections (bacteria, fungi or viruses) or in the most severe cases, osmotic stress, through extensive tissue damage (Johnson *et al*., 1996; Johnson *et al*., 2004). At high infection levels (>0.5 lice g^{-1} fish), feeding on blood by *L. salmonis* can lead to anemia in Atlantic salmon (Wagner & McKinley, 2004; Hayward *et al*., 2011).

1.5.4 *L. salmonis* as a vector

The attachment of lice and their subsequent feeding can cause the breakdown of the protective mucous layer leading to dermal lesions (Bowers *et al.*, 2000), increasing the host's susceptibility to secondary infections (Nylund *et al.*, 1993; Mustafa *et al.*, 2000b). Studies with infectious salmon anemia (ISA) have demonstrated that sea lice (*L. salmonis*) may be able to carry the infectious virus among fish (Nylund *et al.*, 1993; Rolland and Nylund, 1998). Infectious pancreatic necrosis (IPN) virus (Johnson *et al*., 2004) and salmonid alphaviruses (SAVs) (Petterson *et al*., 2009) have also been isolated from *L. salmonis*. In British Columbia, the pathogenic bacteria *Tenacibaculum maritimum, Pseudomonas fluorescens* and *Vibrio* spp. have been isolated from the exoskeleton of *L. salmonis* and

within the internal gut contents (Barker *et al.*, 2009). In Norway, *Aeromonas salmonicida* has been isolated from homogenized sea lice recovered from Atlantic salmon with furunculosis (Nese and Enger, 1993). More recently, adult *L. salmonis* exposed to infectious haematopoietic necrosis (IHN) virus (Jakob *et al*., 2011) or *A. salmonicida* (Novak *et al*., 2012) through parasitizing infected salmon hosts were capable of acquiring the virus or bacteria and successfully transferring it to naïve hosts. Such studies have led to the hypothesis that sea lice could be vectors of viral and bacterial pathogens of salmonids. If sea lice can be suitable vectors, it is important to understand what factors, at the cellular level of this parasite-host interface, promote such a relationship.

1.6 Research objectives and hypotheses

There were two main objectives to this study. First, I wanted to determine if innate immunity is altered in salmon hosts as a result of secretory products isolated from *L. salmonis*. Specifically, I wished to examine the effect of *L. salmonis* secretions on phagocytic activity of macrophages. Using the macrophage-like cell line, SHK-1, the phagocytosis of bacteria (*Aeromonas salmonicida*) was examined in the presence/absence of *L. salmonis* secretions. I hypothesized that, in the presence of *L. salmonis* secretions, phagocytosis of bacteria would be impaired in SHK-1 cells.

Second, based on the reported differences in susceptibility to *L. salmonis* infection among salmon species, I wanted to determine if macrophages isolated from various salmon species would respond differently following exposure to *L. salmonis* secretions. Macrophages isolated from pink (*O. gorbuscha*), chum (*O. keta*) and Atlantic (*S. salar*) salmon were subjected to phagocytosis and respiratory burst assays in the presence/absence

of *L. salmonis* secretions. I predicted that, in the presence of *L. salmonis* secretions, macrophages isolated from pink, chum and Atlantic salmon would exhibit reduced phagocytosis and respiratory burst responses; however, impairment of macrophage function would be more severe in chum and Atlantic salmon.

2 Modulation of cellular innate immunity by *Lepeophtheirus salmonis* secretory products

2.1 Introduction

The innate immune response is the first line of defense for any animal but plays a significant role in immunity for fish (Woo, 1992; Magnadóttir, 2006). Upon exposure to a foreign antigen, the innate immune response will react rapidly in a non-specific manner (Tort *et al.*, 2003). The components of innate immunity are commonly divided into physical parameters (mucus and skin), cellular factors (macrophages, neutrophils, granulocytes and non-specific cytotoxic cells), and humoral factors (complement, lysozyme or neutral proteases, acute phase proteins, transferrin, chemokines and cytokines) (Woo, 1992; Magnadóttir, 2006; Whyte, 2007).

An important component of teleost innate immunity is the phagocytic cells (neutrophils, macrophages and granulocytes). On the surface of phagocytic cells are toll-like receptors (TLRs) that recognize and bind pathogen associated molecular patterns (PAMPs) (Magnadóttir, 2006; Whyte, 2007; Magnadóttir, 2010). Typical PAMPs include: lipopolysaccharide, peptidoglycan and mannose-binding lectin (Magnadóttir, 2006; Whyte, 2007). When a ligand binds to a TLR, phagocytes are stimulated resulting in phagocytosis, upregulation of proinflammatory cytokines, production of reactive oxygen intermediates (ROIs) and antigen presentation to cells of the specific immune system (i.e., T and B cells) (Watts *et al*., 2001; Basset *et al.*, 2003; Alvarez-Pellitero, 2008).

The sea louse, *Lepeophtheirus salmonis*, is an ectoparasite commonly reported on farmed and wild salmon (*Salmo* and *Oncorhynchus* spp.) in British Columbia (Pike and Wadsworth, 1999; Marty *et al*., 2010) The establishment and infection longevity of this

parasite on salmon hosts is related to the development, or lack thereof, of an inflammatory response at the site of louse attachment (Johnson and Albright, 1992; Jónsdóttir *et al*., 1992). Coho salmon (*Oncorhynchus kisutch*) are capable of eliciting a pronounced inflammatory reaction at louse attachment sites; whereas, among more susceptible species, such as Atlantic salmon (*Salmo salar*), development of inflammation is minimal (Johnson and Albright, 1992). Reduced inflammation at the site of parasite feeding and attachment could be related to immunosuppressive molecules (prostaglandin E_2, trypsin-like protease, cathepsin B) secreted by the louse during feeding (Firth *et al*., 2000; Fast *et al*., 2004; Fast *et al*., 2007; McCarthy *et al*., 2012). Fast *et al*. (2004) showed that expression of IL-1β and MH class 1 significantly decreased in Atlantic salmon head kidney macrophages following incubation with PGE_2 and/or secretions isolated from *L. salmonis*. However, no observations were made on how this affected functionality (i.e., phagocytosis and respiratory burst) of the immune cells.

The aim of this study was to determine if secretory and excretory products (SEPs) isolated from *L. salmonis* affect the acute immune response of fish. Specifically, we examined the effect of SEPs on macrophage function. The first objective was to determine if SEPs had any effect on macrophage phagocytosis using the cell line, SHK-1. Second, to explore any species-related differences, phagocytosis and respiratory burst assays were performed using macrophages isolated from pink (*O. gorbuscha*), chum (*O. keta*), and Atlantic (*S. salar*) salmon. Based on previously reported suppression of IL-1β and MH class 1 in macrophages exposed to SEPs (Fast *et al*., 2004), we expect that SEPs will impair phagocytosis and respiratory burst in salmon macrophages. Also, related to the variability to

L. salmonis infection among salmon species, we expect that impairment of macrophage function will be more severe in species that are more susceptible to sea lice.

2.2 Materials and Methods

2.2.1 Sea lice collection

Adult male and female *L. salmonis* (n ~ 3000) were collected from farmed Atlantic salmon (*Salmo salar*) at a Marine Harvest Canada site located in the Broughton Archipelago, British Columbia (latitude: 50° 42' 46" N; longitude: 126° 42' 03" W) during a fish harvest (November 22, 2010). Upon removal from fish, live sea lice were maintained in aerated, autoclaved saltwater (ASW) on ice until being returned to the lab where they were stored at 10 ^{o}C in ASW for a maximum of 24 hours until needed.

2.2.2 Collection of sea lice secretory products

Following Fast *et al.* (2007), adult *L. salmonis* were washed in ASW, placed in 50 mL centrifuge tubes containing 0.25 mM dopamine (DA) (Sigma) dissolved in ASW. Approximately 125 lice were placed in each tube at a density of 3 lice mL^{-1}. Lice were then incubated at 15 ^{o}C for 45-60 minutes. Following incubation, lice were aseptically removed, using forceps, from the tube and the solution was passed through a 0.45 μm filter to remove large debris (referred to as whole secretions) and stored at -80 ^{o}C until additional filtration could be performed. Next, whole secretions were filtered further using Jumbosep™ Centrifugal Devices (Pall Life Sciences, Ann Arbor, MI) following manufacturer's instructions (2800·g for 20 minutes at 4 ^{o}C) . The Jumbosep™ Centrifugal Devices were fitted with a 30 kilodalton (kDa) membrane and the filtrate retained. A 30 kDa membrane was chosen since the largest known component of *L. salmonis* secretions has a molecular

weight of 22 kDa (Firth *et al*., 2000). The filtrate was dispensed into 1.5 mL Eppendorf tubes and stored at -80 ^{o}C (referred to as fractionated secretory and excretory products or SEPs). To determine that any effects observed on macrophages were not due to the presence of DA, a control was prepared with 0.25 mM DA and ASW in the absence of *L. salmonis* and used as a control in cell culture analysis.

2.2.3 Protein quantification secretions

To determine the relative protein concentration in *L. salmonis* secretions the Quick Start™ Bradford Protein Assay (Bio Rad) was used following manufacturer's instructions. Basically, coomassie brilliant blue G-250 binds to proteins and is detectable at 595 nm. Fractionated secretions (150 µL) and known concentrations (0-100 µg mL^{-1}) of a protein standard (bovine gamma-globulin) (150 µL) were added to a 96-well microplate. Each well then received 150 µL of the 1x Dye reagent and was incubated for a minimum of 5 minutes at room temperature. Following incubation, absorbance was measured once at 595 nm using a SpectraMax 190 microplate reader (Molecular Devices).

2.2.4 PGE_2 concentration in secretions and fractions

The concentrations of PGE_2 in fractionated secretions used for cell culture was determined by a competitive Prostaglandin E_2 ELISA kit (Thermo Scientific) according to the manufacturer's instructions. Absorbance was measured at 405 nm minus the absorbance at 570 nm using a SpectraMax 190 microplate reader (Molecular Devices). Analysis of data was performed using MasterPlex® ReaderFit: Curve-Fitting Software.

2.2.5 Bacteriology

Aeromonas salmonicida (virulent strain 2011-247) was obtained from the Fish Health Unit of the Pacific Biological Station, Nanaimo, British Columbia (Department of Fisheries and Oceans Canada) and used in all experiments. To ensure virulence, naïve Atlantic salmon (*Salmo salar*) were challenged with live A. *salmonicida*; upon death, *A. salmonicida* was isolated from the head kidney of fish. Pure *A. salmonicida* colonies were grown on Difco™ Tryptic Soy Agar (TSA) at 22 ^{o}C for 48 hours, suspended in sterile saline and transferred into 2 mL Eppendorf tubes. The cell suspension was centrifuged, supernatant removed, and tubes re-filled with trypticase soy broth containing 15 % glycerol. The culture was stored at -80 ^{o}C until needed. Prior to use, frozen samples were thawed at room temperature (2-4 hours) plated on TSA and grown for 48 hours at 22 ^{o}C.

For phagocytosis assays, *A. salmonicida* colonies were suspended in phosphate buffered saline (PBS) and adjusted to a concentration of ~1.0 x 10^{7} cells mL^{-1} using MacFarland Turbidity Standards (Whitman, 2004). To confirm the concentration obtained, a dilution series was prepared, 0.1 ml of each subsequent dilution was drop inoculated, spread on TSA and enumerated. The bacterial suspensions were prepared approximately 0.5-1.0 hour prior to use in assays.

2.2.6 Cell culture

The Atlantic salmon head kidney (SHK-1) cell line is a continuous cell line derived from Atlantic salmon (*S. salar*) head kidney leucocytes. The cell line is considered to have the same properties as macrophages; however, it is unable to successfully kill bacteria after phagocytosis (Dannevig *et al*., 1997). SHK-1 cells were cultured at 20 ^{o}C in 25 cm^{2} tissue-

culture treated flasks with Leibovitz-15 (L-15) medium supplemented with L-glutamine (Gibco), 400 µL mercaptoethanol (1000x, Gibco) and 5 % heat inactivated fetal bovine serum (FBS) (Gibco). Upon reaching confluence, cells were subcultured by addition of 0.05 % trypsin-EDTA (Gibco) for 1.5-4.0 minutes followed by repeated agitation using a pipette to dislodge cells from the surface of the flask. Cell viability and density was determined by trypan-blue exclusion (0.4 %, Gibco) (Strober, 2001) and direct count using a haemocytometer (10 x magnification) respectively.

2.2.7 Diff-Quik phagocytosis assay

SHK-1 cells (passage number 64-70) were suspended in L-15 medium supplemented with L-glutamine, 400 µL mercaptoethanol (1000 x), 5 % heat inactivated FBS, 0.5 % gentamycin (10 mg mL^{-1}) (Sigma) and 1.5 % HEPES (1 M) buffered solution (Gibco). These cells were plated (~2.0 x 10^5 cells ml^{-1}) in four-well glass chamber slides (1 mL working volume) (Lab-Tek® II) and incubated for 24 hours at 20 ^oC. After incubation, the media was aspirated and cells were rinsed twice with room temperature phosphate-buffered saline (PBS) to remove non-adherent cells. Cells were then challenged with one of the following treatments (100 µL $well^{-1}$): (i) SEPs; (ii) live *A. salmonicida* (A. sal) cells (10^7 cells ml^{-1}); (iii) SEPs + live A.sal cells; (iv) PBS; (v) DA solution, or (vi) DA solution + live A. sal cells. Once each treatment had been applied to the cells, 1.0 mL of L-15 supplemented with L-glutamine, 400 µL mercaptoethanol (1000x) and 5 % heat inactivated FBS was added to each well and cells were incubated for 3.0 hours at 20 ^oC. Ten replicates were prepared for each treatment. After incubation, the media was removed, slides were rinsed twice with room temperature PBS, air-dried and stained with Diff-Quik®. After staining, 200 cells per well were examined using a microscope (1000 x) and the number of phagocytic cells and number

of internalized bacteria were recorded. The total number of live cells was also determined per well for all treatments. Cells were not counted if there appeared to be severe damage to the cell membrane or nucleus (i.e., nuclear membrane was no longer intact).

2.2.8 Fish

Atlantic salmon (*Salmo salar*, n = 750 - 800, $\bar{x}$ weight = 15.9 g) were transferred (August 8th, 2010) from Freshwater Farms Hatchery (Marine Harvest Canada) in Duncan, British Columbia via an aerated 1000L plastic tank (D.O. = 11.7 mg L^{-1}; T = 13.5 ^{o}C; pH = 7.9), to the Pacific Biological Station, Nanaimo, BC, Canada. Fish were held in two 1800 L freshwater tanks maintained at 12-13 ^{o}C. The fish were smolted by increasing the seawater: freshwater ratio by ¼ every 4 to 5 days. For use in trials, fish (n = 150, $\bar{x}$ weight = 508 g) were transported (March 14th, 2012) to Vancouver Island University (VIU) and maintained in 1800 L tanks with recirculated seawater treated via UV sterilizers at 9-11 ^{o}C. Photoperiod was kept at 12-hour ambient light and 12-hour dark and fish were fed daily, at 1.5 % of their body weight, a pelleted diet (BioOregon®).

2.2.9 Primary culture

Following euthanization in 300 mg L^{-1} tricaine methanesulfonate (TMS), head kidney was immediately removed aseptically using a sterile scalpel and forceps and placed in L-15 medium supplemented with 2 % heat inactivated FBS, 10 units mL^{-1} heparin and 100 units mL^{-1} gentamycin. The kidney tissue was dissociated by pressing it through a 250 μm stainless steel mesh screen followed by 3-5 minutes in a tissue stomacher (Lab-Blender 80) and 3 mL of the resulting cell suspensions were layered on top of 3 mL Histopaque 1077 (Sigma) in a 15 mL sterile centrifuge tube. Cells were centrifuged at 400·g for 30 minutes at

15 ^{o}C, the supernatant was discarded, the opaque cell layer removed and placed in a new, sterile 15mL centrifuge tube. The cells were then washed by centrifugation in room temperature PBS (250·g at 15 ^{o}C) for 10 minutes and the supernatant was removed. The resulting cell pellet was resuspended twice more in PBS and centrifuged (250·g at 15 ^{o}C) for 10 minutes. The final cell pellet was resuspended in 5 mL L-15 media supplemented L-glutamine and 5 % heat inactivated FBS and 0.05 mg mL^{-1} gentamycin (Sigma). Prior to plating the number of viable cells was determined by trypan blue exclusion (Strober, 2001) and the cell density adjusted to 5.0 x 10^5 cells mL^{-1}.

2.2.10 Macrophage respiratory burst assay

The production of intracellular superoxide anion (O_2^-) by isolated macrophages from three salmon species (pink, chum and Atlantic) was determined microscopically and colorimetrically.

2.2.10.1 Microscopic respiratory burst assay

Pink (*Oncorhynchus gorbuscha*) (n = 9, $\bar{x}$ weight = 101.9 g) and chum (*O. keta*) (n = 7, $\bar{x}$ weight = 90.65 g) salmon were kindly donated from L.M. Braden at the Pacific Biological Station, Nanaimo British Columbia. Pink, chum and Atlantic salmon (n = 5, $\bar{x}$ weight = 496.8 g) macrophages were isolated (section 2.2.9) and inoculated (1.5 mL) in 8-well plates (Nunc) containing 22 mm circular glass coverslips and incubated 12 hours at 20 ^{o}C. The media was then removed and the cells rinsed twice with room-temperature PBS to remove non-adherent cells. Adherent cells were challenged with one of the following treatments (100 µL well^{-1}): (i) SEPs; (ii) live A. sal cells (10^7 cells mL^{-1}); (iii) SEPs + live A. sal, or (iv) PBS. Once each treatment had been applied to the cells, 100 µL of nitro-blue tetrazolium (NBT) (1 mg mL^{-1}

in PBS) and 1 mL L-15 supplemented with L-glutamine and 5% heat-inactivated FBS was added to each well and cells were incubated for 3.0 hours at 20 ^{o}C. Five replicates of each treatment were included. After incubation, cells were rinsed twice with room temperature PBS, air-dried, fixed with cold methanol (5 minutes), and counter-stained with 1 % safranin O solution (30-60 seconds). The percentage of cells containing blue formazan particles was determined by evaluating 100 randomly selected cells using a microscope (1000 x).

2.2.10.2 Colorimetric respiratory burst assay

Pink, chum and Atlantic salmon macrophages (section 2.2.10.1) were inoculated (1.0 mL) in a 24-well plate and incubated 12 hours at 20 ^{o}C. The media was then removed and the cells rinsed twice with room-temperature PBS to remove non-adherent cells. Adherent cells were challenged and incubated as in section 2.2.10.1. After incubation, cells were rinsed twice with room temperature PBS, then once with cold (4 ^{o}C) methanol, and air-dried. Intracellular NBT was then dissolved by adding 120 µL of 2M potassium hydroxide (KOH), then 140 µL of dimethyl sulfoxide (DMSO) (Sigma), followed by gentle shaking for 10 minutes at room temperature. The solution was then transferred to a 96-well plate and absorbance was read once at 620 nm using a SpectraMax 190 microplate reader (Molecular Devices).

2.2.11 Phagocytosis assay (isolated macrophages)

Macrophages were isolated from pink, chum and Atlantic salmon macrophages (section 2.2.10.1) were inoculated (1.0 mL) in 8-well plates (Nunc) containing 22 mm circular glass coverslips and incubated 12 hours at 20 ^{o}C. The media was then removed and the cells rinsed twice with room-temperature PBS to remove non-adherent cells. Adherent

cells were challenged with one of the following treatments (100 µL well^{-1}): (i) SEPs; (ii) live A. sal cells (10^7 cells mL^{-1}); (iii) SEPs + live A. sal, or (iv) PBS. Plates were incubated for 3.0 hours at 20^o C. Five replicates were included for each treatment. After incubation, cells were rinsed twice with room temperature PBS, air-dried, and stained with Diff-Quik®. After staining, 200 cells per well were examined using a microscope (1000 x) and the number of phagocytic cells and number of internalized bacteria were recorded.

2.2.12 Phagocytosis response curve

To test if various concentrations of *L. salmonis* secretions have different effects on cells, SEPs were diluted ten-fold in PBS to a final concentration of 10^{-4} of the original solution. SHK-1 cells (passage number 66) were suspended in L-15 medium supplemented with L-glutamine, 400 µL mercaptoethanol (1000 x), 5 % heat inactivated FBS, 0.5 % gentamycin (10 mg mL^{-1}) (Sigma), and 1.5 % HEPES (1M) buffered solution (Gibco). Cell density was adjusted to 2.5 x 10^5 cells mL^{-1} and plated in four-well glass chamber culture slides (1 mL working volume) (Lab-Tek® II) and incubated for 24 hours at 20 ^{o}C. After 24 hours, media was aspirated and the cells were rinsed twice with room temperature PBS. Cells were then challenged with (100 µL): (i) SEPs (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4}); (ii) live A. sal cells (10^7 cells mL^{-1}); (iii) SEPs (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4}) + A. sal cells, or (iv) PBS. After application of the treatment, cells were incubated with media (section 2.2.7) for 3.0 hours at 20 ^{o}C. After incubation, the media was aspirated, the cells were rinsed twice with room temperature PBS, air dried and stained with Diff-Quik®. After staining, the first 200 individual cells were observed using a microscope (1000 x) and percentage of damaged cells (section 2.2.7) assessed for all treatments. Additionally, for treatments that contained live A.

sal cells, the number of phagocytic cells and the number internalized bacteria were recorded. Five replicates were completed for each treatment.

2.2.13 Data analysis

Phagocytosis was quantified using the formula (Campbell *et al*., 1995):

$$\text{Phagocytic index (PI)} = \begin{pmatrix}\text{Percent of macrophages}\\ \text{containing}\\ \text{at least one bacterium}\end{pmatrix} \times \begin{pmatrix}\text{Mean number of}\\ \text{bacteria}\\ \text{per positive cell}\end{pmatrix}$$

This phagocytic index accounts for the number of macrophages that are phagocytic and the intensity of phagocytic activity (Campbell *et al*., 1995).

Normality of data sets containing more than two means was confirmed using Shapiro-Wilk and Anderson-Darling tests. If normal, data were statistically analyzed by one-way ANOVA; if data were not normal, significance was determined using Kruskal-Wallis one-way ANOVA. A *p*-value of 0.05 or less was considered statistically significant. Data sets containing only two means were statistically analyzed by either a Student's T-tests (for equal variance) or Welch's T-test (for unequal variance). Variance was determined by equal variance test. All statistical analysis was performed using NCSS[8] statistical software.

2.3 Results

2.3.1 Protein and PGE_2 concentration in secretions

The concentration of protein in fractionated secretory products from *L. salmonis* (SEPs), as determined by Bradford assay, ranged from 27.70 to 39.49 ($\bar{x}$ = 31.19) µg mL^{-1}. The protein concentration of a control dopamine solution was negligible, with a range of 0 to 0.687 ($\bar{x}$ = 0.116) µg mL^{-1}. Using a competitive ELISA kit, the concentration of PGE_2 in SEPs was determined to be in the range of 63.79 to 223.8 ($\bar{x}$ = 123.5) pg mL^{-1}.

2.3.2 Diff-Quick phagocytosis assay- SHK-1 cells

There was no significant difference (T = -1.453, p = 0.1636) in the percent of cells positive for at least one bacterium between cells treated with *A. salmonicida* (A. sal) or SEPs + A. sal (Figure 1). However, cells treated with SEPs + A. sal had significantly more (T = -2.670, p = 0.0156) bacteria per positive cell (3.310 ± 1.055) than cells treated with A. sal (2.304 ± 0.5548). Similarly, cells exposed to SEPs + A. sal had a greater (T = -2.296, p = 0.0339) phagocytic index (223.2 ± 103.0 %) than cells exposed to A. sal alone (136.5 ± 60.28 %). There was also no significant differences (df = 3, F = 0.73, p = 0.5407) in the total number of undamaged SHK-1 cells per well among treatments ($\bar{x}$ = 3760 cells) (Figure 2).

There was no significant difference in the total number of live cells (T = 0.4386, p = 0.6703), percent of cells positive for bacteria (T = 0.8338, p = 0.4349), mean number of bacteria per positive cell (T = -0.0655, p = 0.9490), or the phagocytic index (T = 0.1119, p = 0.9131) for SHK-1 cells exposed to dopamine + A. sal when compared to cells challenged with A. sal (Figure A.1).

2.3.3 Respiratory burst NBT assay- isolated macrophages

The nitroblue tetrazolium (NBT) microscopic assay determined that macrophages isolated from pink, chum and Atlantic salmon undergo oxidative respiratory burst in response to phosphate-buffered saline (PBS), SEPs, SEPs +A. sal, and A. sal (Figure 3). Among species, the greatest (df = 2, F = 12.61, p = 0.0011) percent of NBT-positive cells following stimulation with SEPs was observed in pink salmon macrophages (Figure 4) (31.8 ± 14.8 %) compared to chum (4.0 ± 2.45 %) and Atlantic salmon (10.8 ± 5.02 %) macrophages. Conversely, when challenged with A. sal, Atlantic salmon macrophages had significantly

more (df = 2, F = 7.55, p = 0.0075) NBT-positive macrophages (26.8 ± 4.55 %) than pink salmon (12.4 ± 2.70 %) and chum salmon (11.2 ± 11.0 %). However, when exposed to SEPs + A. sal, there was no difference (df = 2, F = 2.44, p = 0.1289) in macrophage response among the three species (Figure 3). Curiously, pink salmon macrophages had a pronounced response (df = 2, F = 7.74, p = 0.0069) to phosphate-buffered saline (PBS) (14.8 ± 4.44 %) compared to chum (5.6 ± 3.05 %) and Atlantic salmon macrophages (4.6 ± 5.68 %).

When comparing data within species, pink salmon macrophages exposed to SEPs had significantly more (df = 3, F = 5.49, p = 0.0087) NBT- positive cells compared to other treatments. For Atlantic salmon macrophages, the A. sal treatment had significantly more (df = 3, F = 13.79, $p < 0.01$) NBT-positive cells compared to other treatments. There were no differences among any treatments for chum salmon macrophages (df = 3, F = 1.35, p = 0.2938) (Figure 3).

2.3.4 Respiratory burst colorimetric assay- isolated macrophages

Pink salmon macrophages produced the greatest amount of intracellular O_2^- following stimulation with SEPs (df = 2, F = 49.50, p = <0.01), SEPs + A. sal (df = 2, χ^2= 12.2, p = 0.002), and PBS (df = 2, χ^2= 6.75, p = 0.034) (Figure 5) compared to chum and Atlantic salmon macrophages. There was no difference in the intracellular O_2^- production among species following stimulation with A. sal (df = 2, χ^2= 5.66, p = 0.059) or in control wells (no cells) (df = 2, F = 3.74, p = 0.0881).

Within species, pink salmon macrophages produced significantly more (df = 4, F= 14.43, $p < 0.01$) intracellular O_2^- following stimulation with SEPs than any other treatment. However, there were no differences in intracellular O_2^- production among treatments for

chum salmon (df = 4, χ^2= 7.29, p = 0.121) and Atlantic salmon macrophages (df = 4, χ^2= 6.19, p = 0.185) (Figure 5).

2.3.5 Phagocytosis assay-isolated macrophages

When exposed to A. sal, macrophages isolated from chum salmon had significantly more (df = 2, F = 9.39, p = 0.0035) cells containing at least one bacterium than macrophages isolated from Atlantic salmon; however, there was no difference when compared to pink salmon (T = 2.292, p = 0.0511) (Figure 6). Curiously, there was no difference (df = 2, F = 1.78, p = 0.2109) in the mean number of bacteria per positive cell among the three salmon species when exposed to A. sal. Despite this result, the phagocytic index of chum salmon macrophages was significantly higher (df = 2, F = 10.85, p = 0.0020) compared to the pink and Atlantic salmon macrophages following exposure to A. sal. When exposed to SEPs + A. sal, chum salmon macrophages had significantly more (df = 2, F = 18.74, $p < 0.001$) cells positive for at least one bacterium than pink and Atlantic salmon. There was no difference (df = 2, F = 2.64, p = 0.1191) among species in the mean number of bacteria per positive cell with SEPs + A. sal. Again, chum salmon had a significantly greater (df = 2, F = 12.11, p = 0.0013) phagocytic index compared to the other two species when challenged with SEPs + A. sal.

When comparing treatments within species, pink salmon macrophages had significantly more cells positive for bacteria (T = 5.361, $p < 0.01$) and a greater phagocytic index (T = 3.733, p = 0.0058) when challenged with A. sal than when challenged with SEPs + A. sal. However, there was no difference in the mean number of bacteria per positive cell (T = 0.2987, p = 0.7728). For chum salmon macrophages, there was no difference in

percentage of cells positive for bacteria (T = -0.7376, p = 0.4819), mean number of bacteria per positive cell (T = -0.5112, p = 0.6230), and phagocytic index (T = -0.5581, p = 0.5925) between cells exposed to A. sal or SEPs + A. sal. Similarly, Atlantic salmon macrophages had no difference in the percent of cells positive for bacteria (T = -1.021, p = 0.3370), mean number of bacteria per positive cell (T = -0.8807, p = 0.4042), and phagocytic index (T = -1.138, p = 0.2881) when challenged with A. sal or SEPs +A. sal.

2.3.6 Dose response- SHK-1 cells

When exposed to any dilution of SEPs in the presence of *A. salmonicida*, the percentage of undamaged SHK-1 cells was significantly lower (df = 11, F = 79.05, $p < 0.01$) than those cells exposed to PBS, SEPs or *A. salmonicida* alone (Table A.1).

SHK-1 cells challenged with SEPs (10^{-1}) + A. sal and SEPs (10^{-2}) + A. sal had the highest percentage of cells positive for bacteria at 76.8 ± 4.44 % and 77.6 ± 6.35 %, respectively; which were significantly higher (df = 5, F = 4.53, p = 0.0059) than cells exposed to SEPs (10^{-4}) + A. sal (Figure 7). Similarly, SEPs (10^{-1}) + A. sal and SEPs (10^{-2}) + A. sal had a significantly greater (df = 5, F = 5.81, p = 0.0012) phagocytic index than SEPs (10^{-4}) +A. sal. The three least dilute (10^{0}-10^{-2}) preparations of SEPs +A. sal, had significantly more (df = 5, F = 5.64, p = 0.0014) bacteria per positive cell and higher phagocytic indices (df = 5, F = 5.81, p = 0.0012) than A. sal alone.

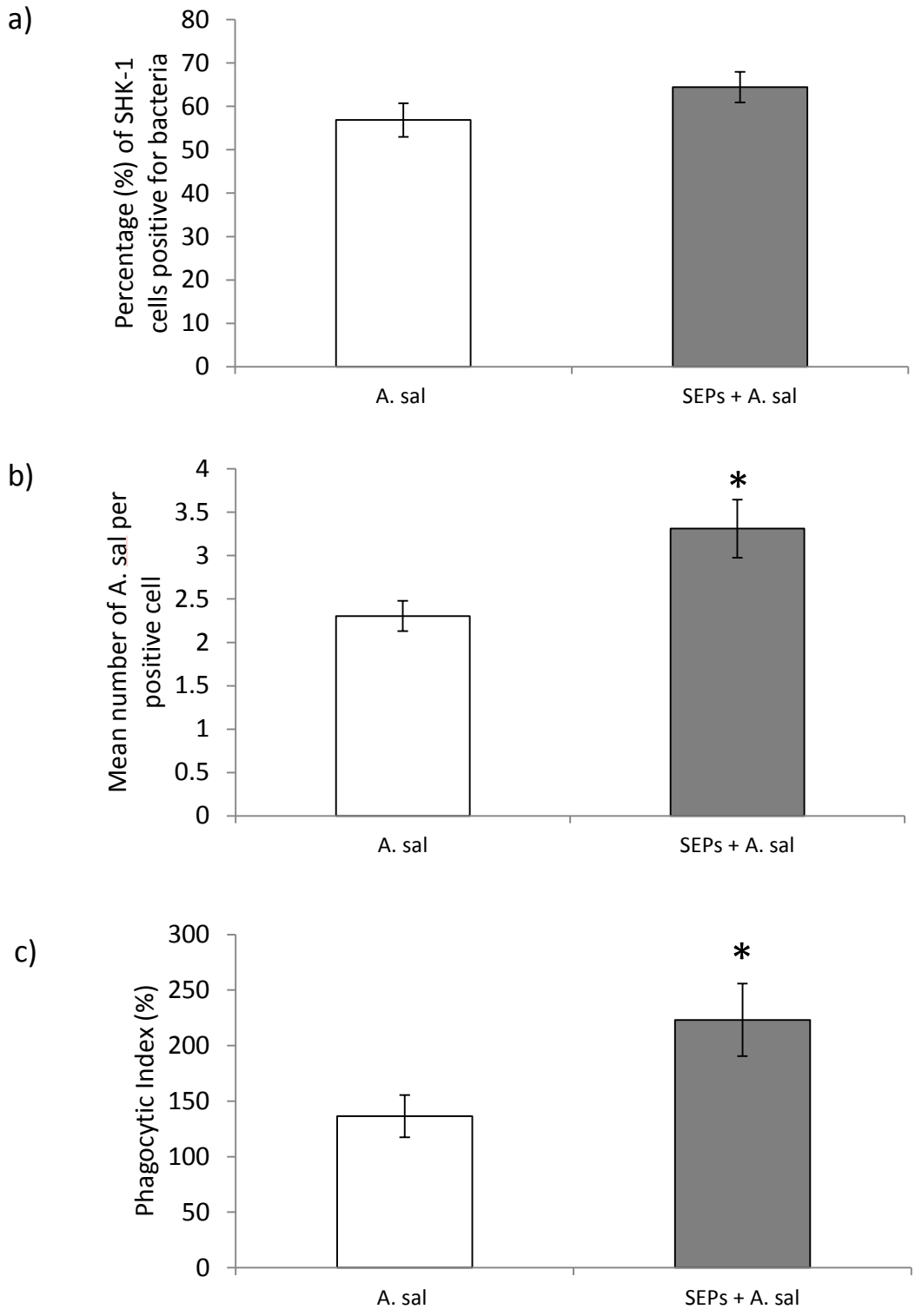

Figure 1. Phagocytic activity of SHK-1 cells: a) percentage of SHK-1 cells positive for at least one bacterium, b) number of bacteria per positive cell, and c) phagocytic index following exposure to A. salmonicida (A. sal) or SEPs + A. sal. Values represent mean (± SE) of 10 replicates per treatment; for each treatment, 200 individual cells were examined per well. Differences between treatments were determined using a two-sample T-test ($p < 0.05$). Symbol (*) represents differences between treatments.

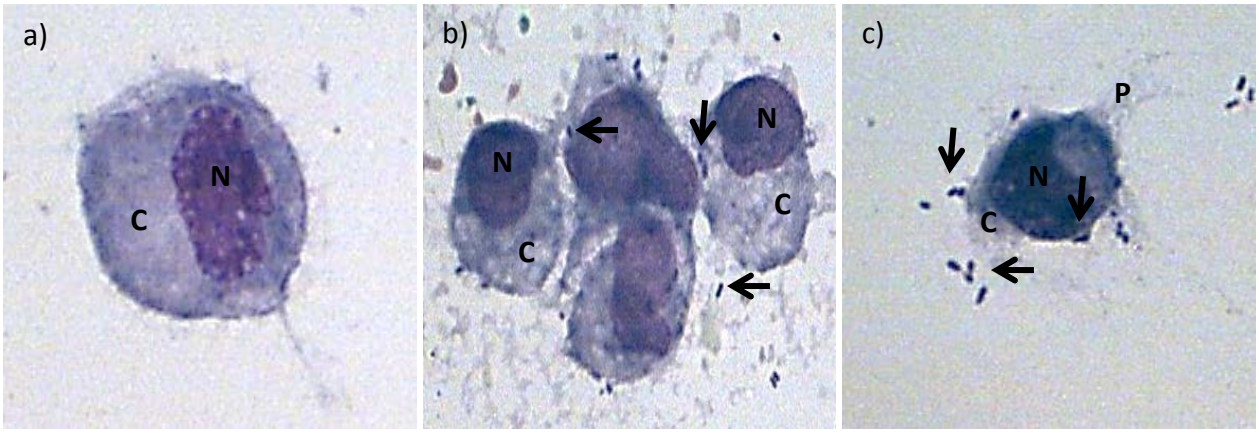

Figure 2. Micrographs of SHK-1 cells stained with Diff-Quik® following exposure to: a) SEPs, b) SEPs + A. sal, and c) A. sal. Arrows represent A. sal cells, N = nucleus, C = cytoplasm, and P = pseudopodia.

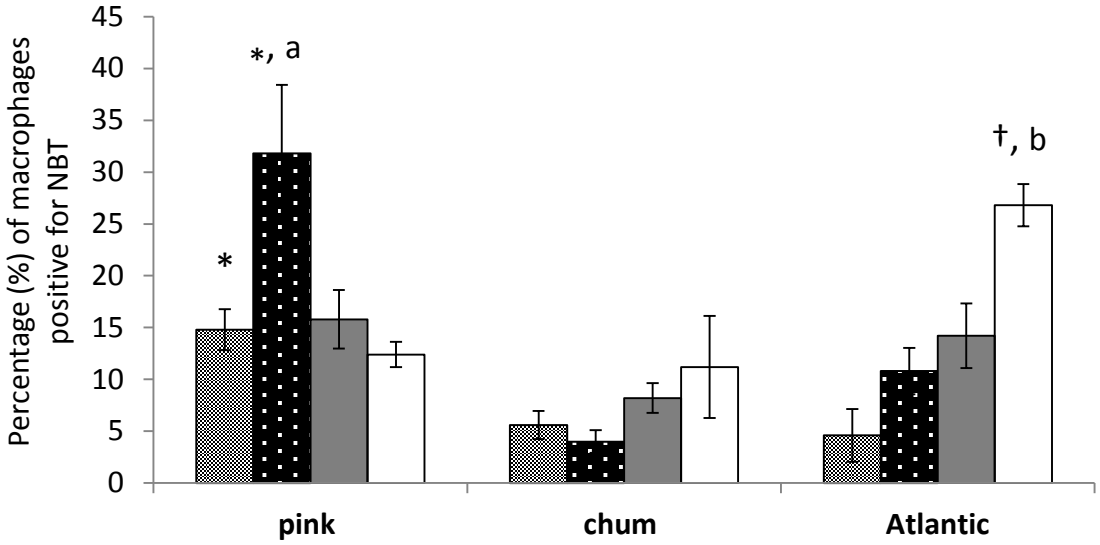

Figure 3. Percentage of pink, chum and Atlantic salmon macrophages positive for nitroblue tetrazolium (NBT) following exposure to: PBS (▨), SEPs (▪), SEPs + A. sal (■), or A.sal (□). Values represent mean (±) SE calculated from 5 replicates per treatment; for each replicate, presence of formazan was determined by microscopically evaluating 100 randomly selected macrophages. Differences among species for each treatment were determined by one-way ANOVA ($p = 0.05$). Within species, significant differences among treatments were determined by one-way ANOVA ($p = 0.05$). Symbols (*, †) represent differences among species. Lowercase letters (a, b) represent significant differences within species.

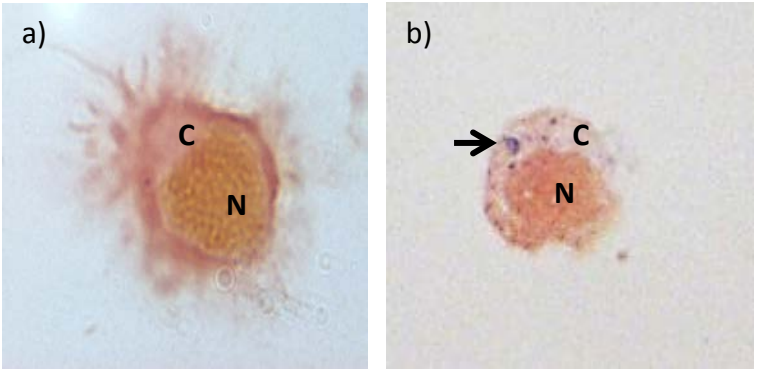

Figure 4. Micrographs of pink salmon macrophages following exposure to SEPs: a) NBT-negative cell, b) NBT-positive cell. Arrows represent formazan deposits within cell, N = nucleus, and C= cytoplasm.

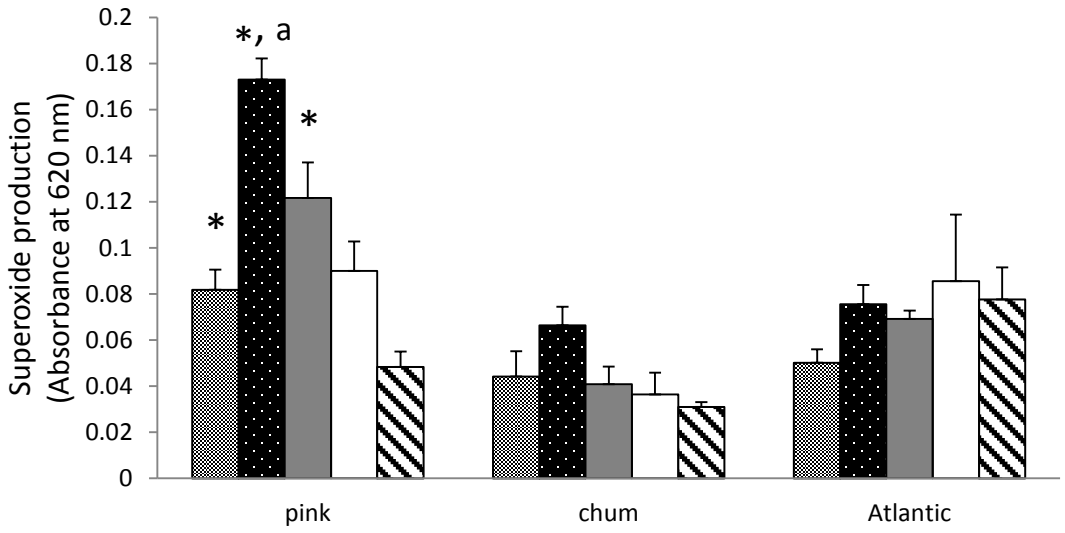

Figure 5. Superoxide (O2-) production in pink, chum and Atlantic salmon macrophages following incubation with PBS (▨), SEPs (▪), SEPs + A. sal (▩), or A. sal (□). Control wells (▧) contained no cells. Values represent mean (± SE) calculated from 5 replicates per treatment. Differences among species for each treatment and within species among treatments were determined using Kruskal-Wallis one-way ANOVA and one-way ANOVA ($p < 0.05$). Symbol (*) represents significant differences among species for a given treatment. Lowercase letter (a) represents significant differences among treatments within species.

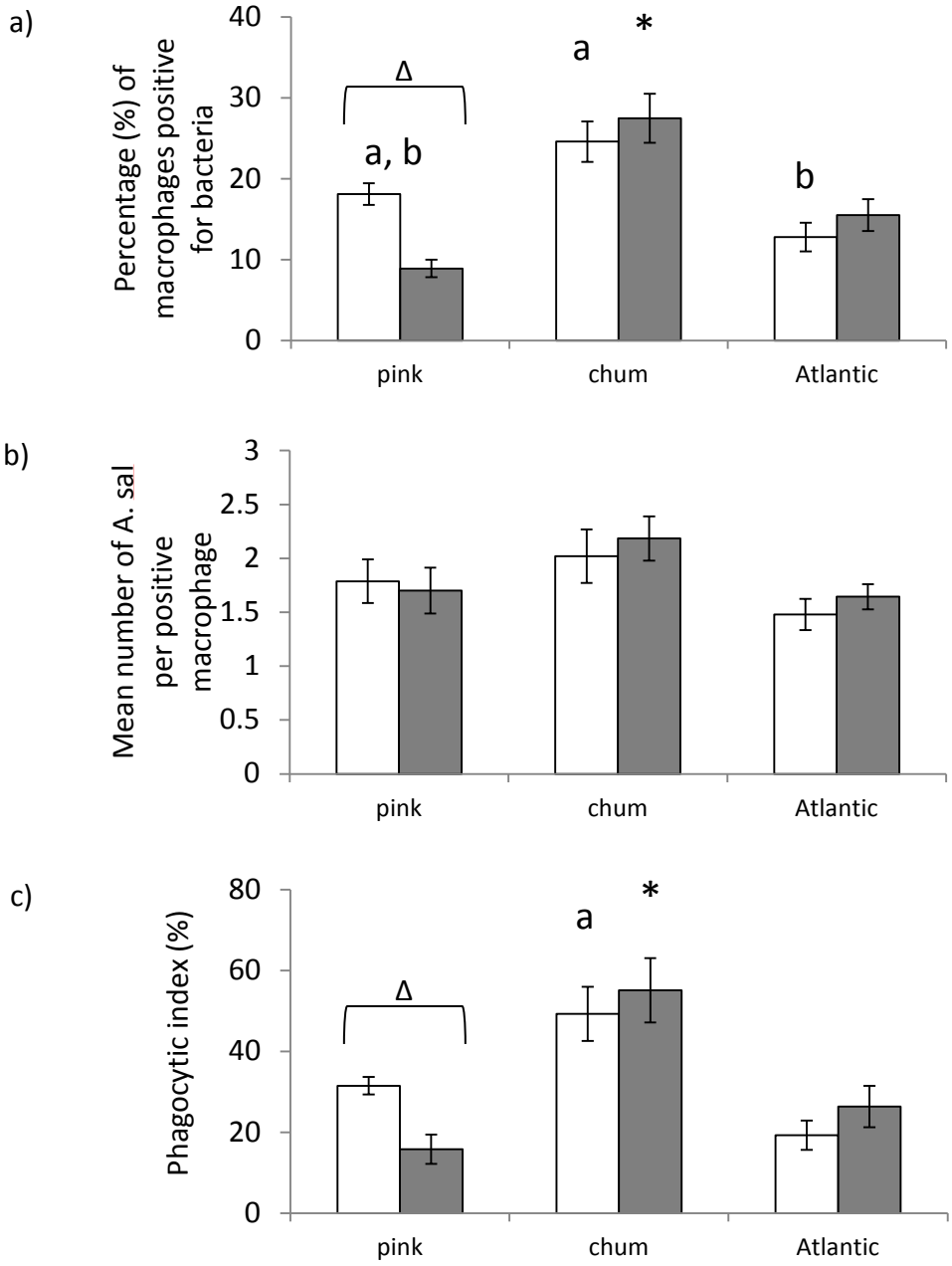

Figure 6. Phagocytic activity of macrophages isolated from pink, chum and Atlantic salmon: a) percentage of macrophages positive for at least one bacterium, b) number of bacteria per positive cell, and c) phagocytic index following exposure to A. sal (□) or SEPs + A. sal (■). Values represent mean (± SE) calculated from 5 replicates per treatment for each species; for each replicate, 200 individual macrophages were examined for the presence of internalized bacteria. Differences among species for each treatment were determined using one-way ANOVA ($p < 0.05$). Two-sample T-tests were performed to determine differences ($p < 0.05$) between treatments within species. Lowercase letters (a, b) represent significant differences among species when macrophages were exposed to A. sal; symbol (*) represents significant differences among species when macrophages were exposed to SEPs + A. sal. Significant differences within species are joined by a bracket and indicated by a Greek letter (Δ).

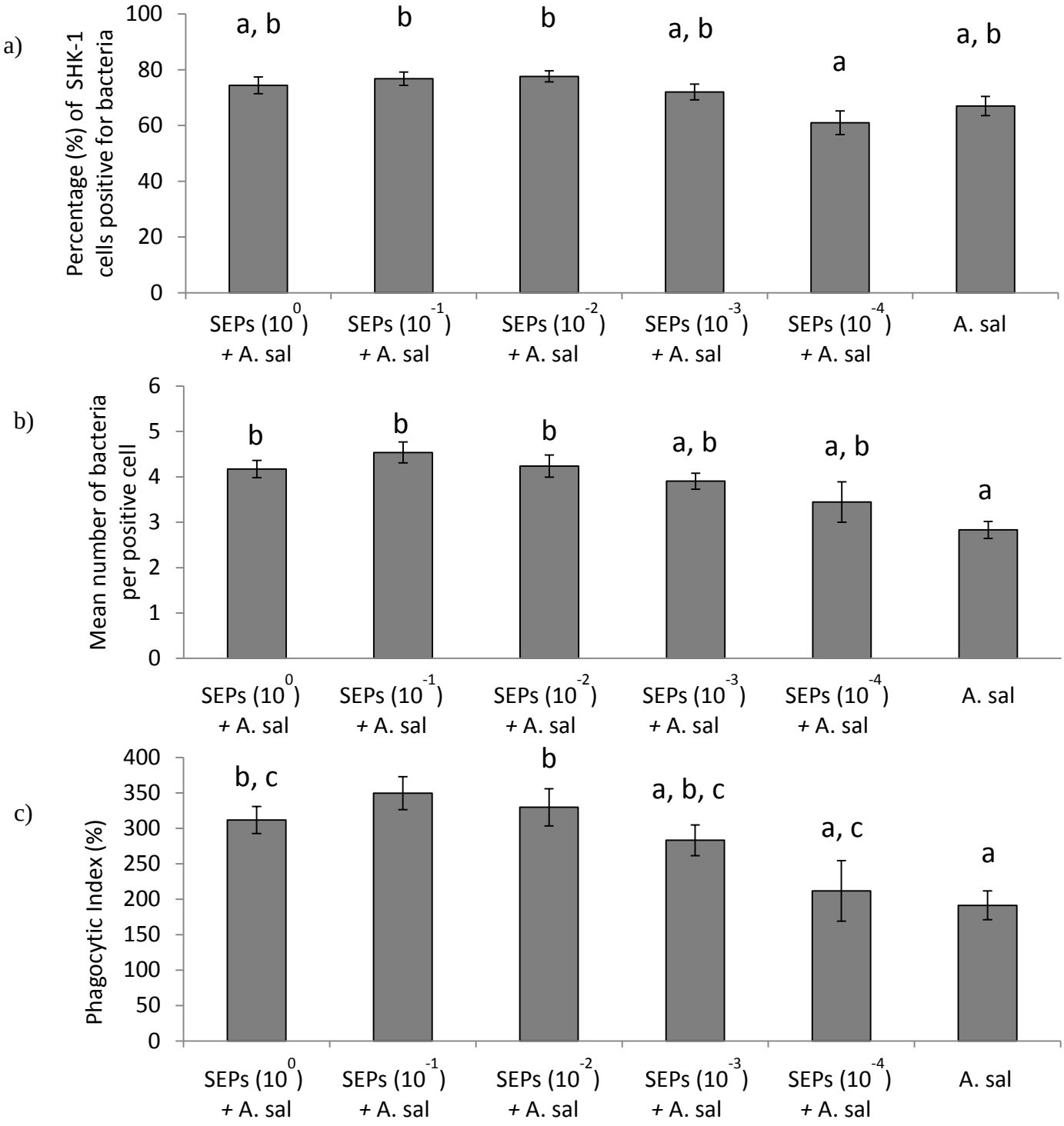

Figure 7. Phagocytic activity of SHK-1 cells: a) percentage of SHK-1 cells positive for at least one bacterium, b) number of bacteria per positive cell, and c) phagocytic index following incubation with various concentrations of SEPs + A. sal or A. sal (see text for further detail). Values represent mean (± SE) calculated from 5 replicates per treatment; for each replicate, 100 individual macrophages were examined and the number of internalized bacteria recorded. Significant differences among treatments were determined using one-way ANOVA ($p < 0.05$). Lowercase letters that differ (a, b, c) represent significant differences among treatments.

2.4 Discussion

It was expected that secretions isolated from *L. salmonis* (SEPs) would impair phagocytic activity and respiratory burst of macrophages. With the exception of pink salmon macrophages, phagocytic index was enhanced in macrophages following incubation with SEPs. In contrast, respiratory burst (measured as superoxide production) was highest in pink salmon macrophages and minimal in chum and Atlantic salmon macrophages incubated with SEPs. Second, we hypothesized that SEPs would have a more significant effect on phagocytosis and respiratory burst in macrophages isolated from species more susceptible to *L. salmonis* infections. In the present study, we found that alteration of macrophage function by SEPs varies significantly among *L. salmonis*-resistant species (i.e., pink salmon) and *L. salmonis*-susceptible species (i.e., chum and Atlantic salmon). This variation in macrophage response to SEPs could indicate possible mechanisms of *L. salmonis*-resistance in some salmon species.

Many cellular, genetic and ecological studies have alluded to the negative impacts of this parasite on salmonids. Genetic expression profiles of salmonids parasitized by *L. salmonis* have identified possible pathways of susceptibility to infestation (Skugor *et al*., 2008; Tadiso *et al*., 2011; Braden *et al*., 2012). Impairment of cellular transcription factors (e.g., NF-κB) (Tadiso *et al*., 2011; Braden *et al*., 2012) and the acute-phase response (Braden *et al*., 2012) have been observed in *L. salmonis*-infected salmon.

Reduced expression of NF-κB has been observed in pink, chum and Atlantic salmon following *L. salmonis* infection (Tadiso *et al*., 2011; Braden *et al*., 2012). NF-κB is a transcription factor that mediates expression of pro-inflammatory cytokines (TNF-α and IL-

1β) and plays a role in the differentiation of macrophages into either pro-inflammatory or anti-inflammatory (Hayden and Ghosh, 2011). Release of TNF-α and IL-1β by activated macrophages leads to activation of NF-κB, presenting a positive feedback mechanism for production of these cytokines and development of inflammation (Gilroy and Lawrence, 2008; Hayden and Ghosh, 2011). In the present study, macrophages isolated from chum salmon and SHK-1 cells exhibited an increased phagocytic index when concurrently incubated with *L. salmonis* secretions (SEPs) and the Gram negative, *Aeromonas salmonicida.* After phagocytosis, macrophages will release TNF-α and IL-1β which have a wide range of biological activities including: chemotaxis, enhancement of cellular immune response (T- and B- cells), cytokine production and control of the acute phase response (APR). These are all processes important in the regulation of local and systemic inflammation (Auger and Ross, 1992). Inhibition of NF-κB can result in impaired inflammatory reactions and can leave the host susceptible to secondary infections (Gilroy and Lawrence, 2008; Hayden and Ghosh, 2011), the latter being a reported consequence of *L. salmonis* infection (Mustafa *et al*., 2000b).

In contrast, macrophages from pink salmon had the lowest phagocytic index of the three species studied. Transcriptomic profiles created for pink, chum and Atlantic salmon showed that while expression of NF-κB is suppressed in all three species initially, pink salmon were the only species to show a significant upregulation following a 48 hour infection (Braden *et al*., 2012). Similarly, pink salmon had increased expression of C-reactive protein (CRP), an important acute phase protein (APP), following a 24 hour *L. salmonis* infection (Braden *et al*., 2012). Activation of NF-κB and the development of an APR are indicators of inflammation (Kushner, 1982; Gilroy and Lawrence, 2008). The decreased

expression of these two components in chum and Atlantic salmon suggests that the process of inflammation may be suppressed or delayed. CRP has been identified as a non-immunoglobulin activator of the complement cascade (Volanakis, 1982). In the present study, the enhanced superoxide (O_2^-) production observed in pink salmon macrophages following incubation with SEPs could be a result of complement activation. Mouse macrophages that internalized particles coated with the complement factors C1q and iC3b demonstrated increased O_2^- production (Dykstra *et al.*, 2011). Similarly, increased O_2^- production was observed in human polymorphonuclear leucocytes (PMNs) following stimulation with C5a, in the absence of phagocytosis (Goldstein *et al.*, 1975). Activation of NADPH oxidase, and subsequent production of O_2^-, can occur as a result of receptor-ligand interactions or through soluble stimuli (Underhill and Ozinsky, 2002) and production of O_2^- by macrophages incubated with SEPs could be a result of the latter.

Increased phagocytic index in chum salmon macrophages, Atlantic salmon macrophages, and SHK-1 cells in the present study could indicate alternative activation of macrophages. Alternatively activated macrophages (AAMs) are induced in response to the presence of Th2 cytokines (IL-4, IL-13), in contrast to classically activated macrophages (CAMs) which arise in response to pro-inflammatory Th1 cytokines (IFN-γ, IL-1, IL-12, TNF-α) (Roitt, 1997; Noël *et al.*, 2004; Varin and Gordon, 2009). Properties of AAMs include enhanced endocytosis and phagocytosis but a lack of bactericidal activity (e.g., impaired NO production) (Varin and Gordon, 2009). In the present study, chum and Atlantic salmon macrophages both had a higher phagocytic index than pink salmon macrophages when incubated with SEPs but failed to elicit a significant respiratory burst response. Mouse macrophages infected with *Leishmania mexicana* amastigotes also had reduced expression of

pro-inflammatory IL-12, a cytokine released from CAMs, and impaired NF-κB-DNA binding (Cameron *et al*., 2004). Reduced IL-12 production and impaired NF-κB signaling was attributed to parasite-derived cysteine proteases (i.e., cathepsin) (Cameron *et al*., 2004).

Conflicting results to the present study have been reported. Macrophages isolated from rainbow trout and Atlantic salmon infected with *L. salmonis* had reduced phagocytic capacity and respiratory burst activity (Mustafa *et* al., 2000a; Fast *et al*., 2002). This inhibition was observed after chronic *L. salmonis* infection (14 and 21 dpi), which corresponded to the later stages of parasite development (chalimus IV and pre-adult) (Mustafa *et* al., 2000a; Fast *et al*., 2002). The suppression of phagocytic capacity and respiratory burst were observed when the highest plasma cortisol and plasma glucose was detected in the fish (Mustafa *et al*., 2000a); however, the same increase in cortisol was not observed in Fast *et al*. (2002). Elevated cortisol levels in fish have been shown to decrease phagocytic ability and respiratory burst in macrophages (reviewed in Wendelaar Bonga, 1997). Differences between these studies and the present study could be associated with experimental design. In Mustafa *et al*. (2000a) and Fast *et al*. (2002), head kidney macrophages were isolated from sea lice-infected fish and used in phagocytic and respiratory burst assays. In the present study, macrophages used in assays were isolated from the head kidney of experimental fish and then concurrently exposed to SEPs and bacteria. The results obtained during the present study may be more indicative of phagocytic activity and respiratory burst response in macrophages at the primary site of interaction (i.e., the skin) between host and parasite; whereas, previous studies may allude to systemic responses of the host following sea lice infection. Different responses have been reported between the skin and kidney for various fish species following infections with *Gyrodactylus* spp. (Lindenstrøm

et al., 2003; Lindenstrøm *et al*., 2006), *Chondracanthus goldsmidi* (Covello *et al*., 2009) and *Argulus siamensis* (Saurabh *et al*., 2011).

L. salmonis produces secretory products that contain pharmacologically active substances in response to host mucus. To date, *L. salmonis* secretions (SEPs) have been shown to contain prostaglandin E_2 (PGE_2), trypsin-like proteases, and cathepsin L proteases (Firth *et al*., 2000; Fast *et al*., 2004; McCarthy *et al*., 2012). In the present study, the presence of PGE_2 and proteins in SEPs was confirmed. Although the production of these components was not quantified per louse, SEPs did increase phagocytosis in SHK-1 cells in a dose-dependent manner that demonstrated threshold levels.

Data from the response curve indicates that there may be an optimal concentration of PGE_2 and/or proteins present in SEPs that exert an effect on phagocytosis in SHK-1 cells. The phagocytic index was greatest in the most concentrated SEPs and began to decrease following dilution. The phagocytic index of most diluted SEPs (10^{-4}) was similar to cells incubated with *A. salmonicida* alone; this result suggests that a biologically optimal concentration of PGE_2 and protein in SEPs is 1.2×10^{-2} pg mL^{-1} and 3.1 ng mL^{-1}, respectively.

The present study provided the first evidence of a direct effect of *L. salmonis* secretions on functionality of macrophages. An observable difference in phagocytosis and respiratory burst response was reported for macrophages isolated from various salmon species, providing further insight into the innate immune responses that may contribute to species susceptibility observed for *L. salmonis* infections. There are disadvantages to studying the effects of *L. salmonis* secretions on macrophages *in vitro,* principally,

elimination of the full milieu of systemic components that would typically be encountered *in vivo* (Freshney, 2000). However, advantages include elimination of indirect effects produced by other cell populations (i.e., neutrophils, NCC's, lymphocytes) and full control of the experimental environment (i.e., temperature, exposure time and concentrations). Furthermore, comparisons between freshly isolated macrophages from the head kidney of Atlantic salmon and SHK-1 cells should be made with caution as inherent changes with SHK-1 cells will develop through repeated passages *in vitro* (Freshney, 2000). Conversely, primary cell isolates can be used to generate data that closely represents living systems since the cells have been freshly isolated from a living organism (Freshney, 2000). In the present study, macrophages isolated from salmon species were used within 24 hours of being removed from the fish and it can be assumed that they were completely functional.

Phagocytic index increased in SHK-1 cells and macrophages isolated from chum and Atlantic salmon following incubation with SEPs but O_2^- production was minimal. This suggests that the anti-microbial mechanisms of these cells may be affected by SEPs and could indicate an alternative activation of macrophages. Conversely, pink salmon macrophages exhibited increased O_2^- production in response to SEPs but a reduced phagocytic index. In pink salmon, the development of a respiratory burst response by macrophages and increased expression of APPs following acute *L. salmonis* infection (Braden *et al*., 2012) suggests that this species has the capacity to mount an inflammatory response against the parasite; whereas, in chum and Atlantic salmon, inflammation may be delayed or impaired, potentially increasing susceptibility to secondary infections.

3 Concluding Discussion

3.1 Effect of *L. salmonis* on the immune response

Pathology is typically associated with pre-adult and adult stages of *Lepeophtheirus salmonis* (Jónsdóttir *et al*., 1992). Adult stages of this parasite attach to the surface of salmon and graze the host, feeding on the mucus, epidermal cells and blood (Mustafa et al., 2000a). The attachment of lice and their subsequent feeding can cause the breakdown of the protective mucous layer leading to dermal lesions (Johnson *et al*., 1996).

The cytokines, TNF-α and IL-1β, are typically released upon tissue injury or infection leading to a cascade of signaling pathways involved in the onset of inflammation (Gilroy and Lawrence, 2008). Receptor interactions result in the recruitment of signaling proteins that lead to the activation of cellular transcription factors, such as NF-κB (Gilroy and Lawrence, 2008). In response to TNF-α and IL-1β, the IκB regulatory complex will degrade, resulting in activation of the NF-κB transcription pathway (Skaug *et al*., 2009). NF-κB dimers are responsible for the transcription of genes related to cell proliferation and differentiation, apoptosis and inflammation (Senftleben and Karin, 2002), providing a positive feedback mechanism to strengthen the inflammatory response (Gilroy and Lawrence, 2008). Following acute exposure (i.e., 24 hours) to *L. salmonis*, expression of NF-κB is suppressed in pink (*Oncorhynchus gorbuscha*), chum (*O. keta*) and Atlantic (*Salmo salar*) salmon at the site of infection but upregulated at non-attachment sites; suggesting louse-induced local suppression (Braden *et al*., 2012). By 48 hours exposure, expression of NF-κB was upregulated in louse-infected skin for pink salmon but not in chum or Atlantic salmon (Braden *et al*., 2012).

In epithelial surfaces, a functioning NF-κB pathway is essential for the development of a proper immune response and protection of the host from invading pathogens (Senftleben and Karin, 2002). Impaired NF-κB control mechanisms can result in an improper balance between inflammation and immune responses, through increased or suppressed transcription of target genes, which can result in failure to respond to an antigen (Senftleben and Karin, 2002). In the present study, following exposure to *L. salmonis* secretions (SEPs), an increase in phagocytic index was observed for chum and Atlantic salmon macrophages and SHK-1 cells; however, respiratory burst, determined by superoxide (O_2^-) production, was not different from control cells. In contrast, pink salmon macrophages produced significantly more O_2^- in response to SEPs than cells isolated from chum and Atlantic salmon, but did not exhibit an increase in phagocytosis.

Increased O_2^- production is a defense mechanism employed by macrophages to kill invading organisms (Iles and Forman, 2002). Initiation of respiratory burst involves the assembly of the NADPH oxidase complex which is activated through receptor-ligand interactions or through soluble stimuli (Iles and Forman, 2002; Underhill and Ozinsky, 2002). Superoxide produced during the respiratory burst is rapidly converted to hydrogen peroxide (H_2O_2) (Iles and Forman, 2002). Hydrogen peroxide is involved in the activation of signaling pathways, including NF-κB and MAPK, in macrophages (Iles and Forman, 2002). Therefore, increased expression of NF-κB expression in pink salmon, 48 hours post-infection, could be the result of the host overcoming louse suppressive mechanisms, increased respiratory burst response by macrophages, or a combination of the two. Related to transcription factors, Braden *et al*. (2012) observed a significantly greater expression of C/EBPβ in pink salmon than in chum and Atlantic salmon following *L. salmonis* infection

and postulated that the presence of the parasite may partially block activation of C/EBPβ by MAPK in susceptible species (i.e., chum and Atlantic salmon). Activation of C/EBPβ is partially regulated through phosphorylation by MAPK (Kim *et al*., 2007); activation of MAPK by H_2O_2 produced by pink salmon macrophages may account for the marked increase in C/EBPβ expression observed following sea lice infection.

C/EBPβ increases the expression of acute phase proteins (APPs) and initiates the acute phase response (APR) (Poli, 1998). APPs include: C- reactive protein (CRP), serum amyloid A (SAA), and serum amyloid P (SAP) (Cray *et al*., 2009). CRP acts as an opsonin, activating complement and increasing phagocytosis, as well as a regulator of cytokine production and chemotaxis (Du Clos, 1996; Cray *et al*., 2009). SAA functions in chemotaxis and has a regulatory role in inflammation (Cray *et al*., 2009). SAA also binds outer membrane protein A (OmpA), a conserved protein among Gram negative Enterobacteriaceae, suggesting an antibacterial function (Raida and Buchmann, 2009). Like CRP, SAP activates the classical complement pathway but does not function as an opsonin (Du Clos, 1996). The increase in C/EBPβ observed by Braden *et al*. (2012) in *L. salmonis*-infected fish suggests an attempt to mount an APR; however, CRP was only upregulated in pink salmon at sites of louse attachment and was found to be down-regulated in lice-infected Atlantic salmon (Tadiso *et al*., 2011).

CRP activates the classical complement cascade by binding C1q (a complement component) at the same time it prevents the assembly of the membrane attack complex (MAC) through recruitment of a complement regulatory protein, factor H (Devitt and Gregory, 2008). In that regard, complement activation, via increased CRP expression, may play an important role in the resolution of inflammation through apoptotic cell clearance

(Devitt and Gregory, 2008). In addition to activating the complement cascade, CRP has been shown to stimulate production of O_2^- in rat macrophages (Devaraj *et al*., 2009).

The early development of an APR and inflammation, indicated by the increased expression of CRP, C/EBPβ and NF-κB, suggests that pink salmon are capable of mounting an inflammatory response during *L. salmonis* infection but inflammation may be lacking or delayed in chum and Atlantic salmon. Macrophages play a major role in inflammation; they are responsible for antigen presentation, phagocytosis and immunomodulation through the release of cytokines and growth factors (Fujiwara and Kobayashi, 2005). In response to inflammatory signals, macrophages will become activated, enhancing their killing power (Fujiwara and Kobayashi, 2005). The greater production of reactive oxygen intermediates (ROIs) by pink salmon macrophages in the present study provides further support that these cells were activated via pro-inflammatory stimuli. Inflammation at the site of louse attachment is believed to be a mechanism by which *L. salmonis*-resistant hosts are capable of shedding the parasite quickly (Johnson and Albright, 1992). Similarly, activation of complement through increased CRP could indicate an important mechanism in louse-rejection observed for pink salmon (Jones *et al*., 2007). Incubation of *Gyrodactylus derjavini* with complement factor C3 from rainbow trout (*Oncorhynchus mykiss*) has been shown to induce parasite death (Buchmann, 1998). In the present study, the depressed antimicrobial mechanisms (i.e., O_2^- production) observed in chum and Atlantic salmon macrophages, in the presence of SEPs, may explain the susceptibility to secondary infection previously reported for Atlantic and chinook (*O. tshawytscha*) salmon (Johnson and Albright, 1992).

3.2 Possible pathways of resistance to *L. salmonis*

The increase in phagocytic index observed for SHK-1 cells and chum and Atlantic salmon macrophages in the present study may be an example of alternative macrophage activation. Alternative activation of macrophages occurs through IL-4 and IL-13 induced expression of macrophage mannose receptor (MRC1) and its function; whereas, IFN-γ (a classical activator of macrophages) results in decreased expression of MRC1 (Gordon and Martinez, 2010). Alternatively activated macrophages (AAMs) are involved in the maintenance of tissue homeostasis or tissue remodeling, wound healing and reduction of inflammatory responses (Chen *et al.*, 2012). AAM's also exhibit increased endocytic and phagocytic activity, expression of MH class II, and are capable of antigen presentation (Noël *et al.*, 2004). However, AAM's demonstrate poor bactericidal activity as they fail to produce nitric oxide (NO) (Noël *et al*., 2004; Varin and Gordon, 2009). Recently it has been shown that head kidney macrophages isolated from carp (*Cyprinus carpio*) are capable of becoming activated via classical or alternative pathways (Joerink *et al*., 2006). Similarly, human monocytes stimulated with IL-13, exhibited decreased expression of interleukin-1 converting enzyme (ICE) and pro-IL-1β (Gordon and Martinez, 2010). Decreased expression of IL-1β was previously observed in SHK-1 cells following exposure to SEPs (Fast *et al*. 2007), but the increased phagocytosis observed in the present study imply that SEPs can induce an alternative pathway of macrophage activation. Mouse macrophages incubated with *Leishmania mexicana* amastigotes showed reduced production of IL-12, indicating impairment of Th1 response and generation of a Th2 response; AAMs being a product of the latter (Cameron *et al*., 2004; Noël *et al*., 2004). In addition, presence of *L. mexicana* resulted

in elimination of NF-κB transcriptional activation and was related to the parasite-derived cysteine proteases (Cameron *et al*., 2004).

Upon presentation of antigens, $CD4^+$ cells will differentiate into either T-helper 1 cells (Th1) or T-helper 2 cells (Th2) (Wagner *et al*., 2008).The Th1 cells primarily secrete IFN-γ and mediate the development of a cytolytic cellular immune response (Wagner *et al*., 2008). In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13 which promotes B-cell proliferation and result in the development of a humoral antibody response (Wagner *et al*., 2008). Arthropod ectoparasites, particularly ticks (family Ixodidae), have been shown to decrease production of IL-2 and IFN-γ, important cytokines in the development of a Th1 like response, as well as IL-1β and TNF-α (Wikel, 1999; Brossard and Wikel, 2004; Kovář, 2004). Expression of cytokines that promote a Th2 response have been reported to be unaffected (Wikel, 1999; Brossard and Wikel, 2004) or enhanced (Leboulle *et al*., 2002; Brossard and Wikel, 2004; Kovář, 2004) in response to saliva or components of saliva from ticks. Interestingly, increased expression of IL-10 (in *L. salmonis*-infected pink, chum and Atlantic salmon) (Braden *et al*., 2012) and IL-4 (in lice-infected Atlantic salmon) (Skugor *et al*., 2008) along with decreased expression of IFN-γ dependent proteins in infected Atlantic salmon (Tadiso *et al*., 2011) suggest a similar bias toward Th2 responses in lice infections. The polarization towards a Th2 immune response is associated with susceptibility to tick infection in mammal hosts (Ganapamo *et al*., 1995). Studies on the tick, *Rhipicephalus sanguineus*, feeding on non-natural hosts (e.g., guinea-pigs) showed the host was capable of developing resistance that was associated with a Th1 response (Steen *et al*., 2006) The suppression of a Th1 response has also been linked to increased transmission of pathogens from tick to host. Salivary gland extracts from the tick, *Dermacentor andersoni*, are capable

of suppressing macrophage production of IL-1β and TNF-α as well as T-lymphocyte derived IL-2 and IFN-γ (Wikel, 1999). Reconstitution of TNF-α, IL-2 and IFN-γ in mice, during infection with *Borellia burgdorferi* (the spirochete bacterium associated with Lyme disease)-infected ticks, resulted in a marked increase in protection against tick transmission of the pathogen (Wikel, 1999).

The ability to develop a Th1 like response in salmon species infected with *L. salmonis* might represent a possible pathway of resistance. Juvenile pink salmon showed a weakly significant (p= 0.056) increase in expression of gamma-interferon-inducible lysosomal thiol reductase (an enzyme induced by IFN-γ) following infection with *L. salmonis* (Sutherland *et al*., 2012). This could indicate that pink salmon may have the ability to polarize the Th1 subset of cells and mount a cellular immune response to *L. salmonis*. Along with IFN-γ, the cytokines IL-12, IL-18 and lymphotoxin-α could indicate development of a Th1 response (Szabo *et al*., 2003; Secombes, 2008). To date, there are no detailed studies examining the expression of these cytokines in various salmon species infected with *L. salmonis* but the adaptive cellular response in salmonids following sea lice infection certainly warrants further research.

3.3 Local vs. systemic response to *L. salmonis* infection

Results obtained using the present method may be more indicative of an acute phagocyte response at the site of host and parasite interaction (i.e., the skin); whereas, previous studies may allude to suppression of systemic responses of the host due to chronic sea lice infection.

Among three striped trumpeter (*Latris leneata*) infected with the gill ectoparasite, *Chondracanthus goldsmidi*, there was upregulation of TNF-α, IL-1β and IL-8 in the gills of parasitized fish; whereas, there was no expression of IL-1β and IL-8 in head kidney tissue (Covello *et al*., 2009). Similarly, increased expression of IL-1β has been reported in the skin of rainbow trout and Atlantic salmon infected with *Gyrodactylus* spp. (Lindenstrøm *et al*., 2003; Lindenstrøm *et al*., 2006). Studies with recombinant rainbow trout IL-1β (rIL-1β) showed that an intraperitoneally-injected dose of 1 µg rIL-1β $fish^{-1}$ resulted in enhanced percent phagocytosis in rainbow trout leukocytes (Hong *et al*., 2003). Gene expression studies on rohu (*Labeo rohita*) heavily infected with *Argulus siamensis* identified upregulation of TNF-α and toll-like receptor 22 (TLR 22) in the fish's skin (Saurabh *et al*., 2011). However, there was no difference in TNF-α expression in the kidney between infected and control fish and expression of TLR 22 was suppressed in infected fish kidney (Saurabh *et al*., 2011). Saurabh *et al*. (2011) concluded that *A. siamensis* was not capable of inducing a systemic inflammatory reaction in the kidney of infected fish and the observed suppression of TLR-22 as well as β2-microglobulin (β2-m) in kidney tissue suggest a possible reason for a poor immune response observed in *Argulus*-infected fish. Expression of β2-m was suppressed in Atlantic salmon infected with *L. salmonis* (Skugor *et al*., 2008) also suggesting depressed antigen processing. Both TLR-22 and β2-m are involved in the recognition and presentation of pathogen components. β2-m is available as a free form and a cell surface associated form with major histocompatibility class I (MH I) complex where it functions to present peptides of phagocytized viral and bacterial proteins to T cells (Saurabh *et al*., 2011).

Transcriptomic profiles created for size classes (0.3, 0.7 and 2.4 g) of pink salmon determined that the smallest pink salmon (0.3 g) were the most susceptible to pathology

caused by *L. salmonis* infection (Sutherland *et al*., 2011). It was concluded that increased susceptibility in 0.3 g salmon was a result of cell stress (e.g., inhibited cell proliferation) which could be a result of parasite-induced nutrient diversion through increased inflammation and tissue remodeling (Sutherland *et al*., 2011). In the 0.3 and 0.7 g groups, there was up-regulation of genes related to tissue remodeling, matrix mellatoproteinase (MMP) 9 and MMP13. However, in 0.7 g salmon, genes related to cell proliferation were not suppressed and no reduced growth or mortality occurred as a result of lice infection (Sutherland *et al*., 2011). Skugor *et al*. (2008) found that for Atlantic salmon, chronic *L. salmonis*-infections result in MMP-dependent tissue remodeling without accompanying cell proliferation. Suppressed cell proliferation could be another indicator of systemic stress placed on host fish in response to *L. salmonis* infection. Induction of salmon MMPs occurs via inflammatory stimuli and stress (Tadiso *et al*. 2011). Both MMP9 and MMP13 have been shown to be upregulated in Atlantic salmon skin (Skugor *et al*., 2008; Tadiso *et al*., 2011) and spleen (Tadiso *et al*., 2011), during prolonged *L. salmonis* infection (i.e., longer than 10 days), indicating systemic distress which could explain immunosuppressive observations of sea lice infections. Similarly, upregulation of MMP13 occurred in pink, chum and Atlantic salmon at louse attachment sites with pronounced expression occurring in pink salmon at 48 hours (Braden *et al*., 2012). Matrix mellatoproteinases are also responsible for cleaving membrane associated pro-TNF, leading to the release of soluble TNF-α from macrophages (Bradley, 2008). Increased TNF-α has been reported in skin and head kidney of salmonids during *L. salmonis* infection (Fast *et al*., 2006; Jones *et al.,* 2007; Braden *et al*., 2012)

Expression of prostaglandin D synthase (PGDS) was reduced in louse attachment sites for pink, chum and Atlantic salmon following a 48 hour infection (Braden *et al*., 2012).

Reduced expression of PGDS was also observed in Atlantic salmon skin, with a significant reduction occurring at 33 dpi, corresponding to the appearance of pre-adult stages of *L. salmonis* on fish (Skugor *et al*., 2008). The product of PGDS, prostaglandin D_2 (PGD_2) is an anti-inflammatory molecule (Harris *et al*., 2002) so reduced expression at the site of *L. salmonis* attachment implies preference for pro-inflammatory molecules (e.g., PGE_2) at these sites (Braden *et al*., 2012). Sources of PGE_2 at the site of louse attachment could be host derived as macrophages produce PGE_2 following inflammatory stimuli, resulting in phospholipase A_2 driven release of arachidonic acid from membrane phospholipids (Harris *et al*., 2002). This is followed by oxygenation by cyclooxygenase (COX) enzymes and subsequent production of prostaglandins (Harris *et al*., 2002). It is also plausible that exogenous PGE_2 (i.e., parasite derived) is responsible for the shift from anti-inflammatory to pro-inflammatory prostaglandins at *L. salmonis* attachment sites. Evidence of a exogenous source of PGE_2 include the decreased expression of COX-2 in pink salmon between 24 and 48 hours of lice infection (Braden *et al*., 2012) and the reduced expression of prostaglandin E synthase (PGES) and phospholipase A_2 in lice-infected Atlantic salmon (Tadiso *et al*., 2011).

3.4 Dose response

Data from the dose response assay indicates that there may be a biologically optimal concentration of PGE_2 and/or proteins present in SEPs that exert an effect on phagocytosis of SHK-1 cells. Phagocytic activity was greatest in SHK-1 cells exposed to undiluted SEPs and decreased as concentration of SEPs decreased. Phagocytic index of cells challenged with SEPs diluted 10, 000 x was no different than cells exposed to *A. salmonicida* alone, suggesting that at a PGE_2 concentration below 1.235×10^{-2} pg mL^{-1} and/or a protein concentration below 3.119 ng mL^{-1}, effects on SHK-1 cells are negligible.

Fast *et al*. (2004) found that adult *L. salmonis* can produce 0.21-6.4 ng PGE_2 $louse^{-1}$ after 24 hours off a host fish. Prostaglandin production was even higher (14.5 ng $louse^{-1}$) if secretions were collected from adult lice immediately following removal from a host (Fast *et al*., 2004). In the present study, PGE_2 production was not determined per louse so conclusions cannot be made on effects a single louse may have on cellular immune response, specifically phagocytic activity of macrophages. Fast *et al*. (2005) found that at the lowest concentration tested (3.3×10^{-12} M), PGE_2 reduced expression of MH class I, MH class II and IL-1β in SHK-1 cells following stimulation with LPS. In contrast, PGE_2 had a stimulatory effect on TNF-α expression, increasing expression above control levels at the lowest concentration tested (1.0×10^{-10} M) (Fast *et al*., 2005). Based on ELISA analysis of SEPs used in the present study, the concentration of PGE_2 in undiluted SEPs, was approximately 3.5×10^{-10} M, suggesting that, in addition to altering immune gene expression (Fast *et al*., 2005), PGE_2 can influence macrophage activity.

Reported effects of SEPs on gene expression are not solely related to the presence of PGE_2. Atlantic salmon macrophages stimulated with LPS and PGE_2 (1.0×10^{-8} M) exhibited no change in MH class I expression but when incubated with SEPs (0.66 µg) there was a significant upregulation (Fast *et al*., 2007). Furthermore, when macrophages were incubated with SEPs and PGE_2, MH class I expression was significantly reduced to levels similar to controls (Fast *et al*., 2007). Expression of IL-1β was also reduced in SHK-1 cells incubated with SEPs in the absence of PGE_2 (Fast *et al*., 2007).

Atlantic salmon infected with *L. salmonis* showed increased levels of protease activity in their mucus (Ross *et al*., 2000) which was attributed to low molecular weight (17-22 kDa), *L. salmonis*-derived trypsin-like proteases (Firth *et al*., 2000). Further analysis has

revealed five trypsins produced by *L. salmonis* with increased transcription occurring as the parasite reaches pre-adult and adult stages (Kvamme *et al*., 2004). Although it is likely that *L. salmonis* trypsin functions as a digestive enzyme, it may also aid in host immunomodulation. Trypsin proteases produced by the warble fly (*Hypoderma lineatum*) degrade complement component C3 (Boulard, 1989) and proteases released from the protozoan parasite, *Perkinsus marinus*, decrease host defense parameters in the oyster, *Crassostrea virginica* (Garreis *et al*., 1996). Trypsin activity in the secretory-excretory products of the bot fly, *Oestrus ovis*, are important in wound formation, nutrition (degradation of serum albumin and mucin was observed) and possibly impact immunomodulation as cleavage of sheep IgG was also noted (Tabouret *et al*., 2003).

Two cathepsin L proteases have also been sequenced from *L. salmonis* and their enzymatic activity was observed in secretory/excretory products suggesting a role in extracellular digestion of host mucus, blood and skin (McCarthy *et al*., 2012). Cysteine proteases, particularly cathepsin B and L, are found in numerous parasites and function in digestion, tissue and cellular invasion and immunoevasion (including alteration of the cellular immune response and degradation of immune response mediators) (reviewed in Sajid and McKerrow, 2002).

Depending on host species parasitized (i.e., *Oncorhynchus* spp. or *Salmo* spp.), secretions from *L. salmonis* may contain varying levels of pharmacologically active substances and thus may impact immune responses differently. In response to mucus from Atlantic salmon or rainbow trout, significantly more *L. salmonis* released proteases than lice incubated with mucus from coho salmon or winter flounder (*Pseudopleuronectes americanus*) (Fast *et al*., 2003). There was also higher alkaline phosphatase activity observed

in Atlantic salmon mucus and higher protease activity in rainbow trout mucus following incubation with *L. salmonis*, no difference was observed among coho salmon or winter flounder mucus (Fast *et al*., 2003). These reported differences could be due to factors in mucus of susceptible species that stimulates release of proteases from *L. salmonis* or factors that block secretion of these proteases in resistant species (Fast *et al*., 2003).

Additional data obtained during the present study (Appendix B) suggests that there is no difference in respiratory burst response elicited in pink, chum or Atlantic salmon macrophages following incubation with secretions isolated from lice feeding on Atlantic salmon (SEPs) or from lice feeding on the same host fish species that macrophages tested were isolated from (i.e., $SEPs_{pink}$, $SEPs_{chum}$ or $SEPs_{ATL}$). However, phagocytic index was found to be higher in SHK-1 cells exposed to secretions immediately isolated from lice that were feeding on pink salmon compared to secretions from lice feeding on chum salmon. It was speculated that the presence of trypsin in SEPs could lead to degradation of proteins during incubation. However, addition of a protease inhibitor to isolated SEPs did not appear to have any effect on respiratory burst response or phagocytic index when compared to cells incubated with SEPs lacking a protease inhibitor. These results imply that (i) modulation of macrophage phagocytic activity and respiratory burst by SEPs is not related to proteins in *L. salmonis* secretions or (ii) trypsin-like proteases present in SEPs are in an inactive form following isolation. In the mosquito (*Aedes aegypti*) and horsefly (*Stomoxys calcitrans*) trypsin becomes activated after ingestion of a blood meal (Muhlia-Almazán *et al*., 2008). Thus, immunomodulation as a result of *L. salmonis*-derived molecules requires more in-depth studies. Specifically, further analysis of compounds present in these secretions should be completed, followed by identifying their role in the host-parasite interaction.

3.5 Susceptibility to secondary infections

Impairment of immune function would have the potential to increase a host's susceptibility to secondary infection with a pathogen (e.g., *A. salmonicida*). Although it appears that Atlantic salmon macrophage-like cells (SHK-1) and isolated chum and Atlantic salmon macrophages appear to have increased phagocytic activity following exposure to SEPs, they may not possess the ability to effectively destroy the internalized pathogen as evidenced by minimal respiratory burst response in both species. In contrast, macrophages isolated from pink salmon have enhanced production of superoxide after incubation with SEPs, but phagocytic activity appears to be reduced. The results of this study, in conjunction with previous genetic studies, suggest that pink salmon are capable of inducing an inflammatory response to *L. salmonis;* however, in more *L. salmonis*-susceptible species, the development of inflammation may be impaired or delayed leading to increased vulnerability to secondary infection.

Enhanced susceptibility to bacterial and viral pathogens can result from sea lice infection via epidermal disruption caused by the parasite and/or enhanced pathogen invasion in response to decreased host immunocompetence. Based on cellular and molecular studies of sea lice infection, both scenarios are highly likely. Mortality of rainbow trout was enhanced following concomitant challenge with the ectoparasite, *Argulus coregoni,* and the bacteria, *Flavobacterium columnare,* (Bandilla *et al*., 2006). Similarly, infections with *L. salmonis* in rainbow trout led to an increased susceptibility to the microsporidian *Loma salmonae* (Mustafa *et al*., 2000b), and increased mortality was observed in tilapia (*Oreochromis niloticus*) concurrently infected with *Gyrodactylus niloticus* and the bacteria, *Streptococcus iniae* (Xu *et al*., 2007). There is also the possibility that *L. salmonis* can act as

a vector, transmitting pathogens from an infected to a naïve host during feeding. Evidence for the acquisition and transmission of viral (Jakob *et al*., 2011) and bacterial (Novak *et al*., 2012) pathogens between fish by *L. salmonis* have been reported, but data suggests that lice may act as a mechanical rather than biological vector.

3.6 Future Perspectives

Analysis of *L. salmonis* secretory products and understanding their role in immunomodulation of host fish could aid in the development of an anti-louse vaccine. Vaccines developed from a midgut protein of ticks, Bm86, have been shown to provide protection against *Rhipicephalus* spp. in cattle (reviewed in Parizi *et al*., 2012). Bm86 is a concealed antigen, meaning that it does not come in contact with the host's immune system during a parasite infection, but when isolated is capable of eliciting an immune response (Raynard *et al.*, 2002). Similarly, vaccines derived from salivary components of ticks have shown to carry some anti-tick protection in various mammalian hosts (reviewed in Parizi *et al*., 2012). Anti-parasite vaccines developed for blood-feeding arthropods typically work with gut proteins (such as Bm86) because ingestion of host antibody will occur during feeding, followed by binding to antigenic sites of gut cells (Raynard *et al*., 2002). In contrast to obligate haematophagous parasites, ingestion of blood is not mandatory for survival and occurs infrequently in sea lice; therefore, the louse gut may not be the best target for antibody function (Raynard *et al*., 2002). However; louse-derived proteins produced and secreted onto the host during feeding could be ideal targets for anti-louse antibodies. Development of an effective vaccine against *L. salmonis* would reduce the need for bath and oral sea lice treatments. A vaccine would also provide a method of sea lice control that (i) specifically targets *L. salmonis*, reducing risk to other marine invertebrate species, (ii) has no withdrawal

period and (iii) limits the development of resistance (Raynard *et al*., 2002). Secretory products released from *L. salmonis* are capable of stimulating a host immune response and it is feasible that novel proteins present in those secretions may be candidates for vaccine development.

3.7 Conclusions

The overall objective of this thesis was to determine if *L. salmonis* secretory products (SEPs) affected the innate immune response of salmonids. This was achieved by performing a series of phagocytosis and respiratory burst assays on SHK-1 cells and/or macrophages isolated from pink, chum and Atlantic salmon.

The present study provided the first evidence that phagocytic activity increases in SHK-1 cells and chum and Atlantic salmon macrophages following exposure to SEPs. Conversely, phagocytic activity was lower in pink salmon macrophages exposed to SEPs compared to macrophages incubated with bacteria alone. Similar species-specific differences were observed among pink, chum and Atlantic salmon during respiratory burst assays. Production of superoxide was pronounced in pink salmon macrophages incubated with SEPs but minimal in chum and Atlantic salmon macrophages, suggesting that anti-microbial mechanisms in these species may be impaired. It would be interesting to further investigate the interactions between macrophages and SEPs including: microbial killing power, chemotaxis and cytokine release. Macrophages play an important role in maintaining host homeostasis by regulating inflammation, initiating specific immune responses and phagocytizing foreign materials. Immunomodulation by *L. salmonis* may represent a mechanism by which the parasite prevents rejection from more susceptible host species.

Bibliography

Allen, J.E. and Wynn, T.A. 2011. Evolution of Th2 immunity: A rapid repair response to tissue destructive pathogens. PLOS Pathogens. 7:e1002003.doi:10.1371/journal.ppat.1002003.

Alvarez-Pellitero, P. 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic properties. Veterinary Immunology and Immunopathology. 126: 171-198.

Aoki, T., Takano, T., Santos, M.D., Kondo, H., and Hirono, I. 2008. Molecular innate immunity in teleost fish: review and future perspectives. In: Fisheries for Global Welfare and Environment, Memorial book for the 5th World Fisheries Congress. Eds K.Tsukamoto, T. Kawamura, T. Takeuchi, T.D. Beard, Jr and M.J. Kaiser. Terrapub, Tokyo. pp 263-276.

Auger, M.J. and Ross, J.A. The biology of the macrophage. In: The Natural Immune System: The Macrophage. Eds C.E. Lewis and J.O'D. McGee. 1992. Oxford University Press. New York. pp 3-74.

Bandilla, M., Valtonen, E.T., Suomalainen, L-R., Aphalo, P.J., and Hakalahti, T. 2006. A link between ectoparasite infection and susceptibility to bacterial disease in rainbow trout.International Journal for Parasitology. 36: 987-991.

Barker, D.E., Braden, L.M., Coombs, M.P., and Boyce. B. 2009. Preliminary studies on the isolation of bacteria from sea lice, *Lepeophtheirus salmonis*, infecting farmed salmon in British Columbia, Canada. Parasitology Research. 105: 1173-1177.

Basset, C., Holton, J., O'Mahony, R., and Roitt, I. 2003. Innate immunity and pathogen-host interaction. Vaccine. 21: S2/12-S2/23.

Bayne, C.J. and Gerwick, L. 2001. The acute phase response and innate immunity of fish. Developmental and Comparative Immunology. 25: 725-743.

Bird, S., Zou, J., and Secombes, C.J. 2006. Advances in fish cytokine biology gives clues to the evolution of a complex network. Current Pharmaceutical Design. 12: 3051-3069.

Bols, N.C., Brubacher, J.L., Ganassin, R.C., and Lee, L.E.J. 2001. Ecotoxicology and innate immunity in fish. Developmental and Comparative Immunology. 25: 853-873.

Bone, Q. and Moore, R.H. 2008. The immune system. In: Biology of Fishes, 3rd Ed. Taylo r and Francis Group, New York, NY. pp 383-408.

Boulard, C. 1989. Degradation of bovine C3 by serine proteases from parasites *Hypoderma lineatum* (Diptera, Oestridae). Veterinary Immunology and Immunopathology. 20: 387-398.

Bowers, J.M., Mustafa, A., Speare, D.J., Conboy, G.A., Brimacombe, M., Sims, D.E., and Burka, J.F. 2000. The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. Journal of Fish Diseases. 23: 165-172.

Bradley, J.R. 2008. TNF-mediated inflammatory disease. Journal of Pathology. 214: 149-160.

Braden, L.M, Barker, D.E., Koop, B.F., and Jones, S.R.M. 2012. Comparative defense-associated responses in salmon skin elicited by the ectoparasite *Lepeophtheirus salmonis*. Comparative Biochemistry and Physiology, Part D. 7: 100-109.

Brossard, M. and Wikel, S.K. 2004. Tick immunobiology. Parasitology. 129: S161-S176.

Buchmann, K. 1998. Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). Diseases of Aquatic Organisms.32: 195-200.

Buchmann, K. 2012. Fish immune responses against endoparasitic nematodes- experimental models. Journal of Fish Diseases. 35: 623-635.

Cameron, P., McGachy, A., Anderson, M., Paul, A., Coombs, G.H., Mottram, J.C., Alexander, J., and Plevin, R. 2004. Inhibition of lipopolysaccharide-induced macrophages IL-12 production by *Leishmania mexicana* amasitgotes: The role of cysteine peptidases and the NF-κB signaling pathway. The Journal of Immunology. 173: 3297-3304.

Campbell, P.A., Canono, B.P., and Drevets, D.A. 1995. Measurement of bacterial ingestion and killing by macrophages. Current Protocols in Immunology: 12: 14.6.1- 14.6.13.

Chen, W.H., Toapanta, F.R., Shirey, K.A., Zhang, L., Giannelou, A., Page, C., Frieman, M.B., Vogel, S.N., and Cross, A.S. 2012. Potential role for alternatively activated macrophages in the secondary bacterial infection during recovery from influenza .Immunology Letters. 141: 227-234.

Covello, J.M., Bird, S., Morrison, R.N., Battaglene, S.C., Secombes, C.J., and Nowak, B.F. 2009. Cloning and expression analysis of three striped trumpeter (*Latris lineata*) pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, in response to infection by the ectoparasitic, *Chondracanthus goldsmidi*. Fish and Shellfish Immunology. 26: 773-786.

Cray, C., Zaias, J., and Altman, N.H. 2009. Acute phase response in animals: A review. Comparative Medicine. 59: 517-526.

Dannevig, B.H., Brudeseth, B.E., Gjøen, T., Rode, M., Wergeland, H.I., Evensen, Ø., and Press, C. McL. 1997. Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology. 7: 213-226.

Devaraj, S., Dasu, M.R., Singh, U., Rao, L.V.M., and Jialal, I. 2009. C-reactive protein stimulates anion release and tissue factor activity *in vivo*. Atherosclerosis. 203: 67-74.

Devitt, A. and Gregory, C.D. 2008. Innate immune mechanisms in the resolution of inflammation. In: The Resolution of Inflammation. Eds A.G. Rossi and D.A. Sawatzky. Birkhäuser Verlag AG. Basel, Switzerland. pp 39-56.

Díaz, A. and Allen, J.E. 2007. Mapping immune response profiles: The emerging scenario from helminth immunology. European Journal of Immunology. 37: 3319-3326.

Du Clos, T.W. 1996. The interaction of C-reactive protein and serum amyloid P component with nuclear antigens. Molecular Biology Reports. 23: 253-260.

Dykstra, T., Utermoehlen, O., and Haas, A. 2011. Defined particle ligands trigger specific defense mechanisms of macrophages. Innate Immunity. 17: 388-402.

Fast, M.D., Ross, N.W., Mustafa, A., Sims, D.E., Johnson, S.C., Conboy, G.A., Speare, D.J., Johnson, G., and Burka, J.F. 2002. Susceptibility of rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* to experimental infection with sea lice *Lepeophtheirus salmonis*. Diseases of Aquatic Organisms. 52: 57-68.

Fast, M.D., Burka, J.F., Johnson, S.C., and Ross, N.W. 2003. Enzymes released from *Lepeophtheirus salmonis* in response to mucus from different salmonids. Journal of Parasitology. 89: 7-13.

Fast, M.D., Ross, N.W., Craft, C.A., Locke, S.J., Mackinnon, S.L., and Johnson, S.C. 2004. *Lepeophtheirus salmonis*: characterization of prostaglandin E_2 in secretory products of the salmon louse by RP-HPLC and mass spectrometry. Experimental Parasitology. 107: 5-13.

Fast, M.D., Ross, N.W., and Johnson, S.C. 2005. Prostaglandin E_2 modulation of gene expression in an Atlantic salmon (*Salmo salar*) macrophage-like cell line (SHK-1). Developmental and Comparative Immunology. 29: 951-963.

Fast, M.D., Muise, D.M., Easy, R.E., Ross, N.W., and Johnson, S.C. 2006. The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*). Fish and Shellfish Immunology. 21: 228-241.

Fast, M.D., Johnson, S.C., Eddy, T.D., Pinto, D., and Ross, N.W. 2007. *Lepeophtheirus salmonis* secretory/excretory products and their effects on Atlantic salmon immune gene regulation. Parasite Immunology. 29: 179-189.

Finstad B., Bjørn, P.A., Grimnes, A., and Hvidsten, N.A. 2000. Laboratory and field investigations of salmon lice (*Lepeophtheirus salmonis* Krøyer) infestation on Atlantic salmon (*Salmo salar* L.) post-smolts. Aquaculture research. 31: 795-803.

Firth, K.J., Johnson, S.C., and Ross, N.W. 2000. Characterization of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis*) and in whole-body louse homogenate. The Journal of Parasitology. 86: 1199-1205.

Freshney, I. 2000. Introduction. In: Culture of Animal Cells: a manual of basic technique 4th Ed. Wiley-Liss Inc, New York, NY. pp 1-8.

Fujiwara, N. and Kobayashi, K. 2005. Macrophages in inflammation. Current Drug Targets-Inflammation and Allergy. 4: 281-286.

Ganapamo, F., Rutti, B., and Brossard, M. 1995. *In vitro* production of interleukin-4 and interferon-γ by lymph node cells from BALB/c mice infested with nymphal *Ixodes ricinus* ticks. Immunology. 85: 120-124.

Garreis, K., La Peyre, J.F., and Faisal, M. 1996. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defense parameters *in vitro*. Fish and Shellfish Immunology. 6: 581-597.

Gilroy, D. and Lawrence, T. 2008. Resolution of acute inflammation: A 'tipping point' in the development of chronic inflammatory disease. In: The Resolution of Inflammation Eds A.G. Rossi and D.A. Sawatzky. Birkhäuser Verlag AG. Basel, Switzerland. pp 1-18.

Goldstein, I.M., Roos, D., Kaplan, H.B., and Weissmann, G. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. The Journal of Clinical Investigation. 56: 1155-1163.

Gordon, S. and Martinez, F.O. 2010. Alternative activation of macrophages: Mechanism and function. Immunity. 32: 593-604.

Harris, S.G., Padilla, J., Koumas, L., Ray, D., and Phipps, R.P. 2002. Prostaglandins as modulators of immunity. Trends in Immunology. 23: 144-150.

Hayden, M.S. and Ghosh, S. 2011. NF-κB in immunobiology. Cell Research. 21: 223-244.

Hayward, C.J., Andrews, M., and Nowak, B.F. 2011. Introduction: *Lepeophtheirus salmonis*-A remarkable success story. In: Salmon Lice: An integrated approach to understanding parasite abundance and distribution. John Wiley & Sons, Inc. West Sussex, UK. pp 1-28.

Hong, S., Peddie, S., Campos-Pérez, J.J., Zou, J., and Secombes, C.J. 2003. The effect of intraperitoneally administered recombinant IL-1β on immune parameters and resistance to *Aeromonas salmonicida* in the rainbow trout (*Oncorhynchus mykiss*). Developmental and Comparative Immunology. 27: 801-812.

Iles, K.E. and Forman, H.J. 2002. Macrophage signaling and respiratory burst. Immunlogic Research. 26: 95-105.

Jakob, E., Barker, D.E., and Garver, K.A. 2011. Vector potential of the salmon louse *Lepeophtheirus salmonis* in the transmission of infectious haematopoietic necrosis virus (IHNV). Diseases of Aquatic Organisms. 97: 155-165.

Joerink, M., Ribeiro, C.M.S., Stet, R.J.M., Hermsen, T., Savelkoul, H.F.J., and Wiegertjes, G.F. 2006. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. The Journal of Immunology. 177: 61-69.

Johnson, S.C. and Albright, L.J. 1992. Comparative susceptibility and histopathology of the response of naive Atlantic, chinook and coho salmon to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae). Diseases of Aquatic Organisms. 14: 179-193.

Johnson, S.C., Blaylock, R.B., Elphick, J., and Hyatt, K.D. 1996. Disease induced by the sea louse (*Lepeophtheirus salmonis*) (Copepoda: Caligidae) in wild sockeye salmon (*Oncorhynchus nerka*) stocks of Alberni inlet, British Columbia. Canadian Journal of Fisheries Aquatic Science. 53: 2888-2897.

Johnson, S.C., Treasurer, J.W., Bravo, S., Nagasawa, K., and Kabata, Z. 2004. A review of the impact of parasitic copepods on marine aquaculture. Zoological Studies. 43: 229-243.

Jones, S.R.M., Fast, M.D., Johnson, S.C., and Groman, D.J. 2007. Differential rejection of salmon lice by pink and chum salmon: disease consequences and expression of proinflammatory genes. Diseases of Aquatic Organisms. 75: 229-238.

Jones, S.R.M. and Hargreaves, N.B. 2009. Infection threshold to estimate *Lepeophtheirus salmonis*-associated mortality among juvenile pink salmon. Diseases of Aquatic Organisms. 84: 131-137.

Jones, S., Kim, E., and Bennett, W. 2008. Early development of resistance to the salmon louse, *Lepeophtheirus salmonis* (Krøyer), in juvenile pink salmon, *Oncorhynchus gorbuscha* (Walbaum). Journal of Fish Diseases. 31: 591-600.

Jónsdóttir, H., Bron, J.E., Wooteen, R., and Turnbull, J.F. 1992. The histopathology associated with the pre-adult and adult stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases.15: 521-527.

Kania, P.W., Evensen, O., Larsen, T.B., and Buchmann, K. 2010. Molecular and immunohistochemical studies on epidermal responses in Atlantic salmon *Salmo salar* L. induced by *Gyrodactylus salaris* Malmberg, 1957. Journal of Helminthology. 84: 166-172.

Kim, J., Tang, Q., Li, X., and Lane, M.D. 2007. Effect of phosphorylation and S-S bond-induced dimerization on DNA binding and transcriptional activation by C/EBPβ. Proceedings of the National Academy of Sciences of the United States of America. 104: 1800-1804.

Kovář, L. 2004. Tick saliva in anti-tick immunity and pathogen transmission. Folia Microbiology. 49: 327-336.

Kushner, I. 1982. The phenomenon of the acute phase response. Annals of the New York Academy of Sciences. 389: 39-48.

Kvamme, B.O., Skern, R., Frost, P., and Nilsen, F. 2004. Molecular characterization of five trypsin-like peptidase transcripts from the salmon louse (*Lepeophtheirus salmonis*) intestine. International Journal for Parasitology. 34: 823-832.

Leboulle, G., Crippa, M., Decrem, Y., Mejri, N., Brossard, M., Bollen, A., and Godfroid, E. 2002. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. The Journal of Biological Chemistry. 277: 10083-10089.

Leiby, D.A., Kanesa-thasan, N., Scott, P., and Nacy, C.A. 1994. Leishmaniasis in Parasitic Infections and the Immune System. Eds F. Kierszenbaum. Academic Press. San Diego, CA. pp 1-51.

Lindenstrøm, T., Buchmann, K., and Secombes, C.J. 2003. *Gyrodactylus derjavini* infection elicits IL-1β expression in rainbow trout skin. Fish and Shellfish Immunology. 15: 107-115.

Lindenstrøm, T., Sigh, J., Dalgaard, M.B., and Buchmann, K. 2006. Skin expression of IL-1β in East Atlantic salmon, *Salmo salar* L., highly susceptible to *Gyrodactylus salaris* infection is enhanced compared to a low susceptibility Baltic stock. Journal of Fish Diseases. 29: 123-128.

MacArthur, J.I., Fletcher, T.C., and Thomson, A.W. 1983. Distribution of radiolabeled erythrocytes and the effect of temperature on clearance in the plaice (*Pleuronectes platessa* L.). Journal of the Reticuloendothelial Society. 34: 13-21.

Magnadóttir, B. 2006. Innate immunity of fish (overview). Fish and Shellfish Immunology. 20: 137-151.

Magnadóttir, B. 2010. Immunological control of fish diseases. Marine Biotechnology. 12: 361-379.

Manning, M.J. and Nakanishi, T. 1996. The specific immune system: cellular defenses. In: The Fish Immune System: Organism, pathogen and environment. Eds G. Iwama and T. Nakanishi. Academic Press. San Diego, CA. pp 63-105.

Marty, G.D., Saksida, S.M., and Quinn II, T.J. 2010. Relationship of farm salmon, sea lice, and wild salmon populations. Proceedings of the National Academy of Sciences. 107: 22599- 22604.

McCarthy, E., Cunningham, E., Copley, L., Jackson, D., Johnston, D., Dalton, J.P., and Mulcahy, G. 2012. Cathepsin L proteases of the parasitic copepod, *Lepeophtheirus salmonis*. Aquaculture. 356-357: 264-271.

Muhlia-Almazán, A., Sánchez-Paz, A., and Garcia-Carreño, F.L. 2008. Invertebrate trypsins: a review. Journal of Comparative Physiology B. 178: 655-672.

Murphy, K.M. and Reiner, S.L. 2002. The lineage decisions of helper T cells. Nature Reviews Immunology. 2: 933-944.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G.A., and Burka, J.F. 2000a. Effects of sea lice (*Lepeophtheirus salmonis Kröyer*, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). Fish & Shellfish Immunology. 10: 47-59.

Mustafa, A., Speare, D.J., Daley, J., Conboy, G.A., and Burka, J.F. 2000b. Enhanced susceptibility of seawater cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), to the microsporidian *Loma salmonae* during a primary infection with the sea louse, *Lepeophtheirus salmonis*. Journal of Fish Diseases. 23: 337-341.

Nagelkerke, L.A.J., Pannevis, M.C., Houlihan, D.F., and Secombes, C.J. 1990. Oxygen uptake of rainbow trout *Oncorhynchus mykiss* phagocytes following stimulation of the respiratory burst. Journal of Experimental Biology. 154: 339-353.

Narnaware, Y.K., Baker, B.I., and Tomlinson, M.G. 1994. The effects of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout *Oncorhynchus mykiss.* Fish Physiology and Biochemistry. 13: 31-40.

Nese, L. and Enger, O. 1993. Isolation of *Aeromonas salmonicida* from salmon lice *Lepeophtheirus salmonis* and marine plankton. Diseases of Aquatic Organisms 16: 79-81.

Noël, W., Raes, G., Ghassabeh, G.H., De Baetselier, P., and Beschin, A. 2004. Alternatively activated macrophages during parasite infections. Trends in Parasitology. 20: 126-133.

Nolan, D.T., Reilly, P., and Wendelaar Bonga, S.E. 1999. Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in postsmolt Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries Aquatic Science. 56: 947-959.

Novak, C., Barker, D., and McKinley, S. 2012. First evidence of the salmon louse, *Lepeophtheirus salmonis* (Caligidae), as a vector of *Aeromonas salmonicida*. AAC Special Publication. 20: 84-87.

Nylund, A., Wallace, C., and Hovland, T. 1993. The possible role of *Lepeophtheirus salmonis* (Kroyer) in the transmission of infectious salmon anaemia. In: Pathogens of Wild and Farmed Fish: sea lice. Ed by G.A. Boxshall and D. Defaye. Ellis Horwood Limited, West Sussex, UK. 28: 367-373.

Parizi, L.F., Githaka, N.W., Logullo, C., Konnai, S., Masuda, A., Ohashi, K., and da Silva Vaz Jr., I. 2012. The quest for a universal vaccine against ticks: Cross-immunity insights. The Veterinary Journal. http://dx.doi.org/10.1016/j.tvjl.2012.05.023

Petterson, E., Sandberg, M., and Santi, N. 2009. Salmonid alphavirus associated with *Lepeophtheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases. 32: 477-479.

Pike, A.W. and Wadsworth, S.L. 1999. Sealice on salmonids: their biology and control. Advances in Parasitology. 44: 233-337.

Poli, V. 1998. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. The Journal of Biological Chemistry. 273: 29279-29282.

Prabhackar, A. 2010. Immunocytochemistry and Fish. In: Fish Immunology and Biotechnology. Swastik Publications, Sonia Vihar, Delhi. pp 180-203.

Raida, M.K. and Buchmann, K. 2009. Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. Developmental and Comparative Immunology. 33: 35-45.

Raynard, R.S., Bricknell, I.R., Billingsley, P.F., Nisbet, A.J., Vigneau, A., and Sommerville, C. 2002. Development of vaccines against sea lice. Pest Management Science. 58: 569-575.

Richards, D.T., Hoole, D., Lewis, J.W., Ewens, E., and Arme, C. 1996. Stimulation of carp *Cyprinus carpio* lymphocytes *in vitro* by the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae. Diseases of Aquatic Organisms. 25: 87-93.

Ritchie, G. 1997. The host transfer ability of *Lepeophtheirus salmonis* (Copepoda: Caligidae) from farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases. 20: 153-157.

Roitt, I. 1997. The production of effectors. In: Roitt's Essential Immunology 9th Ed. Blackwell Science Ltd. Oxford. pp 179-200.

Rolland, J.B. and Nylund, A. 1998. Infectiousness of organic materials originating in ISA-infected fish and transmission of the disease via salmon lice (*Lepeophtheirus salmonis*). Bulletin of European Association of Fish Pathologists. 18: 173-180.

Ross, N.W., Firth, K.J., Wang, A., Burka, J.F., and Johnson, S.C. 2000. Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Diseases of Aquatic Organisms. 41: 43-51.

Saksida, S.M., Morrison, D., Sheppard, M., and Keith, I. 2011. Sea lice management on salmon farms in British Columbia, Canada. In: Salmon Lice: An integrated approach to understanding parasite abundance and distribution. John Wiley & Sons, Inc. West Sussex, UK. pp 235- 278.

Sajid, M. and McKerrow, J.H. 2002. Cysteine proteases of parasitic organisms. Molecular and Biochemical Parasitology. 120: 1-21.

Salyers, A.A. and Whitt, D.D. 2002. The first line of defense against infection: Prevention and the phagocytic cell response. In: Bacterial Pathogenesis: A Molecular Approach 2nd Ed. ASM Press. Washington, DC. pp 53-68.

Saurabh, S., Mohanty, B.R., and Sahoo, P.K. 2011. Expression of immune-related genes in rohu *Labeo rohita* (Hamilton) by experimental freshwater lice *Argulus siamensis* (Wilson) infection. Veterinary Parasitology. 175: 119-128.

Secombes, C.J. 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Techniques in Fish Immunology. SOS Publications, Fair Haven, NJ. pp 137.

Secombes, C.J., Hardie, L.J., and Daniels, G. 1996. Cytokines in fish: an update. Fish and Shellfish Immunology. 6: 291-304.

Secombes, C.J., Wang, T. Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., and Zou, J. 2001. Cytokines and innate immunity of fish. Developmental and Comparative Immunology. 25: 713-723.

Secombes, C. 2008. Will advances in fish immunology change vaccination strategies? Fish and Shellfish Immunology. 25: 409-416.

Senftleben, U. and Karin, M. 2002. The IKK/NF-κB pathway. Critical Care Medicine, 30: S18- S26.

Sher, A., Pearce, E., and Kaye, P. 2003. Shaping the immune response to parasites: role of dendritic cells. Current Opinion in Immunology. 15: 421-429.

Skaug, B., Jiang, X., and Chen, Z.J. 2009. The role of ubiquitin in NF-κB regulatory pathways. Annual Review of Biochemistry. 78: 769-796.

Skugor, S. Glover, K.A., Nilsen, F., and Krasnov, A. 2008. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*). BMC Genomics. 9: 498.

Smyth, J.D. 1994. Immunoparasitology. In: Introduction to Animal Parasitology 3rd Ed. Cambridge University Press. Cambridge, UK. pp 460-490.

Speert, D.P. 1992. Macrophages in bacterial infection. In: The Natural Immune System: The Macrophage. Eds C.E. Lewis and J.O'D. McGee. Oxford University Press. New York, NY. pp 215-263.

Steen, N.A., Barker, S.C., Alewood, P.F. 2006. Proteins in the saliva of the Ixodida (ticks): Pharmacological features and biological significance. Toxicon. 47: 1-20.

Strober, W. 2001. Trypan blue exclusion test of cell viability. Current Protocols in Immunology: A3B.1-A3B.2.

Sutherland, B.J.G., Jantzen, S.G., Sanderson, D.S., Koop, B.F., and Jones, S.R.M. 2011. Differentiating size-dependent responses of juvenile pink salmon (*Oncorhynchus*

gorbuscha) to sea lice (*Lepeophtheirus salmonis*) infections. Comparative Biochemistry and Physiology, Part D. 6: 213-223.

Szabo, S.J., Sullivan, B.M., Peng, S.L., and Glimcher, L.H. 2003. Molecular mechanisms regulating TH1 immune responses. Annual Review of Immunology. 21: 713- 758.

Tabouret, G., Bret-Bennis, L., Dorchies, P., and Jacquiet, P. 2003. Serine protease activity in excretory-secretory products of *Oestrus ovis* (Diptera: Oestridae) larvae. Veterinary Parasitology. 114: 305-314.

Tadiso, M.T., Drasnov, A., Skugor, S., Afanasyev, S., Hordvik, I., and Nilsen, F. 2011. Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. BMC Genomics. 12: 141.

Tort, L., Balasch, J.C., and Mackenzie, S. 2003. Fish immune system. A crossroads between innate and adaptive responses. Immunologia. 2003; 22: 277-286.

Trust, T.J. 1986. Pathogenesis of infectious diseases of fish. Annual Review of Microbiology. 40: 479-502.

Underhill, D.M. and Ozinsky, A. 2002. Phagocytosis of microbes: Complexity in action. Annual Review of Immunology. 20: 825-852.

Varin, A. and Gordon, S. 2009. Alternative activation of macrophages: Immune function and cellular biology. Immunobiology. 214: 630-641.

Volanakis, J.E. 1982. Complement activation by C-reactive protein complexes. Annals of the New York Academy of Sciences. 389: 235-250.

Wagner, E.K., Hewlett, M.J., Bloom, D.C., and Camerini, D. 2008. Host immune response to viral infection. In: Basic Virology. Blackwell Publishing. Malden, MA. pp 97-118.

Wagner, G.N. and McKinley, R.S. 2004. Anaemia and salmonid swimming performance: the potential effects of sub-lethal sea lice infection. Journal of Fish Biology. 64: 1027-1038.

Watts, M., Munday, B.L., and Burke, C.M. 2001. Immune responses of teleost fish. Australian Veterinary Journal. 79: 570-574.

Weaver, C.T., Harrington, L.E., Mangan. P.R., Gavrieli, M., and Murphy, K.M. 2006. Th17: An effector CD4 T cell lineage with regulatory T cell ties. Immunity. 24: 677-688.

Wendelaar Bonga, S.E. 1997. The stress response in fish. Physiological Reviews. 77: 591-625.

Whitman, K.A. 2004. Bacteriology Culture Media. In: Finfish and Shellfish Bacteriology Manual: Techniques and Procedures. Iowa State Press, Ames, IA. pp 89-90.

Whyte, S.K. 2007. The innate immune response of finfish-A review of current knowledge. Fish and Shellfish Immunology. 23:1127-1151.

Wiegertjes, G.F., Forlenza, M., Joerink, M., and Scharsack, J.P. 2005. Parasite infections revisited. Developmental and Comparative Immunology. 29: 749-758.

Wikel, S.K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. International Journal for Parasitology. 29: 851-859.

Woo, P.T.K. 1992. Immunological responses of fish to parasitic organisms. Annual Review of Fish Diseases. 339-366.

Xu, D-H., Shoemaker, C.A., and Klesius, P.H. 2007. Evaluation of the link between gyrodactylosis and streptococcosis of Nile tilapia, *Oreochromis niloticus* (L.). Journal of Fish Diseases. 30: 233-238.

Yasukawa, H., Sasaki, A., and Yoshimura, A. 2000. Negative regulation of cytokine signaling pathways. Annual Review of Immunology. 18: 143-164.

Yazawa, R., Yasuike, M., Leong, J., von Schalburg, K.R., Cooper, G.A., Beetz-Sargent, M., Robb, A., Davidson, W.S., Jones, S.R.M., and Koop, B.F. 2008. EST and mitochondrial DNA sequences support a distinct Pacific form of salmon louse, *Lepeophtheirus salmonis*. Marine Biotechnology. 10: 741-749.

Yoder, J.A. 2004. Investigating the morphology, function and genetics of cytotoxic cells in bony fish. Comparative Biochemistry and Physiology, Part C. 138: 271-280.

Appendices

Appendix A- Supplementary Data

A.1 Dopamine controls for phagocytosis assays

To ensure that any effects observed on macrophages was not due to the presence of dopamine (DA), a control was prepared with 0.25 mM DA and ASW in the absence of *L. salmonis*. Dopamine was used during the collection of *L. salmonis* secretions and could not be removed from the solution without simultaneously removing any prostaglandin E_2 present because of their close molecular weights.

SHK-1 cells were incubated with DA + *A. salmonicida* and total number of undamaged cells counted. The percentage of cells positive for at least one bacterium, average number of bacteria per positive cell and phagocytic index were also determined and compared to SHK-1 cells incubated with *A. salmonicida* alone.

There was no significant difference ($T = 0.4386$, $p = 0.6705$) in the total number of undamaged cells between incubation with DA + *A. salmonicida* or *A. salmonicida*. There was also no significant difference in percent of SHK-1 cells positive for bacteria ($T = 0.8338$, $p = 0.4349$), mean number of bacteria per positive cell ($T = -0.0655$, $p = 0.9490$) or phagocytic index ($T = 0.1119$, $p = 0.9131$) between the two treatments. Based on these results it was concluded that DA itself had no effect on phagocytic activity of SHK-1 cells.

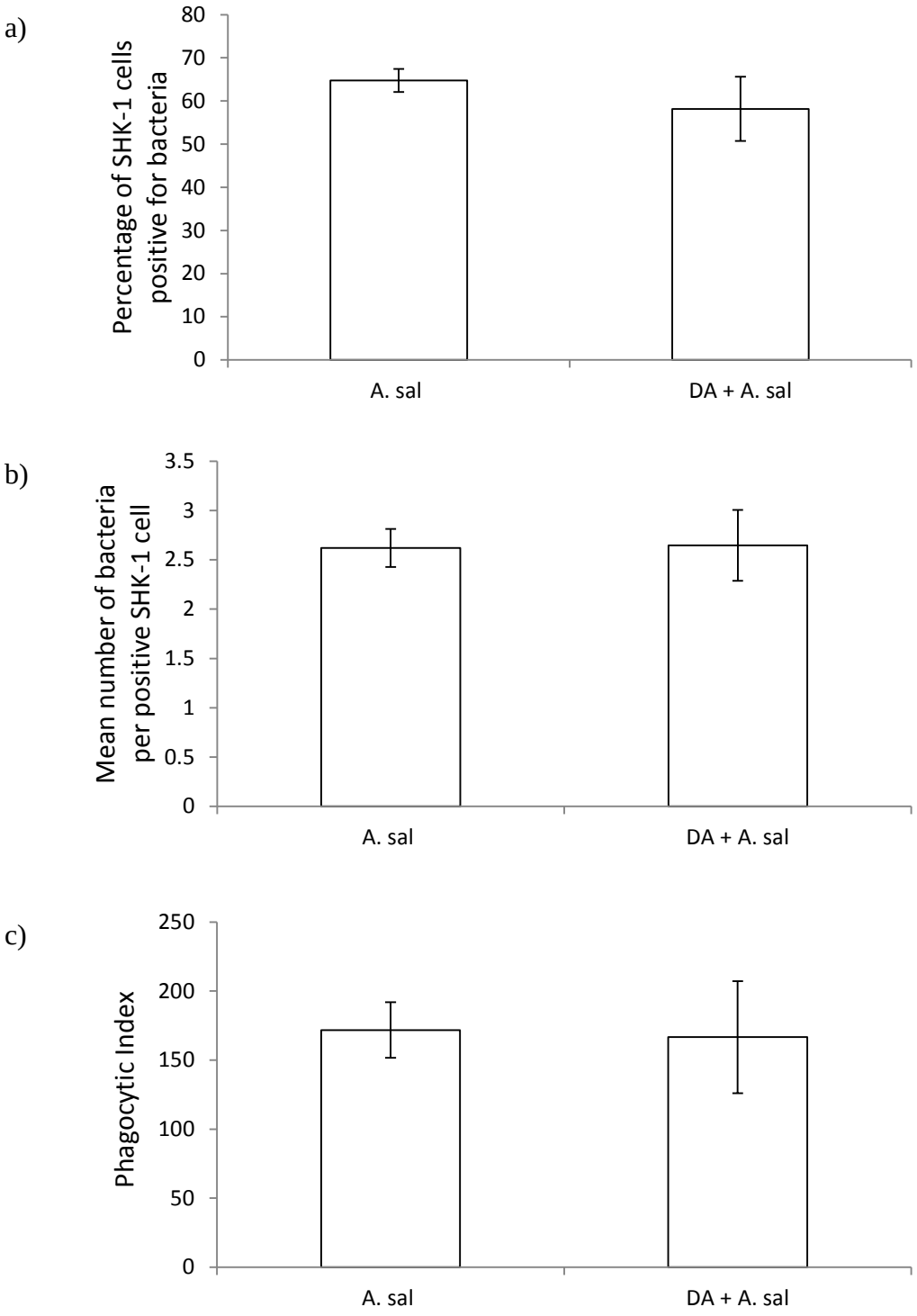

Figure A.1. Phagocytic activity of SHK-1 cells: a) percentage of SHK-1 cells positive for at least one bacterium, b) number of bacteria per positive, and c) phagocytic index following incubation with A. salmonicida (A. sal) or dopamine (DA) + A. sal. Values represent mean (± SE) calculated from 6 replicates per treatment; for each treatment, 200 individual SHK-1 cells were examined. Differences between treatments were determined using a two-sample T-test ($p < 0.05$).

A.2 Dose response-percentage of undamaged cells

For the dose response assay, total number of undamaged cells (i.e. cells that did not exhibit any signs of damage to the nuclear membrane) was determined for each treatment. The total number of undamaged cells was significantly lower ($F = 79.05$, $p < 0.01$) for all treatments where cells were challenged concurrently with any concentration of SEPs + *A. salmonicida*. Cells incubated had significantly ($F = 79.05$, $p < 0.01$) more apparently healthy cells than all treatments with the exception of SEPs (10^{-1}) and SEPs (10^{-2}).

Table A.1. Mean (± SD) percentage of undamaged SHK-1 cells following three hours incubation with listed treatments. Results are expressed as the mean of 5 replicates for each treatment; for each replicate, 200 individual macrophages were examined. Differences in treatments were determined using one-way ANOVA ($p < 0.05$). Lowercase letters (a-f) represent significant differences among treatments.

Treatment	Percent (%) of live SHK-1 cells	Treatment	Percent (%) of live SHK-1 cells
PBS	93.7 ± 3.11 (a)	*A. salmonicida*	75.5 ± 3.82 (b)
SEPs (10^{0})	77.0 ± 7.47 (b, c)	SEPs (10^{0}) + *A. salmonicida*	57.9 ± 2.75 (e)
SEPs (10^{-1})	85.4 ± 5.95 (a, c)	SEPs (10^{-1}) + *A. salmonicida*	58.7 ± 5.33 (e)
SEPs (10^{-2})	87.4 ± 2.41 (a, d)	SEPs (10^{-2}) + *A. salmonicida*	52.5 ± 1.46 (e, f)
SEPs (10^{-3})	81.8 ± 1.68 (b, c, d)	SEPs (10^{-3}) + *A. salmonicida*	52.9 ± 1.82 (e, f)
SEPs (10^{-4})	80.2 ± 2.31 (b, c, d)	SEPs (10^{-4}) + *A. salmonicida*	48.4 ± 4.35 (f)

Appendix B- Protease inhibitor addition to *L. salmonis* secretions

To determine if trypsin-like proteases previously identified (Firth *et al*., 2000) in *L. salmonis* secretions might degrade proteins, thus altering the responses seen throughout this study, SEPs were collected and a protease-inhibitor added. Additionally, SEPs were collected from *L. salmonis* parasitizing various host species (pink, chum and Atlantic salmon) for 24 hours (see methods below). These secretions were then used in respiratory burst assays, using macrophages isolated from pink, chum and Atlantic salmon, and phagocytosis assays, using SHK-1 cells, to examine potential differences between the presence/absence of protease activity in SEPs.

B.1 Collection of secretions

L. salmonis secretory products were collected by L.M. Braden (Pacific Biological Station) (section 2.2) from live lice (n= 1600) 24 hours after removal from farmed Atlantic salmon at a Marine Harvest Canada site located in the Broughton Archipelago, British Columbia (latitude: 50° 24' 56" N; longitude: 126° 25' 08" W) during a fish harvest (June 19, 2012) or following a 24 hour infection on pink, chum or Atlantic salmon. Secretory products were immediately frozen at -80^{o}C. Prior to filtration using Jumbosep™ Centrifugal Devices, *L. salmonis* secretory products were defrosted slowly on ice and a protease inhibitor cocktail added (1:100 v/v)(Sigma). The Jumbosep™ Centrifugal Devices were fitted with a 30 kilodalton (kDa) membrane and the filtrate retained. Secretory products obtained from lice removed from the farmed Atlantic salmon for 24 hours are referred to as $SEPs_{pi}$. Secretory products collected from lice following 24 hour infection on pink, chum and Atlantic salmon are referred to as $SEPs_{pink}$, $SEPs_{chum}$ and $SEPs_{Atl}$ respectively.

B.2 Results

Respiratory burst-isolated macrophages

There was no significant difference in respiratory burst response for pink salmon macrophages (df = 2, χ^2 = 2.693, p = 0.2602), chum salmon macrophages (df = 2, χ^2 = 2.013, p = 0.3655) or Atlantic salmon macrophages (F = 1.69, p = 0.2197) exposed to SEPs, $SEPs_{pi}$ or $SEPs_{pink}$, $SEPs_{chum}$, or $SEPs_{Atl}$, respectively.

When comparisons were made among species, pink salmon macrophages had a significantly greater response to SEPs (F = 47.55, $p < 0.01$) and $SEPs_{pi}$ (df = 2, χ^2 = 9.974, p = 0.0068) than chum and Atlantic salmon macrophages. When exposed to SEPs (isolated after feeding on their respective hosts), chum salmon macrophages had a significantly (df = 2, χ^2 = 7.739, p = 0.0209) lower respiratory burst response than pink and Atlantic salmon macrophages exposed to their corresponding treatments ($SEPs_{pink}$ or $SEPs_{Atl}$) (Figure B.1).

Phagocytosis assay-SHK-1 cells

There was a significantly (F = 18.80, p <0.01) greater percentage of SHK-1 cells positive for at least one bacterium following exposure to SEPs + *A. salmonicida,* $SEPs_{pi}$ + *A. salmonicida,* $SEPs_{pink}$ + *A. salmonicida* or $SEPs_{Atl}$ + *A. salmonicida* compared to $SEPs_{chum}$ + *A. salmonicida* or *A. salmonicida* alone. When compared to *A. salmonicida* alone, the mean number of bacteria per positive SHK-1 cell was only significantly (F = 3.25, p = 0.0222) higher for $SEPs_{pi}$ + *A. salmonicida* and $SEPs_{pink}$ + *A. salmonicida*. Phagocytic index was highest in SHK-1 cells incubated with $SEPs_{pink}$ + *A. salmonicida*; this was significantly (F = 8.62, $p < 0.01$) greater than cells exposed to $SEPs_{chum}$ + *A. salmonicida* or *A. salmonicida* alone. Phagocytic index was also significantly (F = 8.62, $p < 0.01$) greater in $SEPs_{Atl}$ + *A.*

salmonicida and $SEPs_{pi}$ + *A. salmonicida* when compared to *A. salmonicida* alone (Figure B.2).

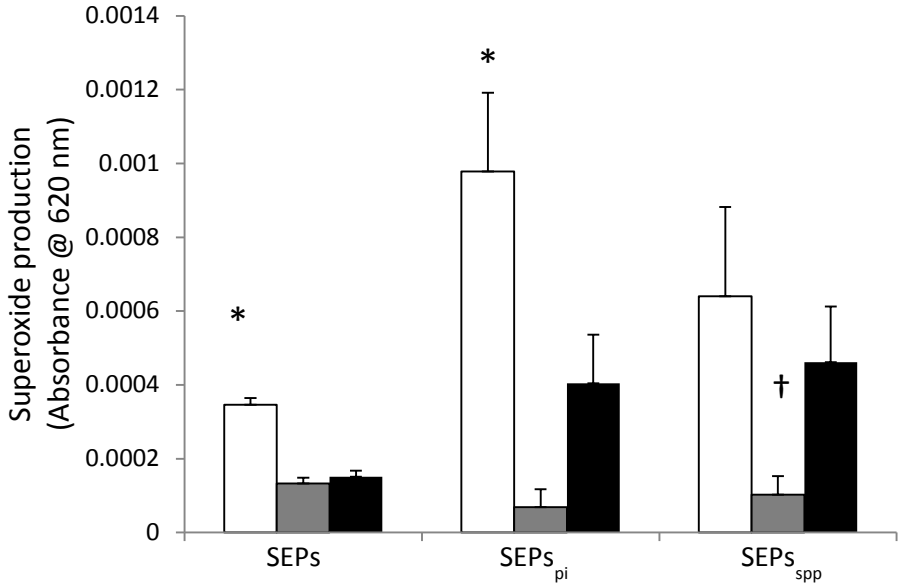

Figure B.1. Superoxide (O2-) production (+ SE) by pink (□), chum (▒) and Atlantic (■) salmon macrophages following incubation with SEPs, SEPspi, or SEPsspp. SEPsspp = L. salmonis secretions isolated off their respective host (pink, chum or Atlantic salmon). Values represent mean (+ SE) calculated from 6 replicates per treatment, with values adjusted to represent absorbance readings for 1000 cells. Differences were determined using one-way ANOVA ($p < 0.05$). Symbols (*, †) represent differences among species.

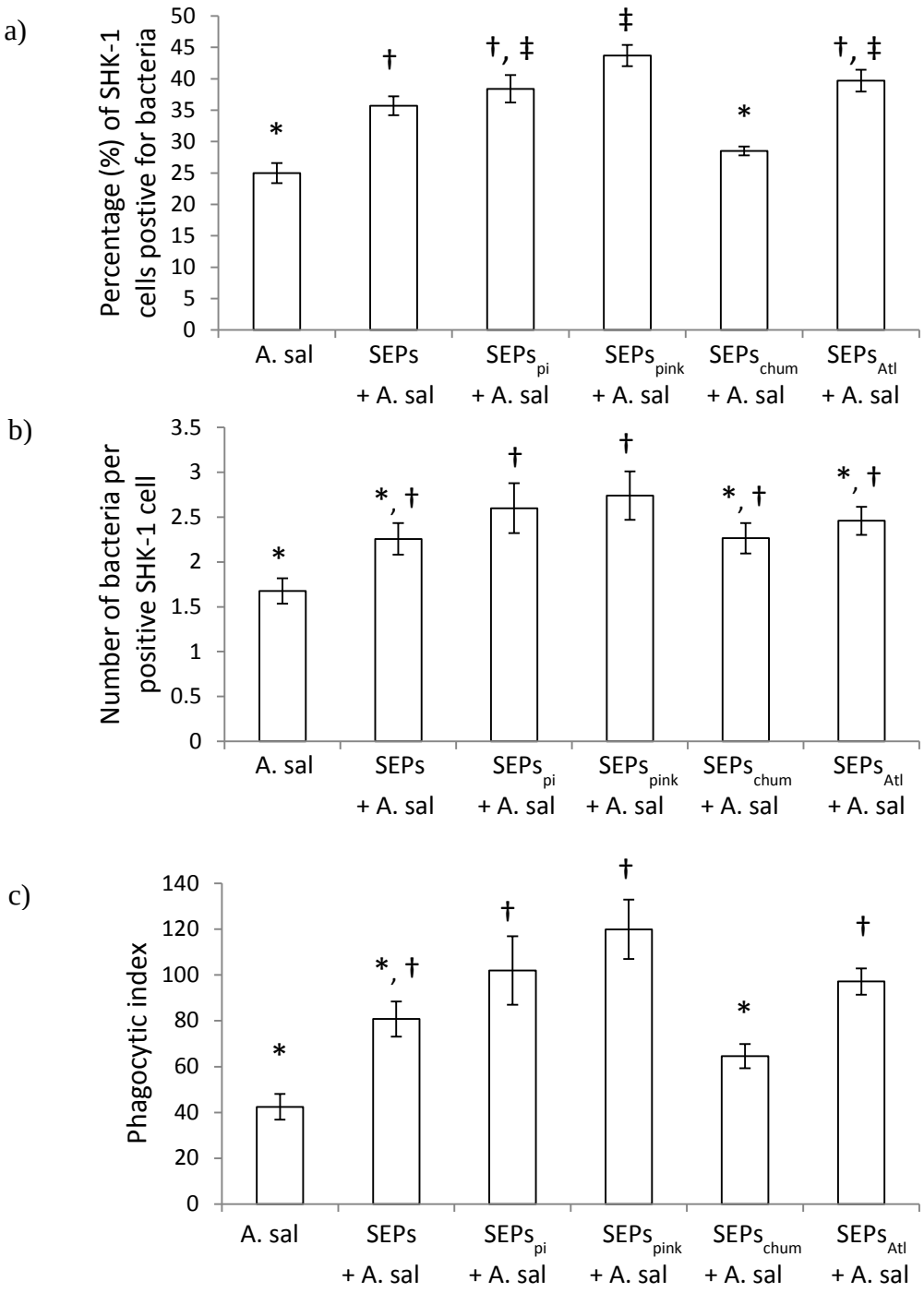

Figure B.2. Phagocytic activity of SHK-1 cells: a) percentage of SHK-1 cells positive for at least one bacterium, b) number of bacteria per positive cell, and c) phagocytic index following incubation with A. salmonicida (A. sal), SEPs + A. sal, SEPspi + A. sal, SEPspink + A. sal, SEPschum + A. sal, or SEPsAtl + A. sal. Values represent mean (± SE) calculated from 6 replicates per treatment; for each replicate 200 individual SHK-1 cells were examined. Differences among treatments were determined using one-way ANOVA ($p < 0.05$). Symbols (*, †, ‡) represent differences among treatments.