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HOST IMMUNOMODULATION BY *LEPEOPHTHEIRUS SALMONIS*

by

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Submitted in partial fulfillment of the requirements
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at

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DALHOUSIE UNIVERSITY

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This work is dedicated to the three most important people in my life. To the two greatest parents one could possibly have and to the most scrumtrelescent person I have ever met, Amy.

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Abstract

Lepeophtheirus salmonis is an ectoparasitic copepod that causes serious disease in wild and farmed salmonids. As the relationship between *L. salmonis* and its hosts is not well understood, this study was undertaken to investigate whether *L. salmonis* immunomodulation of Atlantic salmon (*Salmo salar*) occurs. The presence of prostaglandin E_2 (PGE_2), a potent vasodilator, was identified in the secretions/excretions (SEPs) of *L. salmonis* at similar concentrations to that found in the saliva of other arthropod parasites. Prostaglandin E_2, at physiologically relevant concentrations, inhibited lipopolysaccharide (LPS)-induced expression of numerous immune-related genes in a salmonid macrophage-like cell line.

The presence of other immunomodulatory compounds in the SEPs of these parasites was investigated. Peptides identified from SEP fractions demonstrated significant similarity to six *L. salmonis* expressed sequence tags (ESTs), including trypsin. Many of these ESTs showed increased expression during attached and feeding stages of the parasite. Fractions with and without PGE_2 or trypsin inhibited LPS-induced expression of interleukin-1β (IL-1β).

In chapter 5.0, it was investigated whether a low-level *L. salmonis* infection, in the absence of stress response, affected expression of Atlantic salmon immune-related genes. During low-level infection, head kidney tissue expression of IL-1β and Major histocompatibility (MH) class II increased, while MH class I expression decreased, in infected fish as compared to uninfected fish. Lipopolysaccharide stimulation of macrophages from infected fish did not further increase levels of MH class I and cyclooxygenase-2 (COX-2) expression.

To determine the effects of successive infections of *L. salmonis* on the immunological status of Atlantic salmon a low-level initial infection was carried out 14 d prior to a second infection. The expression of nearly all immune-related genes studied increased following initial infection with *L. salmonis*. However, immunological stimulation did not reduce parasite numbers or provide greater protection against a successive infection. This initial increase in expression of pro-inflammatory genes in infected fish, returned to control levels following re-infection, but was observed to increase late in the infection alongside plasma cortisol and PGE_2 levels.

List of Abbreviations

Ab	antibody
ABC	ammonium bicarbonate
ACN	acetonitrile
ADP	adenosine diphosphate
AMA	ammonium acetate
APC	antigen presenting cell
ATP	adenosine triphosphate
AA	arachidonic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of determination marker
cDNA	complementary deoxyribonucleic acid
C3	complement component 3
Ct	threshold cycle
COX	cyclooxygenase
DA	dopamine
Dpi	days post infection
dpii	days post initial infection
dpri	days post re-infection
EGC	eosinophilic granule cells
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbant assay
EPA	eicosapentanoic acid
ERB	expression relative to β-actin
EST	expressed sequence tag
FBS	fetal bovine serum
HKM	head kidney macrophages
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INVX	invariant chain
ISH	in situ hybridization
kDa	kilodaltons
LC-MS	liquid chromatography mass spectrometry
LPS	lipopolysaccharide
MH	major histocompatability
NFκB	nuclear factor κB
NMW	nominal molecular weight
OD	optical density
ODS	octadecylsilane
PBML	peripheral blood mononuclear leucocytes
PBS	phosphate buffered saline
PG	prostaglandin

P/S	penicillin/streptomycin
RNA	ribonucleic acid
RP-HPLC	reverse phase-high pressure liquid chromatography
RT-PCR	reverse transcription-polymerase chain reaction
RTS-11	rainbow trout spleen cell line
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of mean
SEP	secretory/excretory product
SHK-1	salmon head kidney cell line
SP	secretory product
SPE	solid phase extraction
SPSS	sigma plot sigma stat
SSW	sterile seawater
TGF	transforming growth factor
Th	T helper cell
TNF	tumour necrosis factor
TOF	time of flight

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Final words of wisdom that kept me going throughout,

"A lot of doubters said it couldn't be done by me, those same suckers are now looking from under me...", Ice T, OG.

Chapter 1.0 General introduction

1.1 Parasitic lifestyle of copepods

1.1.1 Copepod life history

The largest and most diverse Sub-Class of crustaceans are the Copepoda, containing over 14, 000 species. Many species of copepod are free-living in fresh and seawater environments, but there are others that are parasitic, including over 1,550 species of the Order Siphonostomatoida (Huys and Boxshall, 1991). While there are numerous orders and families within the Copepoda the following discussion will dwell on those species for which host-parasite interactions are best known. The major groups of parasitic copepods reviewed here are the anchor worms (Order Lernaeaidae), gill maggots (Order Ergasilidae) and sea lice (Order Siphonostomatoida).

As an in depth discussion on the taxonomy of Copepoda is far beyond the scope of this chapter, those interested are directed to the comprehensive reviews by Huys and Boxshall (1991) and Martin and Davis (2001), from which the isolation of Poecilostomatoida as a separate order from Cyclopoida has been followed here. Parasitic copepods are not to be confused with other crustacean parasites, also referred to as fish lice, the Branchiurans (*Argulus spp.*) or Isopods, as they are not copepods and have distinctly different life histories.

The parasitic copepods, which have been most intensively studied are those that have economic impacts on wild or cultured fish. For example, the most widely studied is the salmon louse, *Lepeophtheirus salmonis*, a species that causes indirect and direct losses in cultured salmon in excess of $100 million

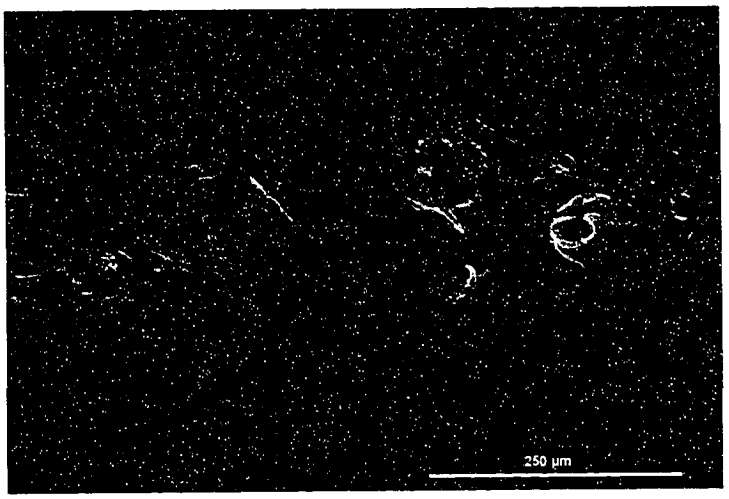
USD annually (Johnson et al., 2004), due to costs associated with parasite control, reduced host growth and carcass downgrading at harvest.

Although lernaeaids, ergasilids and siphonostomatids are quite diverse, they share some basic life history traits. For example, these groups have the same basic body form at the copepodid stage, exemplified by *L. salmonis* (Fig. 1.1). Although there are variations in the number and naming of the developmental stages, members of these groups share a similar life cycle that includes up to 6 naupliar and metanaupliar stages followed by 5 copepodid stages prior to the adults (Huys and Boxshall, 1991). Members of the ergasilidae such as *Ergasilus sieboldi* have this type of life cycle as well (Abdelhalim et al., 1991).

In the siphonostomatoida the number of naupliar stages is reduced and there is a single copepodid stage, which is followed by a series of chalimus stages. The chalimus stages are easily distinguished by attachment to their host by a thread like frontal filament produced from a frontal gland. Some families, such as Lernaeaopodidae (*Salmincola* spp.) escape their egg sac already as copepodids and others, species within the family Caligidae, have pre-adult stages between the chalimus and adult stage (Kabata, 1970).

Parasitic copepods also differ in the number of hosts needed for development. The life cycle of *Lernaea cyprinacea* begins with 3 naupliar non-feeding stages, prior to seeking a host upon which it undergoes its first copepodid moult (Grabda, 1964). This stage is followed by three other moults through copepodid stages before leaving the host as a cyclopoid adult in

Figure 1.1: Copepodid stage of *Lepeophtheirus salmonis* prior to attachment.



search of another host upon which it reproduces (Shields and Goode, 1978). Lernaeaocerids exhibit the presence of an intermediate host in their life cycle (Kabata, 1970). Still for many more species details of the life cycles are not yet known (Kabata, 1970). What is certain is that the rate of development of copepod parasites through the different life stages is largely dependent upon water temperature, and to a lesser extent, the species of host upon which they develop (Johnson, 1993).

The process of host finding is still relatively unknown. The ability of copepod parasites to orientate themselves in the environment to increase the likelihood of encountering potential hosts has been demonstrated in several studies (Boxshall, 1976; Bron et al., 1993a; Heuch 1995; Heuch et al., 1995; Lewis, 1963). Their responses to mechanical and visual cues once in this environment have also been documented (Bron et al., 1993a; Poulin et al., 1990). Most recently, it was revealed that behavioural activation and electrophysiological responses in adult male *L. salmonis* occur in response to host fish odours (Ingvarsdóttir et al., 2002). Whether chemical stimuli are enough to attract these parasites to hosts while they are in the water column or whether this may only be a means of determining a suitable host once in close proximity, has yet to be elucidated.

1.1.2 Attachment and feeding

Most parasitic copepods first attach to their hosts via a modified second antennae and maxillae, which can be reduced and even lost in some species

(Bennett and Bennett, 2001; Bron et al., 1993a; Kabata and Cousens, 1977; Paterson and Poulin, 1999; Robinson and Avenant-Oldewage, 1996; Roubal, 1999). Adults of *Lernaea spp.* clutch the host tissue and then penetrate the tissue with the anterior-cephalic region of their body. After penetration the cephalic region elongates and undergoes metamorphosis to produce an anchor process, which solidifies attachment to the host. The mechanism by which penetration is achieved has not been fully described, but may occur through mechanical disruption (ie. chewing) (Shields and Goode, 1978). Penetration into the host results in extravasated blood occurring between the parasite cuticle and the epidermal sheath that covers the parasite (epithelial cover), substantial tissue compression and necrosis adjacent to developing horns of the anchor process (Shields and Tidd, 1974). Similarly, the siphonistomatoid families Lernaeaoceridae and Pennelidae undergo cephalothoracic expansion and development of antlers anterior to the swimming legs, which develop anteriorly to anchor the parasite to the host tissue (Kabata, 1969; Radhakrishnan and Nair, 1981a). Lernaeopodidae differ from Lernaeaoceridae and Pennelidae in that they remain outside the host and only the inserted bulla (attachment structure) remains within host tissue. These species at first produce a frontal filament, which in later stages becomes associated, and later fused with the 2nd antennae to form the bulla (Kabata and Cousens, 1977). This process is best desribed for *Salmincola californiensis*, and involves the excavation of an implantation cavity, which is then further enlarged by inflation of the bulla (Kabata and Cousens, 1972). In the family Caligidae, as in *S. californiensis*, a frontal filament is

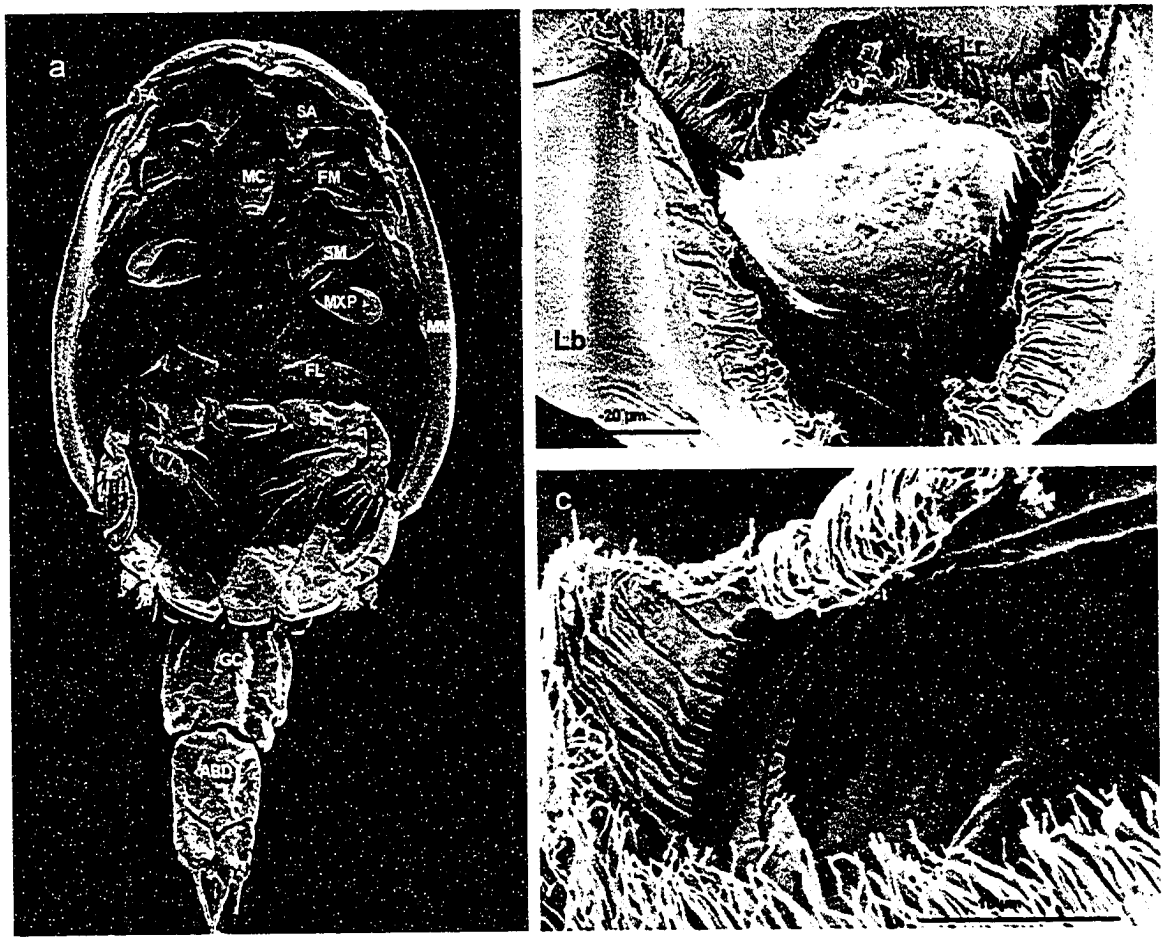
produced by the larval chalimus stages to hold them in place (Bron et al., 1991; Gonzalez-Alanis et al., 2001) (Fig. 1.2). This structure, however, is absent in the adult stage, with the parasites providing purchase to the host surface through suction involving the ventral regions of a dorsoventrally flattened body. This adaptation allows the adult parasites to actively graze over the entire surface of the host (Kabata and Hewitt, 1971).

In the ergasilids, only fertilized females are parasitic. Early developmental stages and the adult males live free in the water column, while parasitic females maintain a non-permanent association with the host by means of powerful claw-like 2nd antennaes. This mechanism of attachment allows them to change attachment sites and graze along the gill filaments (Paperna and Zwerner, 1982).

The lernaeaids and ergasilids can further be differentiated from the Order Siphonostomatoida by the structure of their mouth-parts (Kabata 1970; 1974). These species mainly consist of a buccal orifice that does not protrude above the body surface but is rather like a partly uncovered hole, while the Siphonostomatoids have tubular mouths, called oral cones, that are often folded ventrally when not in the process of feeding (Fig. 1.3a). The oral cone consists of the labrum and labium, which form a tapering tubular structure surrounding the mouth opening (Huys and Boxshall, 1991; Kabata, 1974) (Fig. 1.3b).

Figure 1.2: Hematoxylin and eosin stain of *Lepeophtheirus salmonis* attachment site on Atlantic salmon host. (S) denotes host scale, (E) host epidermal cells, (FF) frontal filament, (Se) frontal filament secretion.

Figure 1.3: Ventral surface of a pre-adult male *Lepeophtheirus salmonis* (a). Increased magnification of the mouth cone structure (b, c). First antennae (FA), Second antennae (SA), mouth cone (MC), first maxilla (FM), second maxilla (SM), maxilliped (MXP), first leg (FL), second leg (SL), marginal membrane (MM), genital complex (GC), abdomen (ABD), labrum (Lr), Labium (Lb), mandible (M), strigil (S), labial fold (Lf).



While modes of feeding are not thoroughly understood for parasitic copepods, Kabata (1974) provides the best explanation of how this may occur in caligid parasites. Briefly, the mouth cone is pressed into the host skin, pushing away the labial fold and exposing the strigil. This initiates contact between the many fine teeth of the strigil (Fig. 1.3c) and the host epithelia. Moving the two halves of the strigil in divergent and convergent directions penetrates the host tissue. The resulting debris passes into the buccal cavity through the motion of the mandibles (Figs. 1.3b,c) (Kabata, 1974). Muscular contractions of the labrum and peristaltic movements of the gut, guide the debris into the esophagus and through the digestive tract. This feeding activity may be assisted by external digestion facilitated by the release of saliva-like secretions containing proteases (Fast et al., 2003; Firth et al., 2000; Kabata, 1970).

In general, all of the developmental stages of parasitic copepods found on the host feed on mucus, skin and/or blood. Food sources may change during development especially in those species that undergo marked morphological changes. For example, early stages of lernaeaid and pennelid species, may only feed on skin and mucus, whereas, following host tissue penetration, the availability of extravasated blood may result in higher incorporation of blood in the meal (Kabata, 1969; Shields and Goode, 1978). It has been suggested, for *L. salmonis*, that mainly the adult female incorporates blood into its meal, whereas adult male and earlier developmental stages feed primarily on skin and mucus (Brandal et al., 1976; Haji Hamid et al., 1998).

In many ways the lifestyles of parasitic copepods mirror those of terrestrial ectoparasitic arthropods, such as ticks, fleas and biting flies. In some species of biting flies only the female is parasitic, needing the blood meal for only a few minutes to complete egg production (Balashov, 1972). The host attachment is longer in parasitic copepods and in some cases adult females show much longer association with the host than males. In the case of species with intermediate hosts, males often do not parasitize the second host as copulation occurs on the first host and their role in fitness is completed. There are terrestrial ectoparasites that remain on their host for extended periods such as the horn fly (*Haematobia irritans*), which feeds intermittently for 24 hr (Cupp et al., 1998). Tick species remain on their host for several weeks and probably the extreme is the cat flea (*Ctenocephalides felis felis*), which in captivity remains on the same host for up to 113 days (Dryden, 1989). Ectoparasites that maintain extended contact with their host must balance the need to feed on host tissues with the detrimental effects of irritation and stimulating either an immune or grooming response from the host. The similarities of parasitic copepods with their terrestrial counterparts and how they manage these responses is discussed in the following sections.

1.2 Host responses and their effects on parasitic copepods

1.2.1 What are the common host responses to infection?

Description of damage to host fish caused by parasitic copepods has mainly involved gross pathology. There has been a lack of histopathological studies until very recently. The response of fish to wounding or the presence of foreign

bodies is well described (Iger et al., 1994). This is depicted as a proliferation of filament cells and wound closure, followed by leucocyte infiltration, moderate increases in intracellular spaces, epidermal thickening and hyperplasia. Often early in the inflammatory response a rapid influx of neutrophils is observed (Ellis, 1977). Following a 2-3 mm incision into the mid-ventral line of juvenile Atlantic salmon skin, neutrophils were still present in the dermis below the insult at 7 days post incision (Roubal and Bullock, 1988). Neutrophil accumulation is thought to involve an up-regulation of lymphocyte-mediated immune systems and to contribute to subsequent eosinophil accumulation (Ellis, 1977; Owhashi et al., 2001). Generally, the host response to infection is not specific for crustacean parasites but is similar to that mounted against any foreign body causing an open wound. This includes tissue deposition around the foreign particle or parasite of a connective tissue capsule, which isolates it from contact with host tissues. Increases in skin thickness and mucus production often accompany this response. The diversity within parasitic copepods, with respect to their life cycle, modes and sites of attachment to the host affect differences in the responses observed from their host species (Table 1.1).

Within the Order Cyclopoida, pathology of freshwater lernaeaids is the best studied (Table 1.1). Prior to host penetration and anchor development, copepodid attachment, feeding and development generally causes minimal host responses (Shields and Tidd, 1974). However, as the parasite infection

Table 1.1: Histopathological studies of parasitic copepod infections

Copepod Order/ family	Parasite species	Host species	Histopathology	Reference
Order: Cyclopoida	*P. spinosus*	*Mytilus spp.*	Exhibit a heavy hemocytic response	Caceres-Martinez and
Family: *Myicolidae*			producing a granuloma-like structure engulfing the copepod	Vasquez-Yeomans, 1997
Family: *Lernaeaidae*	*L. cruciata*	*M. salmoides*	No signs of inflammation against older females; recently metamorphosed females stimulated inflammation, associated with expulsion, EGCs observed	Noga, 1986
	L. cruciata	*M. chrysops*	Thickened epithelia collar near penetration, anterior thoracic region of parasite may become encapsulated; large numbers of leucocytes and neutrophils along margins of penetration	Joy and Jones, 1973
	L. polymorpha	*A. nobilis*	Neutrophils around the periphery of the lesions, only eosinophilic granule cells observed in immune fish	Shariff and Roberts, 1989
	L. cyprinacea	*O. mykiss, C. auratus, H. temmincki*	Copepodid development and attachment produces minimal response, anchors surrounded by fibrous tissue (thick collar) and leucocytic responses below dermis	Berry et al., 1991; Shields and Goode, 1978; Shields and Tidd, 1974; Woo and Shariff, 1990
	L. piscinae	*A. nobilis*	Mainly neutrophils in eye pathology	Shariff, 1981
Order: Siphonostom-atoida	*D. manteri*	*P. leopardus*	Larval stages cause little or no inflammation; adults cause eosinophil and lymphocyte infiltration	Bennett and Bennett 1994; 2001
Family: *Dissonidae*				
Family: *Dichelesthioidea*	*L. kroyeri*	*D. labrax*	Massive mucous cell	Manera and

			proliferation, tissue erosion	Dezfuli, 2003
Family: *Naobranchiidae*	*N. variabilis*	*Tetractenos hamiltoni*	Little gill tissue proliferation, some hyperplastic epithelial cells and little if any infiltration	Roubal, 1999
Family: *Lernaeaoceridae*	*L. branchialis*	*G. morhua*	lymphocyte proliferation, extravasating blood, centres of necrosis	Kabata, 1970; Khan, 1988
	P. wilsoni	*D. hystrix*	Tumourous growth at attachment site results in plasmacyte accumulation	Radhakrishnan and Nair, 1981a
Family: *Pennelidae*	*P. cincinnatus*	*A. stomias*	Large haematoma due to blood vessel damage at attachment site, tumourous growth anchors the parasite	Kabata, 1969; Kabata and Forrester, 1974
Family: *Lernaeaopodidae*	*S. californiensis*	*O. nerka*	Mild reaction to bulla insertion, occlusion of branchial circulation, destruction due to feeding and hypertrophy	Kabata, 1970; Kabata and Cousens, 1977
Family: *Caligidae*	*L. salmonis*	*S. salar*	Little to no inflammation, some mucus cell proliferation, following removal of parasite inflammation occurs around remaining frontal filament, tissue erosion	Johnson and Albright, 1992a; Jones et al., 1990; Jonsdottir et al., 1992; Nolan et al., 1999
	L. salmonis	*O. kisutch*	Acute inflammation early on, encapsulation and shedding of parasite	Johnson and Albright, 1992a
	L. pectoralis	*P. flesus*	Epidermal hyperplasia around the periphery of the feeding sites, localised to dermis	Boxshall, 1977
	C. uruguayensis	*T. savala*	2nd antennae causing tissue proliferation but no leucocyte infiltration into epidermis, some in dermis	Radhakrishnan and Nair,1981b
	C. epidemicus	*A. australis*	No tissue proliferation, however, redundant frontal filaments associated with macrophages and fusion into giant	Roubal, 1994

			cells	
	C. elongatus	*S. salar*	Lesions consisting of necrotic cells, no inflammation and some mild hyperplasia	MacKinnon, 1993
Order: Poecilostom-atoida Family: *Ergasilidae*	*E. labracis*	*M. saxatalis*	Epithelial hyperplasia, mucous cell proliferation and intensive infiltration of macrophages, lymphocytes and eosinophils,	Paperna and Zwerner 1982
	Dermoergasilus spp.	*A. australis*	Infiltrating lymphocytes	Roubal, 1989
	E. lizae	*A. australis*	Inflammation included eosinophils, neutrophils and macrophages but not more so near the parasite	Roubal, 1989

continues, penetration and anchor development results in a thickened epithelial collar formation adjacent to the site of penetration, fibrous tissue around the anchors and in some instances encapsulation of the anterior thoracic region of the parasite. This can be followed by infiltration of large numbers of mononuclear leucocytes and neutrophils along the margins of the penetration site (Berry et al., 1991; Joy and Jones, 1973; Shields and Goode, 1978).

It appears that a high proportion of lernaeaids are rejected soon after penetration and/or prior to achieving full egg producing capacity. Noga (1986) suggests that older *Lernaea cruciata* females attached to largemouth bass (*Micropterus salmoides*) elicited no signs of inflammation, whereas recently metamorphosed-females stimulated inflammation resulting in quick expulsion. The inflammatory response often involved eosinophilic granule cells (EGCs) in close proximity to the parasite. Similarly, Shariff and Roberts (1989) observed neutrophils around the periphery of the lesions caused by *Lernaea polymorpha* on naïve big head carp (*Aristichthys nobilis*), while EGCs were only observed in immune fish.

Similar to the lernaeaids, some members of the Order Siphonostomatoida, such as lernaeaocerids, pennelids and lernaeaopodids use the host response as to secure attachment. *Peniculisa wilsoni* infection, for instance elicits formation of a tumorous growth at the site of attachment (Radhakrishnan and Nair, 1981a). In *Phrixocephalus cincinnatus* infections of Pacific sanddabs (*Citharichthys sordidus*), a hematoma was described, by Kabata (1969), as a result of local hemorrhages. The hematoma was bound together by strands of connective

tissue and the parasite anchor. The lernaeopodid bulla is implanted into soft tissue and stimulates the surrounding tissue to proliferate into an attachment tumor. In these instances, the epithelium grows over the surface of the bulla and adds to the strength of attachment (Kabata and Cousens, 1972). Although this reaction may have little if any adverse effects on the parasite, it can lead to fusion of adjacent gill filaments and occlusion of the branchial circulation (Kabata and Cousens, 1972). Proliferation of epithelial and fibrous tissue also benefits *Dissonus manteri* infections on coral trout (*Plectropomus leopardus*) gills by acting as a renewable food source (Bennett and Bennett, 1994).

Studies on species from the siphonostomatoid families commonly elicit little if any host inflammation (Table 1.1). Probably the best studied of these species is *Lepeophtheirus salmonis*. In *L. salmonis* infections of susceptible hosts, such as Atlantic salmon (*Salmo salar*), little to no inflammation is associated with the attachment and feeding of the copepodid and chalimus stages (Jones et al., 1990; Johnson and Albright, 1992a). However, epidermal hyperplasia, surrounding fibrosis and macrophage infiltration, commonly occurs around the frontal filaments from which the copepods have detached (Jones et al., 1990). When the parasite reached pre-adult/adult stages, Johnson and Albright (1992a) observed mild inflammation with neutrophil infiltration at the site of feeding and attachment in Atlantic and chinook salmon (*Oncorhynchus tshawytscha).* Jonsdottir et al. (1992) reported a limited tissue response from Atlantic salmon to the site of attachment and feeding. However, some inflammation was evident around the periphery of the parasite's cephalothorax, especially in the region

immediately anterior to the parasite. Finally, Nolan et al. (1999) observed mucous cell proliferation at sites of feeding and eventual immature mucous cell discharge. They suggested that the protective role of mucus is reduced through a reduction in its production or a change in its composition. A significantly thinner mucous layer has also been observed in *L. salmonis* infected Atlantic salmon (Horne and Sims, 1998).

In coho salmon (*Oncorhynchus kisutch*), which is a species resistant to *L. salmonis*, infections with all developmental stages result in well-developed host tissue responses. This is similar to responses seen during lernaeaid infections of immune hosts. Johnson and Albright (1992a) observed acute inflammation, primarily due to neutrophil infiltration, in coho salmon gills within the first days of *L. salmonis* infection. On coho salmon fins, epithelial hyperplasia and encapsulation resulted in the eventual loss of copepods. Injection of coho salmon with cortisol prior to *L. salmonis* infection reduced inflammatory responses and epithelial hyperplasia and increased parasite burdens on the host (Johnson and Albright, 1992b).

Other siphonostomatoid infections result in similar host responses. Massive mucous cell proliferation at parasite feeding sites is observed in *Lernanthropus kroyeri* infections of the sea bass (*Dicentrarchus labrax*) (Manera and Dezfuli, 2003). The caligid parasites *Caligus elongatus*, *Caligus epidemicus* and *Lepeophtheirus pectoralis* stimulate little tissue response directly at the attachment site. Despite this, epidermal hyperplasia occurs at the periphery of the feeding site in *L. pectoralis* infection of flounder (*Platichthys flesus*) (Boxshall,

1977) and redundant frontal filaments have been associated with infiltrating macrophages in *C. epidemicus* infection of sea bream (*Acanthopagrus australis*) (Roubal, 1994).

The Order Poecilostomatoida, which includes members of the Family Ergasilidae, elicit comparable host tissue responses. Striped bass (*Morone saxatilis*) infected with *Ergasilus labracis* exhibited epithelial hyperplasia near the mouth and body of the parasite, as well as, mucus proliferation and infiltration of macrophages, lymphocytes and eosinophils (Paperna and Zwerner, 1982). Ergasilid infections of the gills of other host species have shown both proliferation of gill epithelium in areas adjacent to the parasite's mouth-parts, as well as inflammation consisting of macrophage, neutrophil and eosinophil infiltration (Oldewage and van As 1987; Roubal, 1989). The latter study, however, was unable to show a greater abundance of leucocyte infiltration adjacent to the parasite. Finally, Paperna and Thurston (1968) reported massive hyperplastic tissue in the host gills actually trapping *Ergasilus labracis*.

In some instances, marked host tissue responses are thought to play an important role in the maintenance of attachment to the host (anchor worms, etc.). In others, it appears that infections stimulating inflammation in association with the mouth-parts or feeding site, result in rejection of the parasite. Many of these observations are disjointed and were made secondarily to the study of parasite life cycles. In some cases only macroscopic examination of the infection sites was made and very rarely were intensive histological examinations pursued.

The lernaeaid or lernaeaid-like (lernaeaocerids, pennelids and lernaeaopodids) studies did not investigate host responses to the actual feeding site but rather the attachment structure (anchor, bulla). This relationship is completely different from host responses to parasite feeding, as the attachment structure achieves its primary purpose. However, inflammation at the feeding site can interrupt normal feeding for the parasite. In caligid parasite models, host inflammation does not occur at the feeding site, but at its periphery, in highly susceptible hosts. Less susceptible hosts display intense inflammation at the feeding site and a subsequent reduction in parasite burdens. Inflammation can therefore be beneficial or detrimental to parasitic copepods depending on the focal point of the host reaction.

The severity of the inflammatory response is central in other arthropod parasite infections. Sheep blowfly (*Lucilia cuprina*) infections cause skin inflammation and neutrophil influx, mainly mediated by larval excretory/secretory products and tissue damage. In this instance, host resistance is correlated to faster and increased levels of host protein leakage into the infection site and to higher levels of acute phase proteins such as complement component 3 (C3) (O'Meara et al., 1992, 1995).

Infection by the brown dog tick (*Rhipicephalus sanguineus*) shows similarities to parasitic copepod infections. Dogs, do not appear to be irritated by the tick, with only minor skin thickening reported to result from infection. However, guinea pigs , which are not a natural host, display strong localized tissue responses such as intense hyperemia, swelling and fluid exudation (Szabo

and Bechara, 1999). As successive infestations occur so does the magnitude of the guinea pig's response, which elicits an increase in eosinophils and basophils. These two cell types are virtually absent from dog responses to *R. sanguineus* infection. Guinea pigs that are resistant to *Dermacentor variabilis* develop epidermal hyperplasia, oedema and a granulocytic infiltrate and those resistant to *D. andersoni* exhibit strong inflammatory responses consisting of basophil and eosinophil infiltration (Allen, 1973). The lack of a dog host response to *R. sanguineus* infection is unique, as it is not seen upon infection with other ectoparasites, such as fleas (Halliwell and Schemmer, 1987). Szabo and Bechara (1999) suggest that the lack of strong reactions from the host were due to the ability of *R. sanguineus* to manipulate the inflammatory response in the presence of a potentially harmful neutrophil response.

1.2.2 Is there evidence for host responses affecting the biology of parasitic copepods?

The host-parasite relationship is bidirectional, such that parasites will have effects on the host and hosts will respond causing effects on the parasite. The extended attachment and feeding periods of ectoparasitic arthropods provide ample time for their hosts to develop innate and/or acquired immune responses. Wikel (1982; 1996a, b) has previously described effects of hosts on their arthropod ectoparasites including the inhibition of moulting, mortality, reduced engorgement and reduced ova production and viability of the parasite. Mammalian hosts develop resistance, as measured by these parameters, after repeated infestations with ticks. Immunization has proven effective against the

mosquito (*Aedes aegypti*) and the stable fly (*Stomoxys calictrans*) giving further evidence towards host's developing acquired immunity against arthropod ectoparasites (Hatfield, 1988; Webster et al., 1992).

Evidence exists for hosts affecting the biology of parasitic copepods and even developing resistance. Aside from the host responses already mentioned, Shields and Tidd (1974) observed that following goldfish (*Carassius auratus*) maximum tissue response to *Lernaea cyprinacea* copepodids, the copepodids began to feed shallower and on a wider surface area. This suggests the host response was having a negative effect on the parasite. Shields and Goode (1978) later found that 50% of goldfish infected with *L. cyprinacea* had cleared infections by 11 days post infection. These authors mention that later attempts at re-infection of hosts were erratic and attributed this increase in resistance to an acquired immune response. Infection of *L. cyprinacea* on the kissing gourami (*Helostoma temmincki*) also showed that previously infected fish had parasites with decreased numbers of egg strings (Woo and Shariff, 1990). Furthermore, these eggs exhibited a lower hatching success and produced copepodids with lower infectivity than those hatched from eggs produced by females living on previously naïve hosts.

Differential site selection has been suggested as a measure of resistance to some parasitic copepods. Shariff (1981) suggested the occurrence of *L. piscinae* in the eye of the big head carp, was only observed after an immune response had occurred on the body of the host. As the cornea and lens are avascular tissue (Niederkorn, 1990; 1994) they are thought to be a preferred site of

infection, also observed in many other parasitic copepod infections (Bennett, 1994; Benz et al., 2002a; Kabata, 1969; 1970; Neilson et al., 1987). Similarly, Johnson and Albright (1992a) attributed slower development of *L. salmonis* on gills due to an increased intimacy with the immune response.

Different host species have varying effects on *L. salmonis* as well. Slower development of *L. salmonis* and lower egg numbers are observed for copepods living on chinook salmon as compared to Atlantic salmon (Johnson 1993; Johnson and Albright, 1992a). The possibility of an acquired immune response of hosts to *L. salmonis* has also been suggested. Both naturally infected and *L. salmonis*-homogenate-immunized Atlantic salmon have demonstrated humoral antibody responses to several *L. salmonis* antigens (Grayson et al., 1991; Reilly and Mulcahy, 1993). Following immunization with extracts from *L. salmonis*, Atlantic salmon have fewer ovigerous females and subsequently lower numbers of eggs when compared to non-immunized fish (Grayson et al., 1995). This study has lead to some trial work on vaccine targets against *L. salmonis*. Comparable findings are also reported for members of the Ergasilidae. Paperna and Zwerner (1982) found that striped bass were able to reduce egg sac production of *E. labracis*.

Although a body of work suggests impacts of hosts on parasitic copepod biology, further studies conducted under well-controlled experimental conditions are necessary to provide quantitative data on such effects at both the individual and population level. Re-infection studies on naïve fish are necessary to

determine the extent to which acquired resistance plays a role in these host effects.

1.3 The effects of copepods on their hosts

1.3.1 What effects of copepod infection on the host have been documented?

When dealing with production animals the possibility of major economic losses due to parasitic infection are always of concern. The cattle industry, for decades, has battled tick infestations, with an estimated 80% of the world's cattle being at risk from ticks and tick borne diseases (Pegram et al., 1993). Tick-infested cattle exhibit reduced weight gain, milk production, and higher mortality rates for calves in comparison to non-infested individuals (Norval, 1990; Pegram et al., 1993; Taylor and Plumb, 1981). Ectoparasitic arthropods can have effects on future generations of their host species through behavioural alterations and reduced fecundity (Fitze et al., 2004).

Effects of parasitic copepods on their hosts are also documented. Similar effects range from decreases in host growth, weight, fat content and reproduction to increased susceptibility to secondary infection and mortality. Unfortunately, however, some deficiencies exist in these studies with respect to their design. Any complete discussion on copepod parasite effects on the host must first state these problems so that the information can be put into context.

Examples of decreased host weight and fat content in the wild due to copepod infection are numerous. Abrosov and Bauer (1959) observed decreased weights of 2-5 year old whitefish (*Coregonus peled*) infected with

Ergasilus sieboldi in Lake Sebezhskoye. Mann (1952; 1970) observed decreased weights and fat content in European whiting (*Merlangius merlangus*) infected with a *L. branchialis*. However, these studies lack proper controls. Making firm conclusions fro this work is problematic due to the lack of knowledge with regards to the length of exposure of the host to the parasite. And for those studies that show decreased weight of infected fish there are those that show the opposite. Manera and Dezfuli (2003) observed higher body weights in sea bass (*D. labrax*) infected with *L. kroyeri* when compared to uninfected sea bass.

As mentioned by Kabata (1970) in many cases we do not know the full life span of the parasite, its rate of development, growth or length of infection of each particular host. We often cannot be sure of those hosts, which recovered recently from copepod infection (eg. macroscopic examination) but still retain physiological after effects, thereby affecting results for 'control' animals. Especially in fish, which exhibit compensatory growth, the sampling time with regards to the cycle of infection may decide whether positive or negative growth are associated with the infection. A further issue is that investigators in these studies did not always examine fish for other parasites such as endoparasitic helminthes and as such the effects cannot always be fully attributable to the ectoparasitic copepod (Kabata, 1970). Although drawing hard conclusions from naturally infected fish is problematic, they guide experimental manipulations.

Khan and Lee (1989) for example, infected Atlantic cod (*Gadus morhua*) with *L. branchialis* and showed a significant reduction in weight as compared to controls over a one-year period. This effect was increased when more than one

parasite was present. Less efficient food conversion by infected individuals appeared to reduce growth (Khan and Lee 1989). Host species' susceptibility to infection, as well as the level of infection and size of individual hosts are all important in determining the degree of effects the parasite has on the host in any particular infection (Johnson and Fast, 2004). Dawson et al. (1999) observed depressed food consumption in 130 g Atlantic salmon infected with 51.5 *L. salmonis* (<0.4 lice/g host body weight) but no effect on specific growth rate or condition factor. Smaller Atlantic salmon (40 g), however, exhibited decreased growth and condition factor at the late pre-adult stage of *L. salmonis* infection (> 1.0 lice lice/g host body weight) (Grimnes and Jakobsen, 1996). Mucus compositional changes were also observed in host species infected with *L. salmonis*, such as increased lysozyme and alkaline phosphatase enzymes (Fast et al., 2002).

Higher infection levels lead to significantly higher levels of cortisol and other stress parameters (Bjorn and Finstad, 1997; Bowers et al., 2000; Grimnes and Jakobsen, 1996; Ross et al., 2000) as well as osmoregulatory failure (Grimnes and Jakobsen, 1996) and even immunosuppression (Mustafa et al., 2000a). Rainbow trout infected with *L. salmonis* show decreased macrophage function and increased susceptibility to infection with the microsporidian *Loma salmonae* (Mustafa et al., 2000b). Bjorn and Finstad (1997) observed a decreased percentage of lymphocytes in sea trout (*S. trutta*) and Johnson and Albright (1992a) demonstrated secondary infection with rod shaped filamentous bacteria in chinook salmon and Atlantic salmon, each following *L. salmonis* infection.

Faisal et al. (1990) observed a suppression of mitogenic activity in splenic leucocytes of Pacific arrowtooth flounder (*Atheresthes stomias*) infected with *Phrixocephalus cincinnatus*. Consistently elevated cortisol levels, indicative of chronic stress, may be the cause of many of the effects already discussed such as increased disease susceptibility (Johnson and Albright 1992b; Pickering and Duston, 1983; Woo et al., 1987) and decreased growth rates (Barton et al., 1987). One problem is to isolate the direct effects of the parasite on the host from the indirect effects stemming from a chronic stress response of the host. Extensive areas of skin erosion and haemorrhaging on the head and back may occur in heavy parasitic copepod infections (Bergh et al., 2001; Johnson et al., 1996; Pike and Wadsworth, 1999). Lesion development in some cases may be in part due to secondary bacterial infections and resulting tissue necrosis. While mortality is an extreme and often rare effect of parasitic copepod infection, it has been observed under both experimental and natural conditions. Grimnes and Jakobsen (1996) and Ross et al. (2000) observed mass mortalities in Atlantic salmon experimentally infected with *L. salmonis* following parasite moult to pre-adult stage. Johnson et al. (1996) also report high mortalities due to *L. salmonis* in naturally infected sockeye salmon (*Oncorhynchus nerka*). Ueki and Sugiyama (1979) observed mortalities in cultured-black sea bream (*Mylio macrocephalus*) infected with *Alella macrotrachelus*.

1.3.2 *Is there evidence for parasitic immunomodulation of hosts as seen in other ectoparasitic arthropods?*

The effect of copepod infection on their hosts, as just discussed, can be a result of several direct and indirect actions of the parasite. Whereas, active feeding may cause anemia, prevent wound closure and result in osmoregulatory problems, attachment/feeding can also indirectly cause secondary and tertiary effects due to inciting a chronic stress situation in the host. Another consequence of parasite infection is the secretion of compounds into the infection site to assist in feeding or evasion of the host immune response. Haematophagous arthropods, for instance, use several pharmacological strategies that enable them to feed at length on their host, including the production of inhibitors of blood coagulation and platelet aggregations, as well as vasodilatory and immunosuppressive substances (Wikel et al., 1994). Several reviews have been published over the last two decades on terrestrial arthropod parasites and their ability to affect changes in host responses at the feeding site (Balashov, 1984; Barriga, 1999; Gillespie et al., 2000; Ribeiro, 1995; Wikel, 1982; Wikel et al., 1994).

I have discussed the ability of parasitic copepod infection to result in immunosuppression, but previously this was either in the midst of a strong stress response (Bjorn and Finstad, 1997; Bowers et al., 2000; Mustafa et al., 2000a) or in situations in which stress parameters were not measured (Mustafa et al., 2000b).

The parasitic copepod *P. cincinnatus*, which often infects the eye of the Pacific arrowtooth flounder, induces a significant increase in mitogenic activity of blood leucocytes, while at the same time suppressing mitogenic activity in splenic and anterior kidney leucocytes, during unilateral infections (Faisal et al. 1990). A complete suppression of mitogenic responses, however, was only observed in bilateral infections, which are rarely observed in the wild due to their fatal consequences (Kabata and Forrester, 1974). Since plasma/serum cortisol levels were not determined in this experiment, one cannot distinguish any immunomodulatory effect the parasites may have had on leucocytes from the effects of chronically elevated cortisol. Long-term infection often results in elevated cortisol levels in host fish (Bjorn and Finstad, 1997; Bowers et al., 2000). The fish in the Faisal et al. (1990) study were infected with adult *P. cincinnatus*, providing evidence for long-term infection. In an experiment using wild fish acclimated for only one week, stress levels are of particular concern, especially when multiple stressors such as handling and parasitic infection can have an additive effect on cortisol levels and cellular apoptosis in some species (Fast et al., 2002, Nolan et al., 2000; Poole et al., 2000; van der Salm et al., 2000).

Better evidence, however, has been uncovered for immunomodulation or at least pharmacological interaction between parasitic copepods and their hosts. Big head carp infected with *L. polymorpha* exhibited nuclear degenerating red blood cells at the infection site, along the line of entry, at 4 h post penetration (Shariff and Roberts, 1989). A toxic component or digestive enzyme might have

been the cause, as karyorhexis is unusual at such an early stage of hemorrhage. Massive vascularization was also observed around the periphery of the lesion 64h after penetration. Similarly, Benz et al. (2002b) observed localized neovascularization, hemorrhage and epidermal erythrocytosis around the infection site in shortfin makos (*Isurus oxyrinchus*) infected with *Anthosoma crassum*. The authors suggest that *A. crassum* secretes angiogenic factors, anticoagulants and/or vasoactive amines into the site of infection. The presence of coagulative necrosis in all lesions was taken by the authors as evidence for chemical damage (Benz et al., 2002b).

Anti-coagulant activity is a common feature of arthropod parasite saliva. The majority of anticoagulants isolated from arthropods are proteins of 5-65 kDa (Kazimarova et al., 2002). It has been suggested that species' with the strongest anticoagulant activities were those with the widest host spectrum (Kazimarova et al., 2002). Apyrase is an enzyme that hydrolyzes ATP and ADP, thereby inhibiting platelet aggregation. This enzyme is commonly found in the saliva of many hematophagous arthropods (Ribeiro et al., 1986; 1989; 1990). Cupp et al. (1998) showed that horn fly (*Haematobia irritans*) saliva has neither apyrase anti-platelet aggregation nor vasodilative activity but exhibits similar anti-coagulant activity. Many ectoparasitic arthropods exhibit vasodilatory substances in their saliva (Ribeiro, 1995).

Neutrophils present at the periphery of *L. polymorpha* lesions in big head carp during the early acute inflammatory response exhibited no indication of phagocytosis (Shariff and Roberts, 1989). Fast et al. (2002) report decreased

phagocytic capacity and respiratory burst (nitric oxide activity) of head kidney macrophages during the later stages (14-21 dpi) of an *L. salmonis* infection in both Atlantic salmon and rainbow trout, in the absence of a cortisol response.

Reduced host macrophage function is another common theme in other arthropod ectoparasitic infections. Sandfly (*Lutzomyia longipalpis*) saliva has the ability to inhibit macrophage function *in vitro* (Theodus and Titus, 1993). More recently a 7-kDa protein termed maxadilan, was isolated from *L. longipalpis* saliva and shown to inhibit macrophage production of nitric oxide (Gillespie et al., 2000). Ferreira and Silva (1998) demonstrated that *R. sanguineus* saliva inhibited *Trypanosoma cruzi* killing in interferon (IFN)-γ activated macrophages of mice and this was correlated with reduced nitric oxide production. Similarly, salivary gland extract from *Ixodes ricinus* inhibited *Borrelia afzelii* killing by mouse peritoneal macrophages (Kuthejlova et al., 2001) and this was related to a reduction in superoxide anions. Finally, Ribeiro et al. (1990) and Urioste et al. (1994) determined that *Ixodes scapularis* saliva inhibited functions of peritoneal neutrophils *in vitro* and suppressed nitric oxide production from peritoneal macrophages.

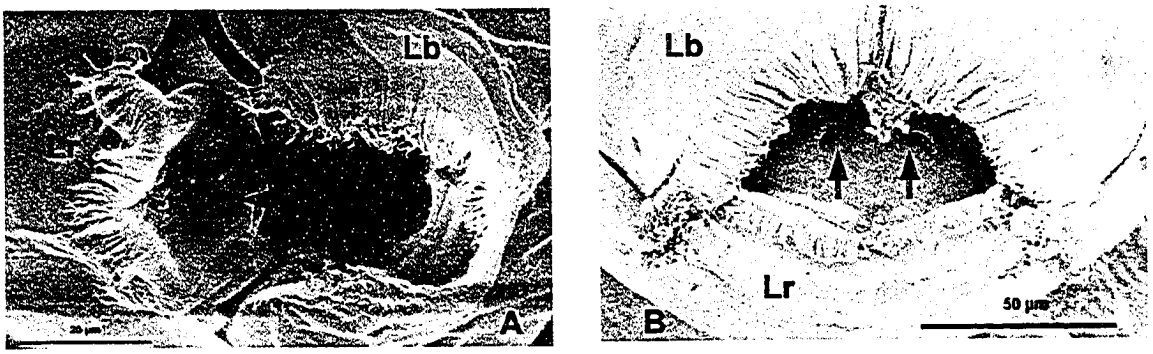
Decreased macrophage function in Atlantic salmon and rainbow trout corresponded to the presence of *L. salmonis* derived low-molecular weight (17-22 kDa) trypsin-like proteases, in infected fish mucus (Fast et al., 2002; Firth et al., 2000; Ross et al. 2000). These tryspin-like proteases are secreted by *L. salmonis* with greater regularity in the presence of susceptible Atlantic salmon and rainbow trout mucus than in the presence of non-host, winter flounder

(*Pseudopleuronectes americanus*), or more resistant host (coho salmon) species' mucus (Fast et al., 2003). Trypsin, which is found in the guts and secretions of other ectoparasitic arthropods (Kerlin and Hughes, 1992; Lecroisey et al., 1983), is capable of decreasing phagocytic capacity in human monocytes (Huber et al., 1968).

Trypsin is not the only potential immunomodulatory compound in the secretions of *L. salmonis*. Bell et al. (2000) detected secretory glands that contained peroxidases and suggested their use in the production of prostanoids. In particular, the ventral median gland of the copepodid stage stained positive for peroxidases (Bell et al., 2000). Ducts leading from this gland pass through the labrum and may exit on its anterior surface. Upon further investigation, using scanning electron microscopy, these pores were not detected to exit on the labrum anterior surface but rather they exited on the opposite side of the labrum, inside the mouth tube (Fig. 1.4). These pores are uniquely situated to allow their secretions to interact with food in the mouth tube and/or host cells at the site of feeding.

Having discussed the host response to parasitic copepod infection we begin to develop an idea of the complexity of interactions between the host and parasite. Host rejection is often associated with intense inflammation at the site of feeding, however, those copepod species that maintain a longer interaction with the host often show little inflammation or inflammation only around the periphery of the attachment site. Some parasitic copepods produce immunomodulatory compounds in their secretions suggesting they are the

Figure 1.4: *Lepeophtheirus salmonis* secretory pores (◄) associated with the oral cone in the copepodid (A) and pre-adult (B) life stages. (Lr) denotes labrum, (Lb) denotes labium.



reason for a lack of host response at the infection site. Eliminating host inflammatory responses at the site of feeding may reduce the negative effects the host's immune response has on the ectoparasite. However, to modulate immune responses throughout the host's entire body would be a major energy investment by the parasite and possibly counterproductive in the long run if it led to increased host mortality. For these reasons it is not surprising to see the effects of these immunomodulatory compounds diminishing distally from the focal point of feeding.

The following research was designed to further elucidate the relationship between *L. salmonis* and its host Atlantic salmon. This was carried out by investigating the interactive components of parasitic secretions and parasitic infection with aspects of the salmonid immune system.

In Chapter 2.0, PGE_2, a potent anti-inflammatory compound was isolated from *L. salmonis* secretions and quantitated. This compound was then tested against a salmonid head kidney cell line, in Chapter 3.0, to observe biological significance at differing concentrations. This work provided evidence that a particular immunomodulatory compound, at physiologically relevant levels, has significant effects on expression of immune-related genes in salmonid fish. In Chapter 4.0, further immunomodulatory capabilities of *L. salmonis* secretions were investigated. This work showed that genes linked to secretory proteins were up-regulated following infection and that immunomodulatory capabilities existed within these secretions beyond that of the already discovered compounds. In Chapter 5.0, the role low-level *L. salmonis* infection plays on host

immune responses was revealed. The ability of salmon head kidney macrophages to respond to LPS challenge under these infection conditions was observed. In Chapter 6.0, high-level infection as well as re-infection with *L. salmonis* and its corresponding effects on salmon physiology and immunology were investigated. The resulting changes in host physiology at the higher infection levels and its effects on immune gene expression were then compared with the results from Chapter 5.0.

Chapter 2.0 *Lepeophtheirus salmonis*: Characterization of prostaglandin E_2 in secretory products of the salmon louse by RP-HPLC and Mass Spectrometry.

2.1 Abstract

Lepeophtheirus salmonis is an ectoparasitic copepod that causes serious disease outbreaks in wild and farmed salmonids. As the relationship between *L. salmonis* and its hosts is not well understood, the current investigation was undertaken to determine whether any immunomodulatory compounds could be identified from secretions of *L. salmonis*. By incubating live *L. salmonis* adults with the neurotransmitter dopamine in seawater, we obtained secretions from the parasite. These were analyzed by RP-HPLC column, as well as LC-MS. *Lepeoptheirus salmonis* secretions contained a compound with the same retention time and mass of PGE_2. The identity of this compound as PGE_2 was confirmed by MS – in source dissociation. The concentrations of PGE_2 in *L. salmonis* secretions ranged from 0.2 – 12.3 ng/individual and varied with incubation temperature and time kept off the host. Prostaglandin E_2 is a potent vasodilator and thought to aid in parasite evasion from host immune responses. This is the first reported evidence of prostaglandin production in parasitic copepod secretions and its implications for the host-parasite relationship are discussed.

2.2 Introduction

Numerous compounds isolated from the secretions of terrestrial arthropod parasites prolong parasitic feeding (anticoagulants), increase blood flow to the site of attachment (vasodilation) and/or assist in evasion of host immune responses (immunomodulators) (Balashov, 1984; Wikel et al., 1994; Ribeiro, 1995; Bowman et al., 1996; Mans et al., 2002). Of these compounds a group of well-studied cyclic fatty acid derivatives with vasodilatory and immunomodulatory capabilities, known as prostanoids, have been discovered in the saliva of many Ixodid tick species such as *Boophilus microplus*, *Amblyomma americanum* and *Ixodes dammini* (Ribeiro et al., 1985; Inokuma et al., 1994; Bowman et al., 1995). Prostaglandin E_2 (PGE_2) is the most commonly found prostanoid in parasitic secretions and is not just limited to ectoparasites but also found in endoparasitic infections with the protozoan, *Entamoeba histolytica*, (Belley and Chadee, 1995) and the fluke, *Fasciola hepatica* (Ali et al., 1999). Prostaglandin E_2 has myriad effects on host cells and tissues including, but not limited to, vasodilation (Williams and Higgs, 1988), decreased proliferation of lymphocytes targeted to the source (Papadogiannakis et al., 1984), decreased adhesion of lymphocytes to endothelial cells (To and Schrieber, 1990), and favoring T-helper cell type 2 (Th_2) responses over Th_1 lymphocyte responses through the down regulation of interleukin (IL)–2 and interferon (IFN)- γ (Betz and Fox, 1991).

With respect to arthropod parasites of aquatic animals, very little is known about the nature of their host-parasite interactions, however, it is reasonable to expect similar mechanisms. Sea lice is a term used primarily for marine parasitic

copepods that belong to the well studied genera *Lepeophtheirus* and *Caligus*. Of all sea lice species, the salmon louse, *Lepeophtheirus salmonis* has received the most extensive study due to its importance as a disease-causing agent of wild and farmed salmonids (Pike and Wadsworth, 1999; Johnson et al., 2004). This parasite is responsible for indirect and direct losses in aquaculture in excess of US$ 100 million annually (Johnson et al., 2004).

Lepeophtheirus salmonis feeds on host mucus, skin and blood of salmonids belonging to the genera, *Onchorynchus*, *Salmo* and *Salvelinus* (Brandal, 1976; Berland and Margolis, 1983). The attachment and feeding activities of sea lice result in lesions that vary in their nature and severity depending upon: the species of sea louse, their abundance, the developmental stages present, host species and a variety of environmental parameters such as water temperature. On most host species, the attachment and feeding activities result in little if any host tissue response (Johnson and Albright, 1992a,b; Kabata and Cousens, 1977). The lack of host tissue response to *L. salmonis* and other copepod parasites has lead to the suggestion that sea lice produce compounds that modulate host immune responses. *Lepeophtheirus salmonis* secretes compounds such as proteases onto the surface of hosts that may aid in feeding and/or avoidance of host immune responses (Ross et al., 2000; Firth et al., 2000; Fast et al., 2002; 2003). The goal of this study was to determine if secretions of *L. salmonis* contain prostanoids. To achieve this goal we used a modification of the techniques used to isolate secretory products from terrestrial arthropod parasites and subjected these secretions to RP-HPLC and LC-MS.

2.3 Materials and Methods

2.3.1 Lepeophtheirus salmonis collection and incubation

Adult male and female *L. salmonis* were collected from farmed Atlantic salmon held at various seawater net cage sites in Atlantic Canada and Maine at various times throughout the year (Table 2.1). Following collection, animals were washed with sterile seawater and maintained off hosts in sterile seawater for differing periods of time prior to incubation with dopamine. The majority of incubations were conducted after 24 h off the host due to the inability of conducting temperature-controlled experiments at the farm cage sites. At one sampling location, however, Atlantic salmon (Grand Manan, N.B.) infected with *L. salmonis* were maintained in a land-based system. This allowed for incubations to be carried out at 10°C immediately following removal from the host and at 3, 12 and 24 h following removal.

Secretory/excretory product (SEP) collection (method 1): Adult male and female *L. salmonis* were incubated at 10°C and 21°C in sterile seawater (SSW) with and without 0.1 –1 mM dopamine (DA) for 30 min - 1h. Anywhere from 17-140 *L. salmonis* were incubated (per beaker) at 2 lice/ml. Lice were pooled to obtain sufficient PGs for analysis. *Lepeophtheirus salmonis* were then removed, the solutions centrifuged (3500 • *g*) for 5 min and the samples processed fresh, when possible, or stored overnight at –80°C.

Table 2.1: Average Prostaglandin E_2 production (ng/louse) by *Lepeophtheirus salmonis* following incubation of several lice with 0.1 mM dopamine in sterile seawater for 30 minutes following 24 h off a host. * indicates incubation at room temperature (21^0C). ** indicates incubation at 10^0C.

Sample Site	Sampling Date	Number of lice	PGE_2/louse
Grand Manan, NB *	May 1, 2002	35	0.21
Aspotogan, NS *	July 18, 2002	140	0.80
Nantucket, MA **	Nov. 9, 2001	33	1.8
Deer Island, NB **	June 27, 2001	17	6.4
Greens Point, NB *	July 28, 2002	55	3.9

Secretory product (SP) collection (method 2): Adult male and female *L. salmonis* were placed on parafilm wax and oriented so that the ventral surface of their cephalothorax covered a 20 µl droplet of 0.1 mM DA in SSW. *Lepeophtheirus salmonis* were incubated for 25 min inside a humidified chamber (10°C). After 25 min each droplet was pipetted off, as well as any remaining solution encountered within the convex of the cephalothorax. Solutions were then centrifuged (3500 • *g*) for 5 min, and processed fresh, when possible, or stored at –80°C overnight. The second method was used to minimize the collection volume as well as to eliminate the possible contribution of excretory products.

2.3.2 *Zymography test of stimulation*

To determine whether SEPs were released in the presence of dopamine we examined a small number of samples obtained using incubation method 1 for protease activity by zymography, following the methods given in Firth et al. (2000) and Fast et al. (2003). Briefly, samples were diluted 1:1 with 4% sodium dodecyl sulfate, 20% glycerol, 125 mM Tris-HCl at pH 6.8 and equal amounts of protein were loaded onto 12% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed (4^0C) three times with Triton X-100, 50 mM Tris-HCl at pH 7.5, and incubated (30^0C) for 19 h in the same buffer containing 50 mM $MgCl_2$ and 6.25 $CaCl_2$. After the incubation gels were stained with 0.1% amido black in MeOH:H_2O:AcOH (45:45:10).

2.3.3 *Sample preparation*

The prostaglandin extraction protocol used was similar to that followed by Powell (1987). Briefly, samples were thawed and diluted 10-20x with 15% ethanol and acidified to pH 3.5 with formic acid. The solution was applied to octadecylsilane (ODS) solid phase extraction columns (C-18 SPE, Waters), which were previously activated with 20 ml of 15% ethanol followed by 20 ml of water (Powell 1987; Ribeiro et al., 1992). The ODS-SPE columns were washed with 20 ml of 15% ethanol and then 20 ml of water. Hexane (Fisher Scientific) (20 ml) was used to elute the neutral lipids. The prostaglandin-containing fraction was eluted with 10 ml of methyl formate (SIGMA) and evaporated to dryness under nitrogen. This residue was dissolved in acetonitrile (Fisher Scientific) and analyzed on RP-HPLC and LC-MS. Samples of SSW and DA were prepared as above as controls.

2.3.4 *RP-HPLC conditions*

An Agilent 1100 HPLC equipped with a diode array detector (monitoring at 210 nm) and a Beckman Ultrasphere column, (ODS 5 µm (2.0 x 250 mm)) was used to detect prostaglandins. The column was kept at room temperature and eluted isocratically with 32.8: 67.2 acetonitrile:water (0.1% acetic acid) for 30 min (Terragno et al., 1981) at a flow rate of 0.2 ml min^{-1}. Solutions of a PGE_2 (1µg/µl) standard, SP/SEPs and control (SSW + DA) were dissolved in acetonitrile and 20 µl were injected individually onto the column.

2.3.5 *Mass Spectrometry conditions*

An API III$^+$ triple quadropole mass spectrometer with an atmospheric pressure ion source (SCIEX) was used to sample negative ions produced from an electrospray interface. It was equipped with a Hewlett Packard 1090 HPLC system that was operated at the same conditions as outlined in the above section. The ionspray interface was maintained at –3.5 kV, and an orifice voltage of -55 V was used. The total ion current for the $[M - H]^-$ ion at 351 m/z was monitored. As the instrument was incapable of doing a true negative ion tandem MS experiment, the orifice voltage was increased to –80 V to achieve optimal fragmentation of prostaglandin standards, *L. salmonis* SEPs and SPs, by in-source dissociation. Identification of PGs in *L. salmonis* SEPs/SPs was made by comparing RP-HPLC peak retention times and their mass spectra with those of prostaglandin standards (SIGMA).

In order to quantify the concentration of prostanoids present in the SEPs/SPs of *L. salmonis* a dilution series of PGE_2 (0.5, 0.8, 1, 2.5, 5, 10, 25, 50, 100 ng μl^{-1}) was used to prepare a standard curve. Prostaglandin $F_{2\alpha}$ $[M - H]^-$ ion at 353 m/z, eluted at 12.56 min and was used as an internal standard. The internal standard (5 ng μL^{-1}) was added to each dilution, as well as each *L. salmonis* sample, to allow for any differences in MS calibration. Prostaglandin concentrations were then used to determine total PGs present in sample products and to calculate the average amount released per individual louse.

2.4 Results

Incubation of *L. salmonis* in SSW containing DA (0.1 mM-1.0 mM) resulted in the production of SEPs as evidenced by the presence of proteases in the incubation media (Fig. 2.1). There was no evidence of any protease activity in SEPs obtained in the absence of DA. Having determined that DA stimulates the production of SEPs, the material was examined for eicosanoids.

Dopamine from the incubation medium was removed from each sample during the water and ethanol washes of the ODS SPE columns so that it was not present in the HPLC chromatograms of the SEP fraction. Analysis of *L. salmonis* SEPs, obtained using the first incubation method, on RP-HPLC (210 nm) revealed one major peak at 16 min that corresponded to the PGE_2 standard (Figs. 2.2a, b). Using RP-HPLC/MS this peak was determined to have a parent ion at m/z 351, which corresponded to PGE_2 and/or PGD_2 (Fig. 2.3). Injection of a PGD_2 standard however revealed that PGD_2 eluted at 19.7 min and not 16.1 min (Fig. 2.2b). The major ions, at m/z 335, 315, 271, 233, 189, 175, observed in the mass spectrum of the SEP peak at 16.1 min (Fig. 2.3a) were identical to those observed for the PGE_2 (Fig. 2.3b) and PGD_2 (data not shown) standards. There were no peaks observed in the SW + DA and SW + *L. salmonis* controls. The controls also lacked major ions between 300-400 m/z (data not shown).

Quantification of PGE_2 in *L. salmonis* SEPs was carried out using LC-MS analysis to assess possible physiological significance (Table 2.1). The levels of

Figure 2.1: Zymogram of protease activity in sterile seawater incubations with live *Lepeophtheirus salmonis* with (+) and without (-) dopamine (1 mM). Numbers alongside the gel indicate molecular masses (kDa). Each lane contains 1 µg of protein from a single incubation.

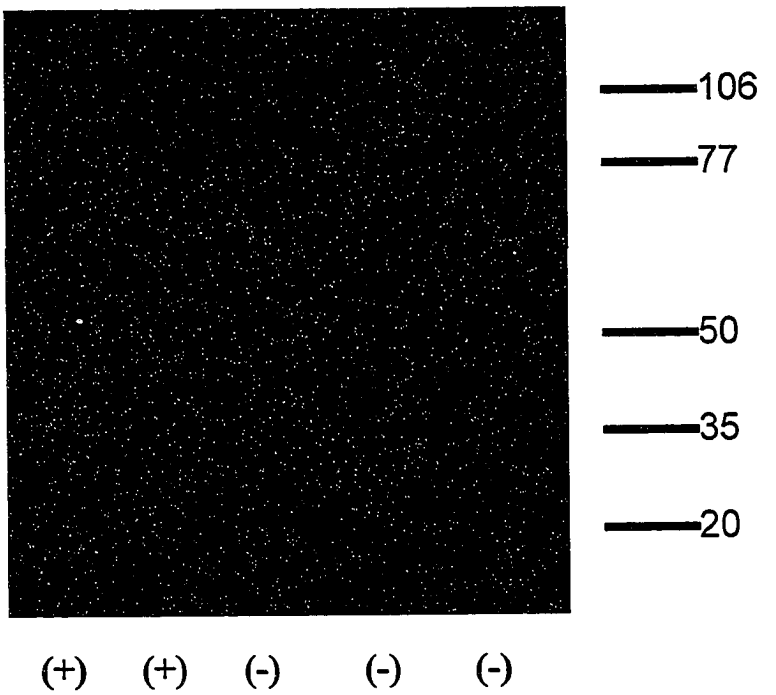


Figure 2.2: Reverse-phased HPLC chromatogram (210 ηm) of PGE_2, PGD_2 (a) and *Lepeophtheirus salmonis* secretory/excretory products (SEPs) (b). The column was kept at room temperature and eluted isocratically with 32.8: 67.2 acetonitrile:water (0.1% acetic acid) for 30 min at a flow rate of 0.2 ml min^{-1}. Injections of 20 µl were made of PGE_2 (1 µg/µl)/PGD_2 (100 ηg/µl) standard and SEPs dissolved in acetonitrile.

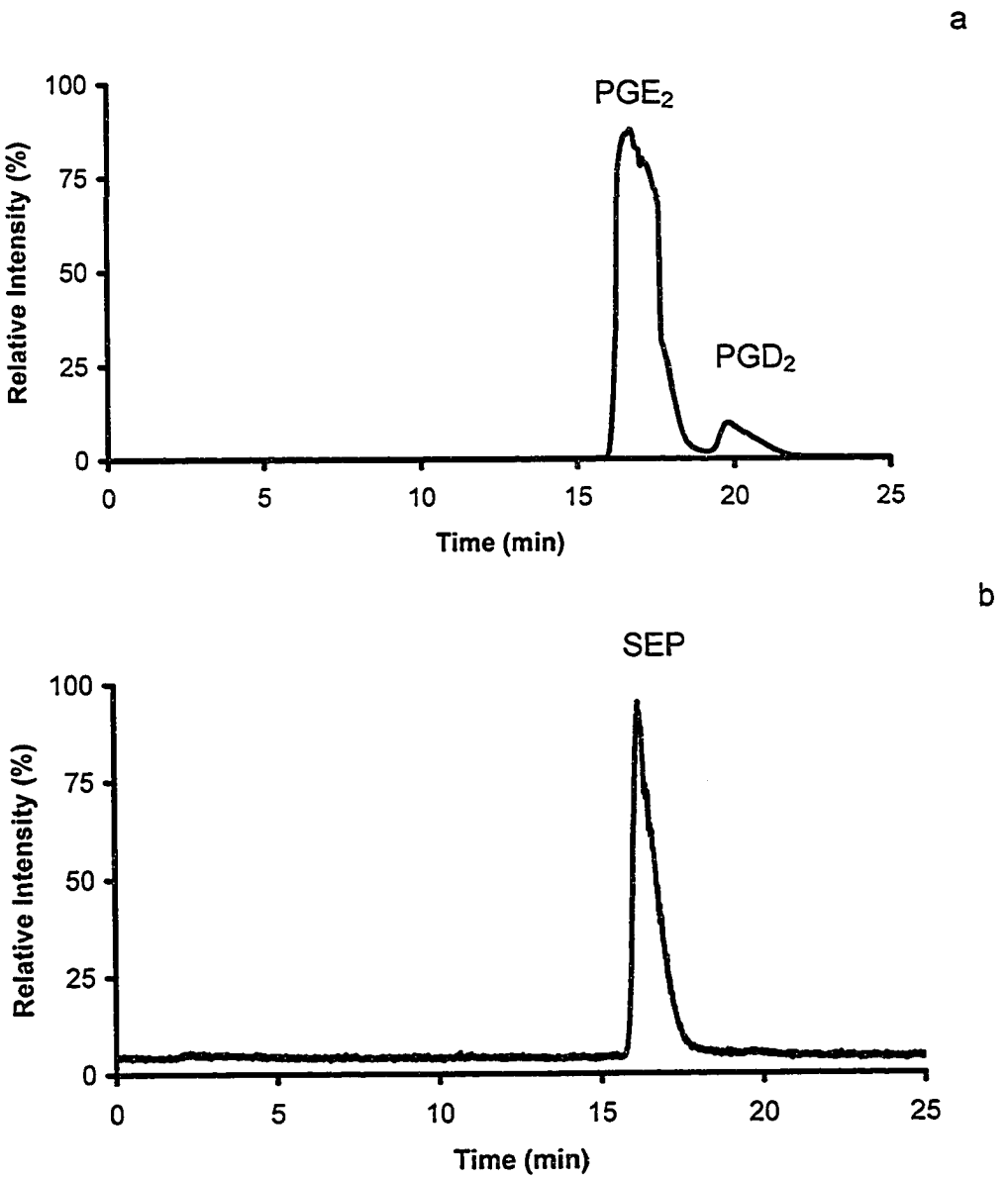
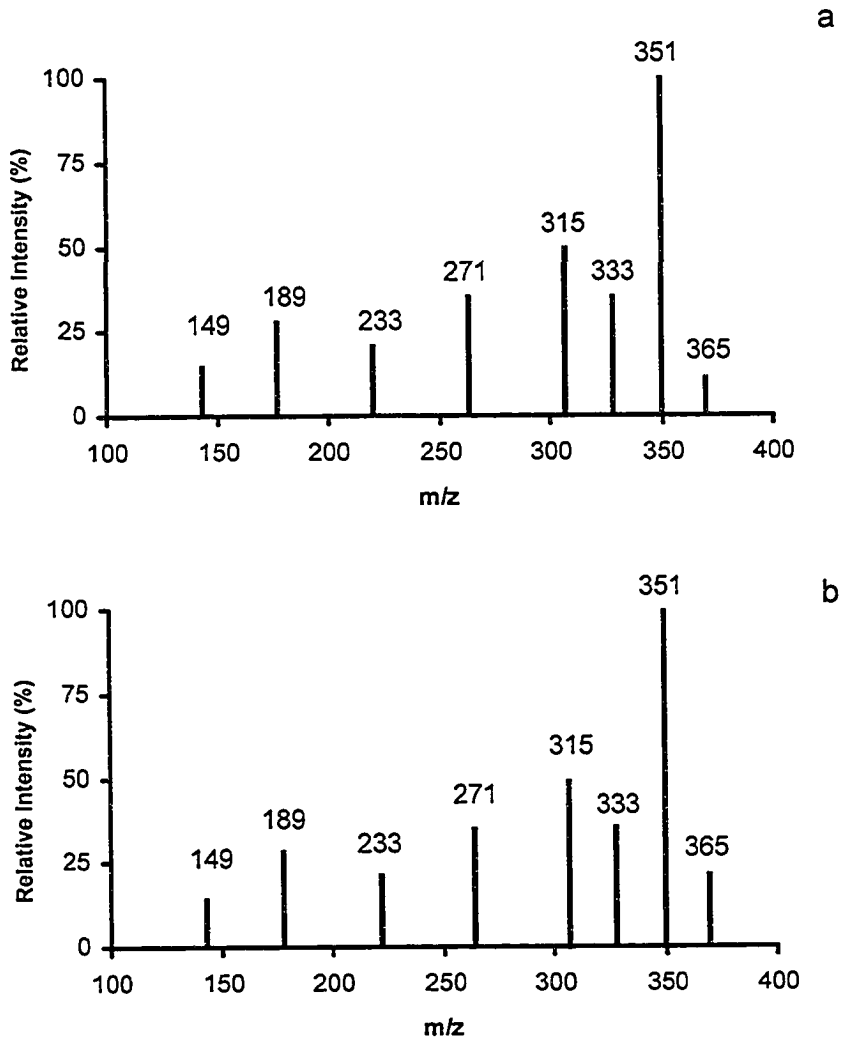


Figure 2.3: Comparison of negative ion MS spectra of *Lepeophtheirus salmonis* secretory/excretory product peak at 16 min (a) and PGE_2 (b).

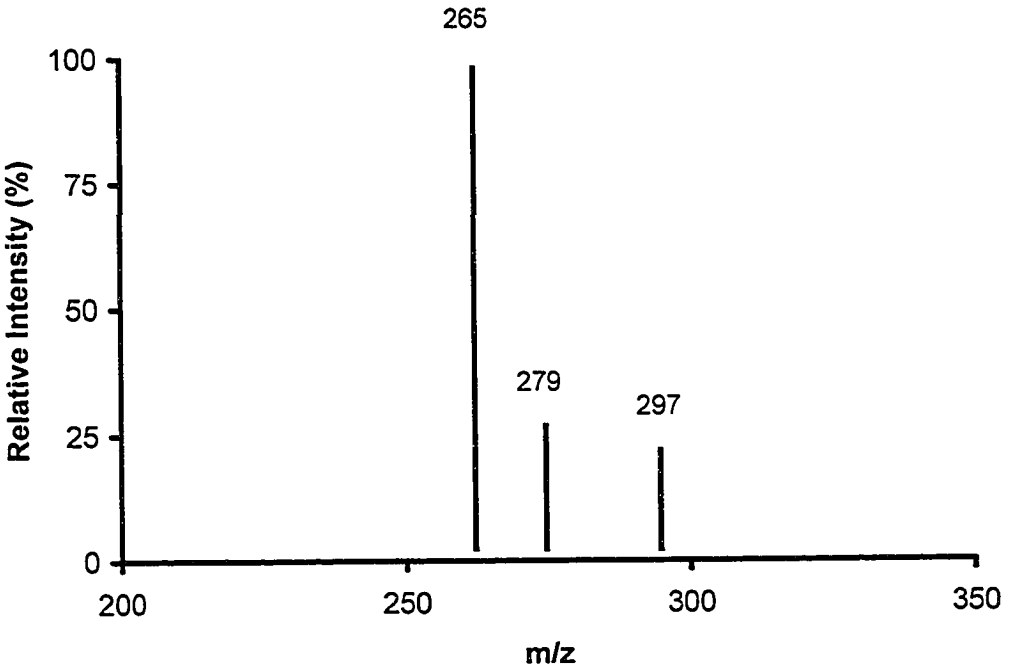


PGE_2 in SEPs obtained from pools of *L. salmonis* that were maintained for 24 h off the host were determined after a 30-minute incubation at either 10 or 21°C with dopamine (Table 2.1). Values obtained for the three pools incubated at 10°C were 1.8, 3.9 and 6.4 ng PGE_2/louse (Table 2.1). Values obtained for the two pools incubated at 21°C were 0.8 and 0.21 ng PGE_2/louse (Table 2.1). *Lepeophtheirus salmonis* that were incubated with dopamine immediately after collection from the host had a PGE_2 level of 14.5 ng/louse in their SEPs. At 3,12 and 24 h off the host PGE_2 levels in the SEPs dropped to 0.2, 0.5 and 0 ng/louse, respectively.

Analysis of the SEPs, by LC-MS and following SepPak treatment, revealed components in addition to the PGE_2. When incubations were carried out on Grand Manan *L. salmonis* at 21°C, peaks at 3.8 and 6.4 min were observed when the column was eluted with 40: 60 Acetonitrile: Water (0.1% acetic acid). Under these conditions PGE_2, PGD_2 and $PGF_{2\alpha}$ had retention times of 7.0, 8.3, and 5.7 min, respectively. The two unknown peaks were determined to be non-prostanoid in nature by the presence of a base peak of m/z 265 (Fig. 2.4) in the two unidentified peaks and the absence of such a peak in the prostanoids examined (Fig. 2.3).

The second incubation method was employed on *L. salmonis* obtained from Aspotogan, NS, and again from Greens Point, NB. Liquid chromatography-MS analysis of these SPs displayed 1.7 and 0.6 ng PGE_2 /louse, from Aspotogan and Greens Point, respectively. The second incubation method revealed

Figure 2.4: The MS spectra of *Lepeophtheirus salmonis* secretory/excretory products with a RP-HPLC retention time of 3.8 and 6.4 min. The spectra were identical for both peaks. The column was kept at room temperature and eluted isocratically with 40: 60 acetonitrile: water (0.1 % acetic acid).



additional PG-like compounds but at much lower concentrations (<10%) than that of PGE_2. These compounds were considered PG-like since they were similar to PGs by LC diode array analysis and had parent ions in the same m/z ratio range as other PGs. Two peaks with later retention times (8.8 and 9.9 min) than PGE_2 and PGD_2 were observed in the SEPs of *L. salmonis* from Aspotogan, NS (Fig. 2.5), and again from Greens Point, NB, (data not shown) following the 30 min incubation with dopamine at 10°C. These peaks had parent ions at m/z of 353 and 357 and had fragmentation ion profiles that differed significantly from those of PGE_2 and PGD_2 standards run under the same conditions (Table 2.2). The identification of these peaks is presently being pursued.

2.5 Discussion

We demonstrated protease activity in samples after topical DA stimulation. These proteases are the same as those reported for *L. salmonis* in the presence of host mucus (Fast et al., 2003). This is taken as evidence of DA stimulating the production of SEPs. The presence of PGE_2 in both the SEP and SP samples indicates that PGE_2 is actively secreted and not excreted into the incubation media.

We have confirmed the presence of PGE_2 in dopamine-induced secretions of *L. salmonis* using LC-MS-in source dissociation. Since structural similarity between PGE_2 and PGD_2 results in nearly identical product ion spectra for these compounds (Yang et al., 2002), we first separated these analytes in the LC phase prior to MS analysis. Following this step we determined that sample LC peaks

Figure 2.5: Selected ion profile chromatogram (353 m/z) of prostanoid-like compounds (8.8 and 9.9 min) found in the secretory products of *Lepeophtheirus salmonis* in comparison to PGE_2 (351 m/z). The column was kept at room temperature and eluted isocratically with 40: 60 acetonitrile: water (0.1 % acetic acid).

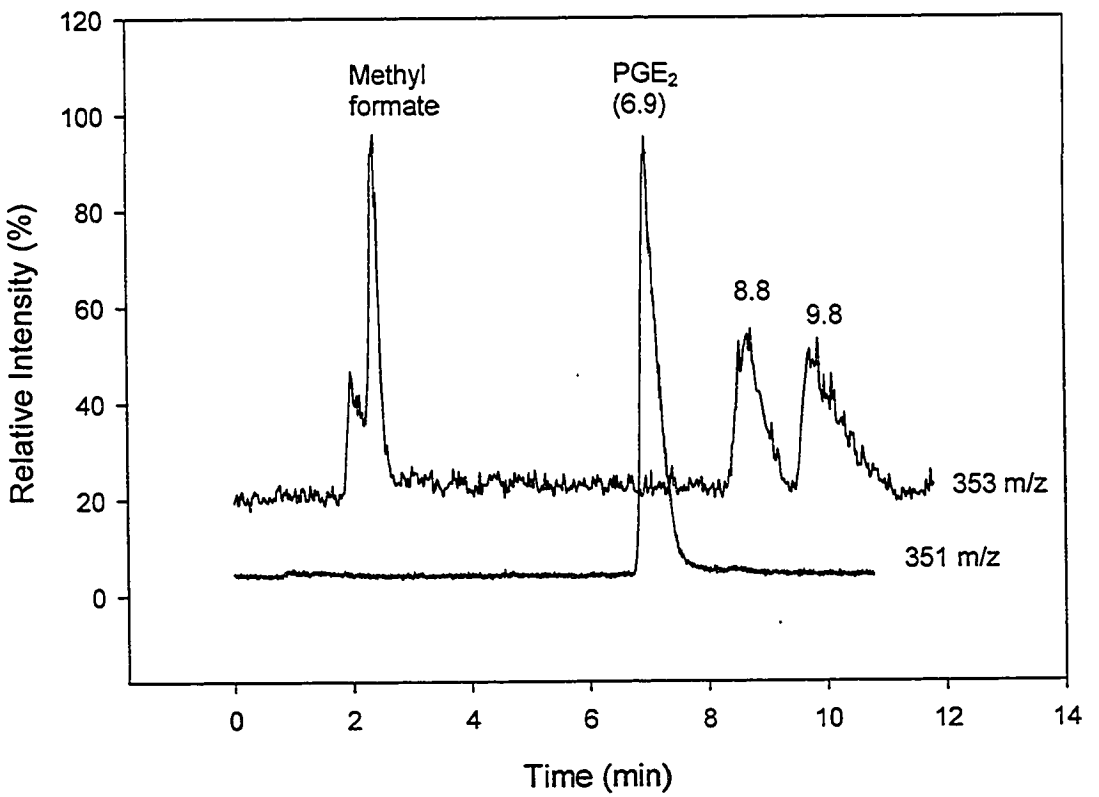


Table 2.2: Mass spectrometry fragment ion profiles and their relative intensities (%) for prostaglandin standards and unknown compounds obtained from *Lepeophtheirus salmonis* collected in Aspotogan, NS and incubated with dopamine (0.1 mM) and seawater. Most abundant ion in bold.

Sample	Fragment Ion Profile (Relative intensity %)
PGE_2	333 (35), 315 (80), 271 (65), 251 (15), 233 **(100)**, 189 (70), 175 (30)
PGD_2	333 (40), 315 **(50)**, 271 (40), 233 (20), 189 (30), 175 (10)
Aspotogan-*L. salmonis* (8.79 min peak)	311 (12), 297 *(95)*, 265 (15), 255 **(100)**, 211 (50)
Aspotogan-*L. salmonis* (9.87 min peak)	323 (5), 311 (25), 271 (10), 255 **(100)**, 239 (8), 211 *(60)*

matched those of the PGE_2 standard based on retention time and analysis of parent (351) and fragment (333, 315, 271, 175) ion profiles. These ion profiles are identical to ion profiles generated for PGE_2 standards in this study and previously published ion profiles for PGE_2 (Margalit et al., 1996).

Prostaglandin E_2 concentrations in secretions of *L. salmonis* are in the same range as PGE_2 production in the saliva of several other arthropod ectoparasites. However, there was a high level of variation observed in PGE_2 production by *L. salmonis* even within groups of animals that had been off the host for the same amount of time. This may be a result of temperature/seasonal differences between times of collection, as well as the health of *L. salmonis* individuals at specific sites. Aljamali et al. (2002) observed a nearly five-fold variability in PG in tick (*A. americanum*) saliva obtained using dopamine stimulation between different samples of the same population. Differences in individual *A. americanum* production also ranged from 0.2-80 ng/salivary gland pair (or per tick) depending on the size and the time elapsed since most recent feeding. Despite the variation it appears that the concentration of PGE_2 in *L. salmonis* SEPs is slightly greater than that seen in the saliva of *B. microplus* and *I. dammini* (ca. 1-2 ng/tick) (Ribeiro et al., 1985; Inokuma et al., 1994) but less than seen in *A. americanum* saliva (ca. 12-13 ng/tick) (Ribeiro et al., 1992). With exception of prostaglandin-like compounds that were observed in two of our samples of SPs, no other PGs could be identified in the secretions of *L. salmonis*. This result is the same as that reported for saliva of most ticks, with exception of *A.*

americanum in which a much smaller amount of $PGF_{2\alpha}$ is present (Aljamali et al., 2002).

Trienoic prostanoids (PGE_3, PGD_3, etc.), which are derived from eicosapentanoic acid (EPA), were not observed in *L. salmonis* secretions despite the fact that the EPA: arachidonic acid (AA) ratio is often quite high in their salmonid hosts (Bell et al., 1996). While AA comprises less of the total polyunsaturated fatty acid content in Atlantic salmon than EPA, PGE_2 production is equal to or higher than PGE_3 in kidney macrophage-enriched leucocyte preparations (Bell et al., 1996). One of the reasons the dinenoic series may be more abundant is that EPA is a much poorer substrate for PG synthetase than AA (Crawford, 1983). In salmonids, such as rainbow trout, PGE_2 is a more potent inhibitor of leucocyte proliferation than PGE_3 (Secombes et al., 1994). For these reasons it is not surprising that *L. salmonis* produces secretions containing primarily PGE_2.

In some samples two unknown peaks that were non-prostanoid in nature were detected. The unidentified m/z 265 adduct seen in these peaks may be the result of dopamine oxidation followed by covalent binding to proteins in solution (Graham, 1978; Mattammal et al., 1995). Peak absorption spectra of this adduct was similar to those obtained for a mixture of dopamine, hemoglobin and PG H synthetase (Mattammal et al., 1995). Prostaglandin H synthetase is a peroxidase required to catalyze the conversion of PGG_2 to PGH_2. This is a necessary step along the cyclooxygenase pathway to produce PGE_2. Although peroxidases were not tested for in the current study, *L. salmonis and Caligus elongatus*, have

glands associated with their oral cone that stain for peroxidase and/or catalase activity with 3',3-diaminobenzidine tetrahydrochloride (Bell et al., 2000). Interestingly, the 265 m/z adduct increased in concentration (21°C incubation) the longer the lice were maintained off a host. This may be an effect of temperature stress on already weakened animals or catabolism of PGE_2 at the elevated temperatures in the presence of other *L. salmonis* enzymes and SEP metabolites. Due to its absence from secretions of *L. salmonis* at 10°C and MS data being inconclusive, further identification of this compound was not pursued.

Prostaglandin E_2 plays a variety of roles in feeding and avoidance of host immune responses in other arthropod parasites. As a vasodilator, PGE_2 would be useful in maintaining blood flow to the site of feeding, since blood constitutes a component of the diet (Brandal, 1976; Bricknell et al., 2003). Prostaglandin E_2 may adversely affect site-specific leucocyte recruitment and function (Papadogiannakis et al. 1984; Papadogiannakis and Johnsen 1987; To and Schreiber 1990). The role of PGE_2 as a systemic immunomodulator, however is unlikely, as it is highly unstable and loses activity (>90%) following one passage through the circulatory system in mammalian models (Piper et al. 1970). These ascribed effects may explain the observation of Jónsdóttir et al. (1992) who reported little tissue response in Atlantic salmon to *L. salmonis* beneath the site of active feeding and attachment yet an inflammatory response in tissues surrounding the lesion.

The lack of significant inflammatory response in Atlantic salmon following *L. salmonis* infection (Johnson and Albright, 1992a) could be caused by PGE_2, as

this compound down-regulates the pro-inflammatory cytokines IL-1_β and TNF_α (Pinge-Filho et al., 1999). Down-regulation of host inflammatory cytokines occurs in several other host-parasite relationships (Fuchsberger et al., 1995; Kopecky et al., 1999; Schoeler et al., 1999, 2001; Ferriera and Silva, 2001; Gwakiska et al., 2001; Hajnicka et al. 2001). Saliva from *Rhipicephalus sanguineus* ticks impairs T cell proliferation and IFN-γ-induced macrophage microbicidal activity (Ferreira and Silva 1998). Prostaglandin E_2 can cause polarization towards a Th_2 lymphocyte response by down-regulating pro-inflammatory cytokines (Betz and Fox 1991). The modulation of Th_2 lymphocyte responses has been observed in other arthropod parasite hosts (Ramchandra and Wikel, 1992; Schoeler et al., 2001) and can also cause delay in the clearance of secondary bacterial infections (Dalton and O'Neill, 2002).

It is possible that the production of host mucus, a component of the diet of *L. salmonis*, may also be increased due to the presence of PGE_2. In rat models, PGE_2 encourages mucin secretion from tracheal (Nettesheim and Bader, 1996) and gastric (Tani et al., 2002) epithelial cells. Nolan et al. (1999) observed increased mucus production by Atlantic salmon skin epithelia following infection with low numbers of adult *L. salmonis* (≤10 lice per fish), however, the potential role of PGE_2 in this observation has yet to be elucidated.

There are several interesting observations that have been made during laboratory infection trials with *L. salmonis*. Infection with high number of *L. salmonis* commonly results in host mortality at the molt to the preadult stage without the development of lesions (Bjorn and Finstad, 1997; Grimnes and

Jakobsen, 1996; Ross et al., 2000). In addition, reduced macrophage function and increased susceptibility to secondary infection has also been reported in infected fish without the presence of a cortisol response (Fast et al., 2002; Mustafa et al., 2000a). It is possible that PGE_2 or other sea louse derived compounds might be responsible at least in part for these observations. For example, the sudden and high level of host mortality reported at the molt to the preadult stage has similarities to a toxic shock response. The role of PGE_2 in toxic shock is unknown, however, prostanoid release has been demonstrated to occur early in the course of shock and its inhibition significantly increases survival in mammalian toxic shock models (Ball et al., 1986; Lefer, 1983; Schade et al., 1991; Terashita et al., 1992).

In summary there is a good body of evidence that *L. salmonis* immunomodulates its hosts at its sites of attachment and feeding. Immunomodulation is most likely achieved by compounds secreted by the parasite including PGE_2, trypsin and as yet undescribed substances. The identification of PGE_2 in secretions of *L. salmonis* is not without precedence, as this compound is important in the host parasite relationships of other arthropod parasites. Further research is required to identify other components of these secretions and to identify the glands or organs associated with their production. Studies on the effects of these immunomodulatory agents on the host immune system will allow us to increase our knowledge of copepod host parasite relationships and may ultimately lead to novel ways of parasite treatment and/or control.

Chapter 3.0: Prostaglandin E_2 modulation of gene expression in an Atlantic salmon (*Salmo salar*) macrophage-like cell line (SHK-1).

3.1 Abstract

Following lipopolysaccharide (LPS)-stimulation of Atlantic salmon (*Salmo salar*) macrophage-like SHK-1 cells, prostaglandin E_2 (PGE_2) exhibited dose-dependent inhibition of the antigen presenting molecules major histocompatibility class I and II and the pro-inflammatory cytokine Interleukin-1β gene expression. Prostaglandin E_2 stimulated cyclooxygenase-2 (COX-2) gene expression at higher concentrations ($1x10^{-6}$ and $1x10^{-8}$ M) and inhibited at lower concentrations ($1x10^{-10}$ and $1x10^{-12}$ M) after 4 h exposure. After 24 h exposure, however, LPS-induced COX-2 expression decreased and was completely inhibited by all PGE_2 concentrations ($1x10^{-6}$ to $1x10^{-10}$ M). Incubation of SHK-1 cells with LPS alone had no effect on tumour necrosis factor α (TNF α)-like gene or transforming growth factor β-like gene expression after 4 h, however, LPS and PGE_2 showed a synergistic effect on TNFα-like gene expression after 24 h. This study provides evidence for the existence of a PGE_2-mediated negative feedback mechanism in the control of PGs through down-regulation of COX-2, as well as for inflammatory responses by the down-regulation of both COX-2 and IL-1β. The differential regulation of immune-related genes under these conditions further demonstrates the usefulness of the SHK-1 cell line for studying aspects of salmonid immunology.

3.2 Introduction

The development of continuous cell lines derived from teleost leucocytes has provided fish immunologists with the ability to study different aspects of cellular immunity and infection conveniently *in vitro*. The SHK-1 cell line is a continuous cell line derived from Atlantic salmon (*Salmo salar*) head kidney leucocytes and characterized as having some of the same properties as the macrophage (Dannevig et al., 1997). As these cells phagocytose fish pathogens but do not exhibit bactericidal activity or a macrophage appearance, they have been likened to a precursor of a melanomacrophage or dendritic leucocyte. The melanomacrophage is a macrophage that contains melanin but retains the ability to phagocytose, even at low temperatures (Dannevig et al., 1997). In order to better characterize this cell line we must obtain a comprehensive understanding of how different physiological conditions regulate SHK-1 cell gene expression.

Previous work has shown that these cells express both major histocompatibility class I (MH class I) and II (MH class II) genes constitutively (Koppang et al., 1999). Antigen presenting cells (APC) and T-cell interactions are regulated by the release of cytokines. Cytokines such as interleukin-1 β (IL-1β) and tumor necrosis factor α (TNFα), for instance, recruit inflammatory leucocytes to sites of injury (Aggarwal, 2003; Dinarello, 1994) and promote phagocytosis of foreign particles, which are then presented to T-cells in conjunction with MH molecules. In contrast, cytokines such as transforming growth factor β (TGFβ) are anti-inflammatory and can inhibit APC phagocytosis (Chantry et al., 1989; Tsunawaki et al., 1988).

The role of eicosanoids (oxygenated derivatives of polyunsaturated fatty acids) in immune modulation has been studied in several fish species (Rowley et al., 1995). However, despite the work done on leucocyte proliferative responses and generation of plaque forming cells in rainbow trout (*Oncorhynchus mykiss*), little work has been applied to other species and nothing has been pursued with respect to effects on gene expression (Knight and Rowley, 1995; Secombes et al., 1994).

Prostaglandin E_2 is a common eicosanoid produced by monocytes and thrombocytes from arachidonic acid (AA) and it is involved in numerous biological processes such as vasodilation, cellular proliferation, leucocyte activation and neutrophil chemotaxis and inflammatory accumulation. The production of PGE_2 during inflammatory events is a result of AA metabolism by the inducible form of cyclooxygenase (COX-2). Over the past decade, the immunomodulatory effects of PGE_2 have been investigated in numerous systems including: rat models of allergic asthma, mast cell cytokine synthesis and host-parasite interactions (Gomi et al., 2001; Inokuma et al., 1994; Leal-Berumen et al., 1995; Martin et al., 2001; Urioste et al., 1994). In mammals PGE_2 down-regulates the pro-inflammatory cytokines tumour necrosis factor α (TNFα), interleukin-12 (IL-12), IL-2, and interferon γ (IFN-γ), and has no effect on others such as IL-4 and IL-5 (Anastassiou et al., 1992; Betz and Fox, 1991; Demeure et al., 1997; Pinge-Filho et al., 1999; van der Pouw Kraan et al., 1995). The role of prostaglandins in fish gene regulation, however, is as yet unstudied.

We investigated the effects of PGE_2 administration on the expression of TNFα and IL-1β in LPS-stimulated and non-stimulated Atlantic salmon head kidney derived cell line cells (SHK-1) using quantitative real-time PCR. To provide further evidence of the applicability of these cells to the study of the salmonid immune system, expression of the other immunologically important genes MH class I, MH class II and COX-2 were studied. These data build upon our knowledge of the characteristics of the SHK-1 cell line, as well as the regulatory role of PGE_2 in immune responses of fish.

3.3 Materials and Methods

3.3.1 Cell Culture

SHK-1 cells were cultured at 18°C in 75 cm^2 tissue-culture-treated flasks (Costar), in L-15 medium (500 mL with 300 mg/L L-glutamine) supplemented with 500 µl gentamicin sulphate (50 mg/mL in distilled water), 365 µl 2-mercaptoethanol (55 mM in Dulbeco's phosphate buffered saline) and 5% fetal bovine serum (FBS). All media components were purchased from Gibco. Confluent flasks with confluent cell populations were passaged weekly by dividing cells and medium evenly between two flasks and adding an equal volume of new media to each flask. Cells used in this study were between passage 58 and 60.

3.3.2 *Cell stimulation*

SHK-1 cells were seeded at approximately $4.0x10^6$ cells/flask in L-15 medium supplemented as described above. Cell stimulation followed the same procedure as Zou et al. (2000) and Brubacher et al. (2000). Briefly, following a 48 h period after passaging, to minimize any manipulation-induced gene expression, 10 ml of fresh media without lipopolysaccharide (LPS) or prostaglandin E_2 (PGE_2), or with LPS and without PGE_2, or without LPS and with PGE_2 or with LPS and PGE_2 was added to each flask. Lipopolysaccharide was added to obtain a final concentration of 1.7 µg/mL and PGE_2 was added to give final concentrations of $3.3x10^{-6}$, $3.3x10^{-8}$, $3.3x10^{-10}$, $3.3x10^{-12}$ M. Treatments were carried out in triplicate and cells were induced under these conditions for 4 h prior to RNA extraction. A second study was conducted under the same conditions, with the following exceptions: Lipopolysaccharide was added to obtain a final concentration of 1.7 µg/mL and PGE_2 was added to give final concentrations of $1.0x10^{-6}$, $1.0x10^{-7}$, $1.0x10^{-8}$, $1.0x10^{-9}$ and $1.0x10^{-10}$ M. Treatments were carried out in triplicate and the cells were incubated for 24 h prior to RNA extraction. The concentrations of PGE_2 and LPS used in these studies did not effect cell viability, morphology or adherence properties as determined by trypan-blue exclusion and microscopy. In all incubations cell viability was > 90% at the end of the incubation period.

3.3.3 *Isolation of RNA and first strand cDNA synthesis*

Total RNA was isolated with the Nucleospin RNA II kit (Clontech) and its concentration determined by spectrophotometery. RNA samples underwent

PCR, were separated and visualized in a 2.5% agarose gel stained with ethidium bromide (3 µg/ml) to verify the lack of DNA contamination in RNA samples. For reverse transcription 1.0 µg of total RNA from each sample was dissolved in molecular biological grade water. Reverse transcription was carried out using the Retroscript (Ambion) kit, with random hexamers, as per supplier's instructions. Complementary DNA was stored at –20°C until use in real-time PCR assays.

3.3.4 Real-time PCR

Real-time PCR primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) and Dr. Michael Zuker's mfold server (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) (Table 3.1). Primers were designed using Atlantic salmon genomic (β-actin: AF012125, MH class I: I51348 and MH class II: X70166) (Grimholt et al., 2003) and National Research Council of Canada - EST databases (COX-2 and IL-1β). The TNFα-like gene and $TGF\beta_{1/5}$-like gene primers were designed from comparisons of the highly conserved regions of those genes in rainbow trout and plaice (*Pleuronectes platessa*) (Hardie et al., 1998; Laing et al., 2000; 2001). The gene product developed for Atlantic salmon TNFα-like gene showed 124/125 (99%) homology to rainbow trout (TNFα 1), while the $TGF\beta_{1/5}$-like gene product exhibited 107/108 (99%) homology to rainbow trout. The specificity of all primers was tested using head kidney cells isolated from Atlantic salmon. The resulting PCR products (100-130 bp) for β actin, MH class I, MH class II, COX-2, IL-1β, TNFα-like gene, and $TGF\beta_{1/5}$-like gene were cloned into a TA-cloning vector (pCR 4-TOPO

Table 3.1: Sequences of oligonucleotide primers used in Real-Time PCR.

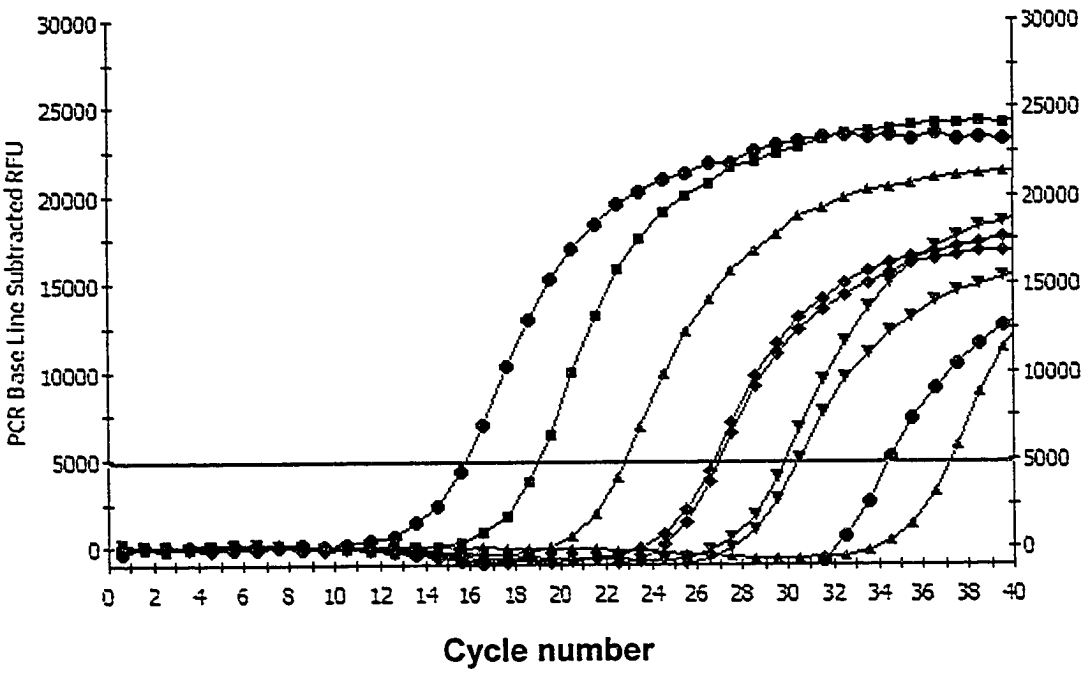
Genes	Primers	Sequences (5`-3`)
β-actin	β actin - forward	CAACTGGGACGACATGGAGA
	β actin - reverse	AGTGAGCAGGACTGGGTGCT
Cyclooxygenase-2	COX-2 – forward	CAGTGCTCCCAGATGCCAAG
	COX-2 – reverse	GCGAAGAAGGCGAACATGAG
MH class I	MH I – forward	TGCTCGTCGTTGCTGTTGTT
	MH I - reverse	TCAGAGTCAGTGTCGGAAGTGC
MH class II	MH II - forward	AAGGCTTGAAGACACGTTGC
	MH II - reverse	CAGTCCAGCAGTAACGTCCA
IL-1β	IL-1β - forward	GGTCCTTGTCCTTGAACTCG
	IL-1β - reverse	ATGCGTCACATTGCCAAC
TNF α	TNF α - forward	GGCGAGCATACCACTCCTCT
	TNF α - reverse	TCGGACTCAGCATCACCGTA
TGF $\beta_{1/5}$	TGF $\beta_{1/5}$- forward	ATCGGAGAGTTGCTGTGTGC
	TGF $\beta_{1/5}$- reverse	GGGCCGATGCAGTAGTTAGC

Invitrogen) and sequenced to confirm their identity. Cloning vectors with the appropriate inserts were then linearized by digestion with NotI (1 h at 37°C) and used as standards for real-time studies.

Quantitative real-time PCR was performed using an iCycler iQ™ real-time detection system and SYBR green kits (Bio-Rad). The SYBR green mastermix kit was used as per manufacturer's instructions with the following exceptions. Supermix was added (25 µl) to template cDNA (2.5 µl), water (20 µl) and specific primers (125 nM final concentration of forward and reverse primers) giving a total volume of 50 µl prior to dividing into separate wells for duplication of readings. Primer concentrations were optimized at 125 nM after testing a range of concentrations from 90-900 nM.

The PCR conditions were as follows: an initial 3-min denaturation step at 95°C, followed by 40 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C) and extension (30 s at 72°C), and finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products such as primer dimers, indiscriminately detected by SYBR green (i.e. SYBR green binds to all double stranded DNA), were evaluated by amplifying 10-fold dilutions of the standards ($5.0x10^{-5}$ to $5.0x10^{-11}$ g/L) and duplicate samples, as well as by performing a blank without cDNA with each run. The relationship between the threshold cycle (Ct) and the log [RNA] was linear (-3.6<slope<-3.2) for all reactions (Fig. 3.1). Copy numbers were estimated based on the molecular weight of clones and OD 260. The copy number in 5 mg/L of β actin, MH class I, MH class II, COX-2, IL-1 β, TNF α-like gene, and TGF $\beta_{1/5}$-like

Figure 3.1: Real-time PCR flourescence of β-actin standard dilution series ($1x10^{-2}$-$1x10^{-8}$). Threshold at which fluorescence was significantly higher than background ca. 5000 RFU. Standard curve of dilution series (all correlation coefficients > 0.96, PCR efficiency between 96 - 105%).



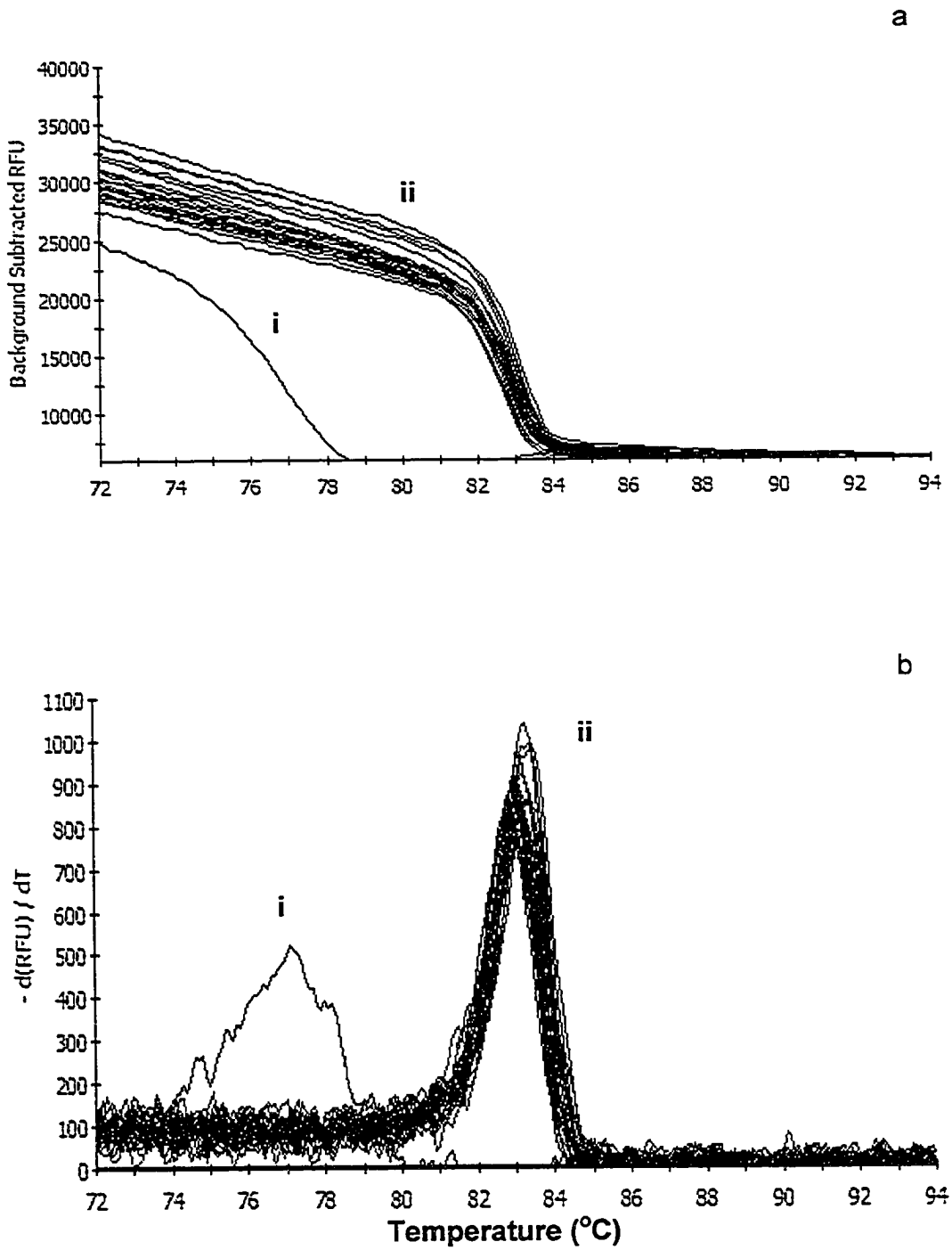
gene were between 2.24 and 2.27 x 10^8. Real-time studies detected as few as 10-15 copies of cDNA in 25 µl reaction mixtures. This is similar to other findings for SYBR-green (BioRad) (M. Matsuoka, NRC-IMB, pers. comm.).

Single product amplification was further verified by gel electrophoresis and melt curve analysis. Melting curves were obtained following 40 cycles of amplification on the iCycler by integrating the signal every 0.1 s during a linear temperature transition from 95°C to 70°C (Fig. 3.2a, b). Fluorescence data was converted by iCycler software in which background fluorescence and the effect of temperature on fluorescence were removed.

3.4 Statistical Analysis

Gene expression is presented as expression changes relative to β actin (ERB). Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). The 4-h incubation trial was duplicated and, as there were no significant differences between these trials, all data were pooled for further analysis. All non-normal data were transformed (ie. Log_{10}) prior to analysis. All values shown are means of trials ± SEM. The statistical significance of differences was assessed using one-way ANOVA ($p<0.05$).

Figure 3.2: Real-time melting curves for the same amplification products (a) along with their respective first-derivative transformed melting peaks (b). A melting curve was acquired after 40 cycles of amplification of TGF-$\beta_{1/5}$ primers in the absence of template (i) compared with template derived from the 24 hr LPS stimulation experiment (ii).



3.5 Results

3.5.1 MH class I

The MH class I gene was constitutively expressed in the SHK-1 cell line albeit at low levels relative to β-actin (Fig. 3.3). There was a significant three-fold increase in the level of MH class I gene expression following a 4-h stimulation with 1.7 µg/mL LPS when compared to the non-stimulated controls. This stimulation was eliminated through the addition of PGE_2 (Fig. 3.3). Virtually no expression of MH class I gene was observed in LPS-stimulated cells that were exposed to the highest concentration of PGE_2 ($3.3x10^{-6}$ M). Major histocompatibility class I expression in cells not stimulated with LPS but exposed to PGE_2 exhibited a high degree of variation especially at the $3.3x10^{-10}$ M concentration of PGE_2 (Fig. 3.3).

There was a significant increase in the expression of MH class I in SHK cells stimulated with 1.7 µg/mL LPS for 24 h when compared to cells stimulated for 4 h and the non-stimulated controls (Fig. 3.4). Similar to the 4-h co-incubation, addition of PGE_2 in the $1X10^{-10}$ M range resulted in a higher average level of MH class I gene expression. However, this was not significantly different from the 24-h LPS-stimulated group (Figs. 3.3, 3.4). Co-incubation with PGE_2 at levels as low as $1X10^{-8}$ M resulted in significant reductions in MH class I expression in cells stimulated for 24-h with LPS when compared with the 24-h LPS-stimulated controls (Fig. 3.4). Co- incubation with PGE_2 generally resulted

Figure 3.3: Real-time PCR analysis of MH class I expression in SHK cells stimulated with (■) and without (□) LPS (1.7 µg/ml) under various concentrations of prostaglandin E_2 for 4 h. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$), † denotes significantly lower than LPS stimulated controls ($p<0.05$), ‡ denotes significantly lower than unstimulated controls ($P<0.05$, $n=6$).

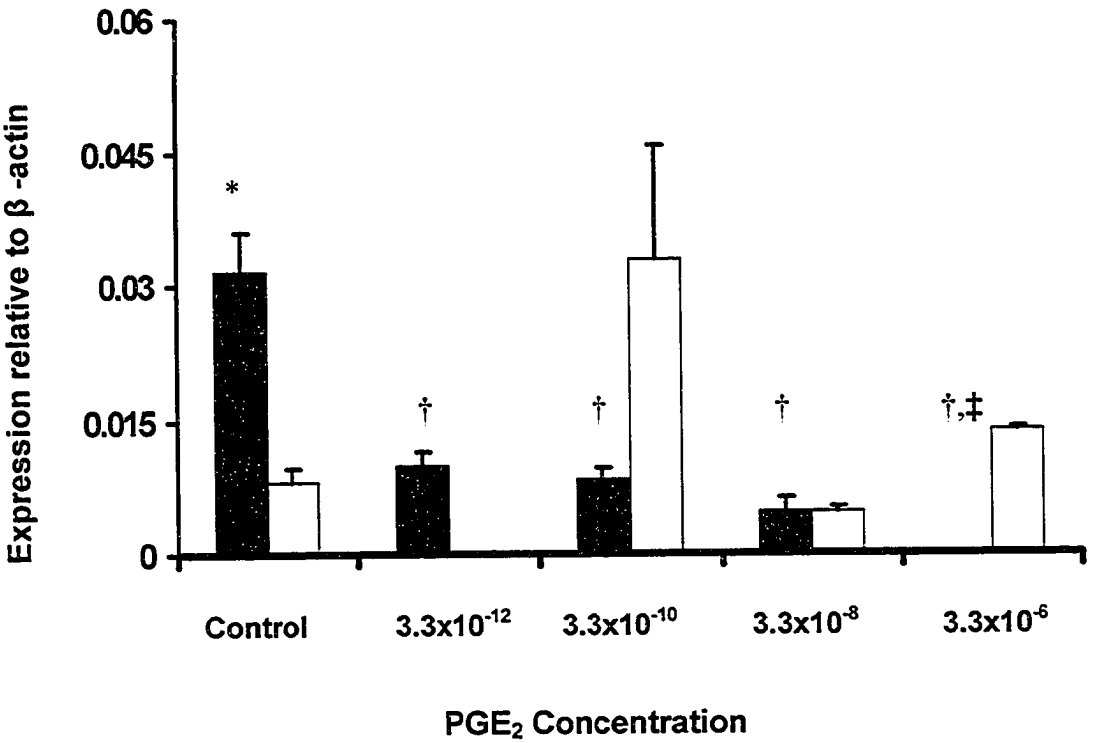
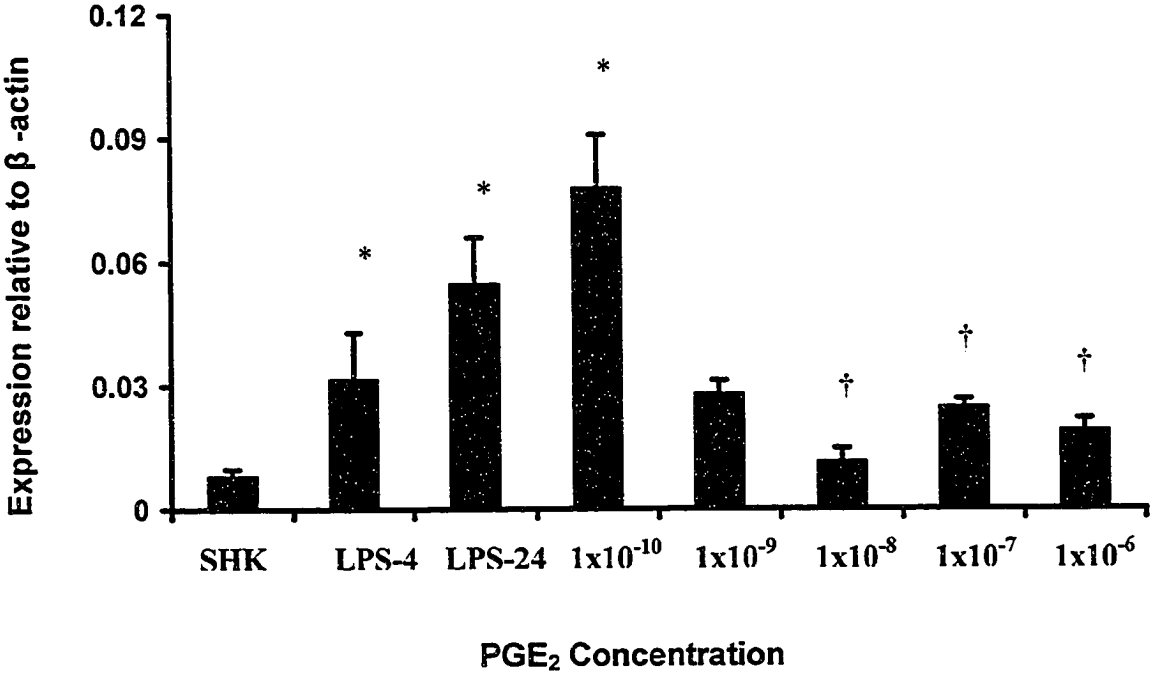


Figure 3.4: Real-time PCR analysis of MH class I expression in SHK cells stimulated with LPS (1.7 µg/ml) under various concentrations ($1x10^{-6}$ to $1x\ 10^{-10}$ M) of prostaglandin E_2. (SHK) are controls without LPS stimulation, (LPS-4) are control cells stimulated for 4 h with LPS, and (LPS-24) are control cells stimulated for 24 h. All other incubations included PGE_2 and were carried out for 24 h. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$), † denotes significantly lower than 24-h LPS stimulated controls ($p<0.05$, n=3 for 24 h, n=6 for 4 h).



in levels of gene expression similar to those seen in the 4-h LPS-stimulated and non-stimulated controls.

3.5.2 MH class II

Constitutive expression of the MH class II gene was observed, but at very low levels relative to β-actin (Fig. 3.5). Stimulation of SHK-1 cells with LPS, for 4 h, did not significantly change expression of MH class II from the constitutive levels seen in the controls. Co-incubation with PGE_2 at concentrations of $3.3x10^{-6}$, $3.3 x10^{-8}$ and $3.3x10^{-12}$ M resulted in significantly reduced MH class II expression in both the LPS-stimulated and non-stimulated controls. Thus, as the concentration of PGE_2 increased so did the inhibition of MH class II gene expression. Co-incubation with $3.3x10^{-10}$ M PGE_2, resulted in significantly reduced expression in the non-stimulated cells when compared to non-stimulated controls. At this concentration, in the LPS-stimulated group, PGE_2 was not inhibitory. The large variation observed in these samples may account for this occurrence rather than PGE_2 acting synergistically at this concentration to cause an increase in MH class II gene expression. Cells stimulated for 24 h with LPS had significantly reduced levels of MH class II expression regardless of whether they were co-incubated with PGE_2 or not when compared to the 4-h LPS-stimulated and non-stimulated controls (Fig. 3.6).

Figure 3.5: Real-time PCR analysis of MH class II expression in SHK cells stimulated with (■) and without (□) LPS (1.7 µg/ml) under various concentrations of prostaglandin E_2 for 4 h. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$), † denotes significantly lower than LPS stimulated controls ($p<0.05$), ‡ denotes significantly lower than unstimulated controls ($P<0.05$, $n=6$).

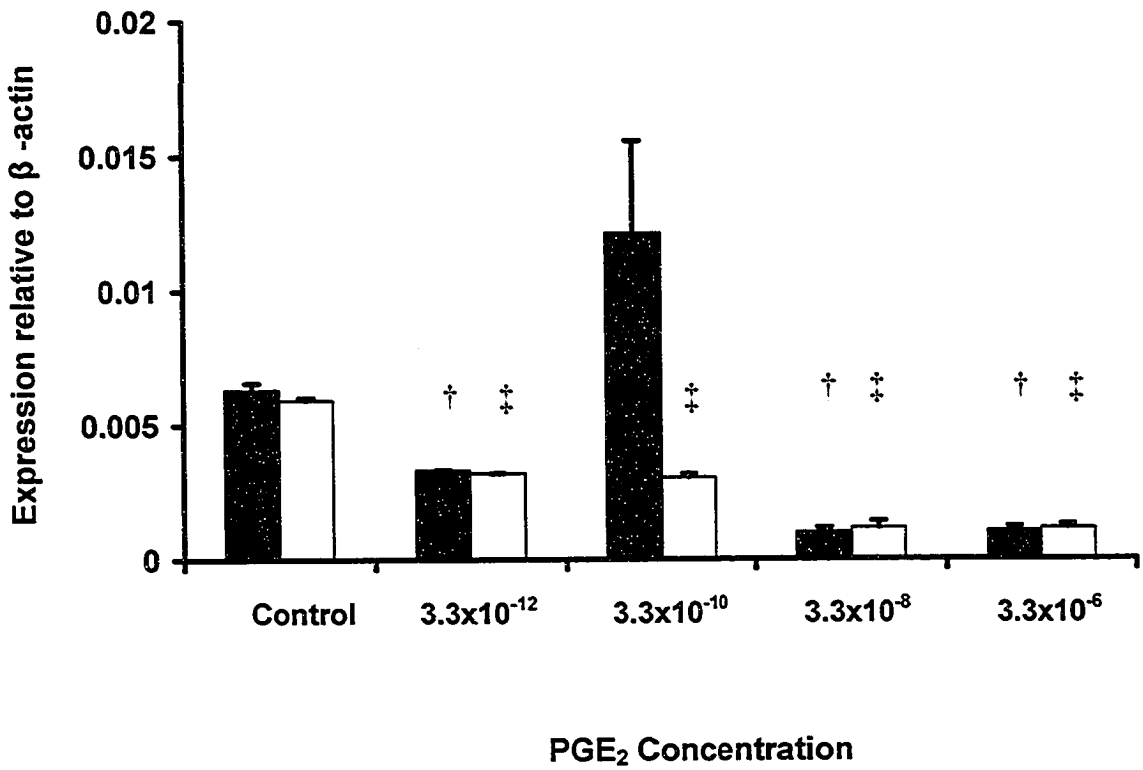
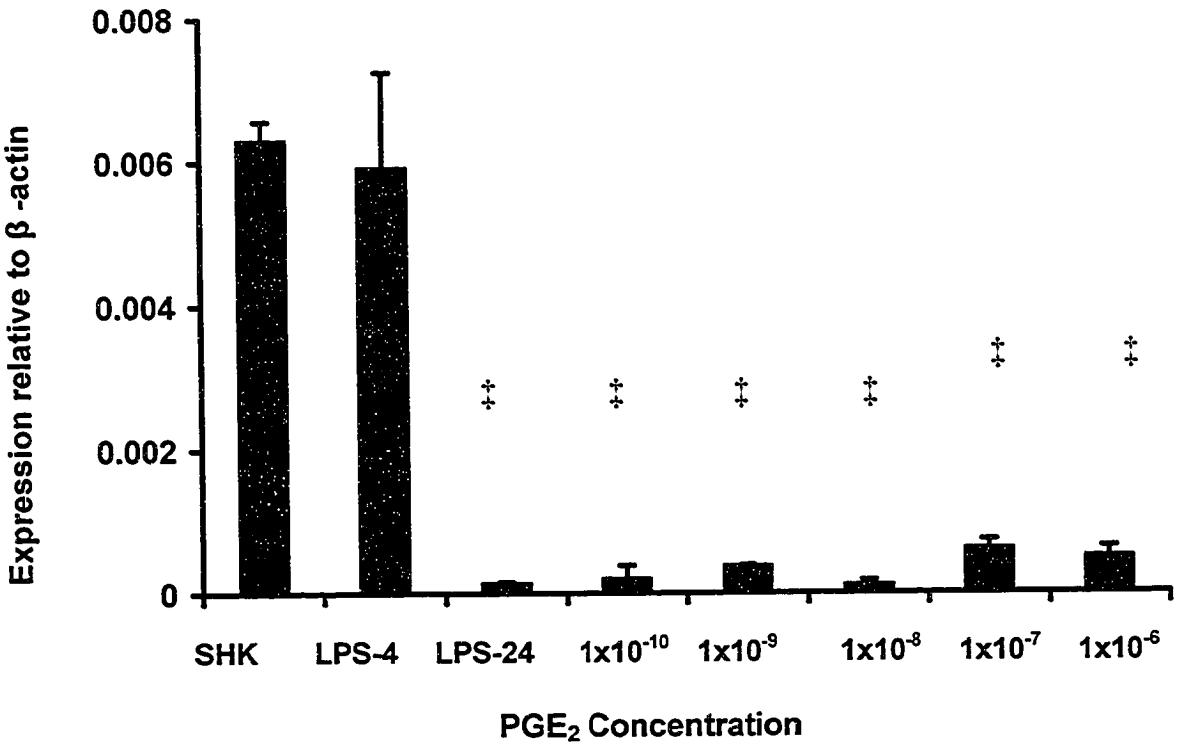


Figure 3.6: Real-time PCR analysis of MH class II expression in SHK cells stimulated with LPS (1.7 µg/ml) under various concentrations ($1x10^{-6}$ to $1x\ 10^{-10}$ M) of prostaglandin E_2. (SHK) are controls without LPS stimulation, (LPS-4) are control cells stimulated for 4 h with LPS, and (LPS-24) are control cells stimulated for 24 h. All other incubations included PGE_2 and were carried out for 24 h. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. ‡ denotes significantly lower than unstimulated controls ($P<0.05$, n=3 for 24 h, n=6 for 4 h).



3.5.3 *IL-1β*

There was very low constitutive expression of IL-1β (0.04 ERB) in the SHK-1 cell line (Fig. 3.7). Stimulation with 1.7 μg/mL LPS for 4-h resulted in a significant increase in IL-1β expression to 1.84 ERB (Fig. 3.7). Co-incubation with PGE_2, at $3.3x10^{-12}$, $3.3x10^{-10}$ and $3.3x10^{-8}$ M concentrations abrogated the stimulatory effects of LPS (Fig. 3.7). Co-incubation with the highest PGE_2 concentration ($3.3x10^{-6}$ M), however, resulted in an increase in IL-1β expression (Fig. 3.7). In the absence of any other stimulus, PGE_2 did not appear to have any effect on IL-1β expression. After 24-h stimulation with LPS, IL-1β expression was 5% of that which was observed after 4-h of LPS stimulation, but still significantly higher than basal levels (Fig. 3.7, 3.8). Co-incubation of LPS with PGE_2 at concentrations ranging from $1.0X10^{-10}$ to $1.0X10^{-6}$ M resulted in significant reductions in IL-1β expression (Fig. 3.8).

3.5.4 *COX-2*

There was extremely low constitutive expression of COX-2; however, following a 4-h stimulation with 1.7 μg/mL LPS, COX-2 expression rose nearly 1000-fold surpassing the level of expression of β-actin (ERB of 66.9) (Fig. 3.9). Co-incubation with PGE_2 at concentrations of $3.3x10^{-10}$ and $3.3x10^{-12}$ M abrogated LPS-induced COX-2 expression. However, following co-incubation with higher concentrations of PGE_2, $3.3x10^{-6}$ and $3.3x10^{-8}$ M, LPS and PGE_2 appeared to act synergistically and increased expression of COX-2 to $2.6x10^{4}$

Figure 3.7: Real-time PCR analysis of IL-1β expression in SHK cells stimulated with (■) and without (□) LPS (1.7 μg/ml) under various concentrations of prostaglandin E_2 for 4h. Total RNA (1 μg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$, n=6).

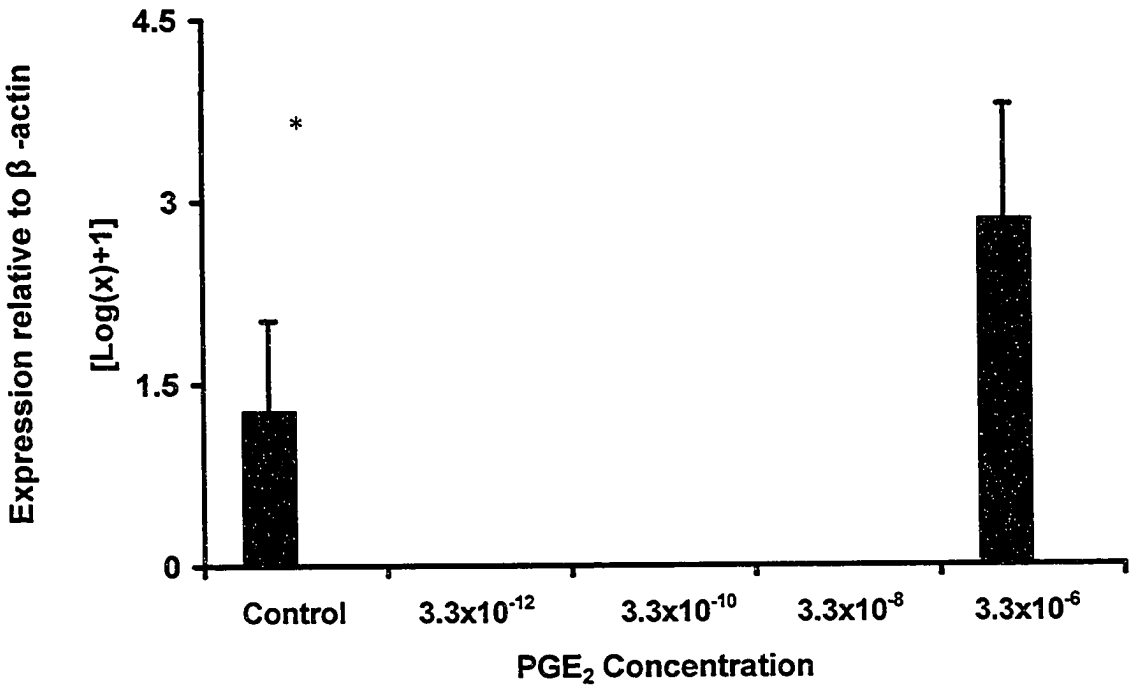


Figure 3.8: Real-time PCR analysis of IL-1β expression in SHK cells stimulated for 24 h with LPS (1.7 µg/ml) under various concentrations ($1x10^{-6}$ to $1x\ 10^{-10}$ M) of prostaglandin E_2. (SHK) are controls without LPS stimulation, and (LPS-24) are control cells stimulated for 24 h. All other incubations included PGE_2. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene.
‡ denotes significantly lower than unstimulated controls ($P<0.05$, n=3 for 24 h, n=6 for 4 h).

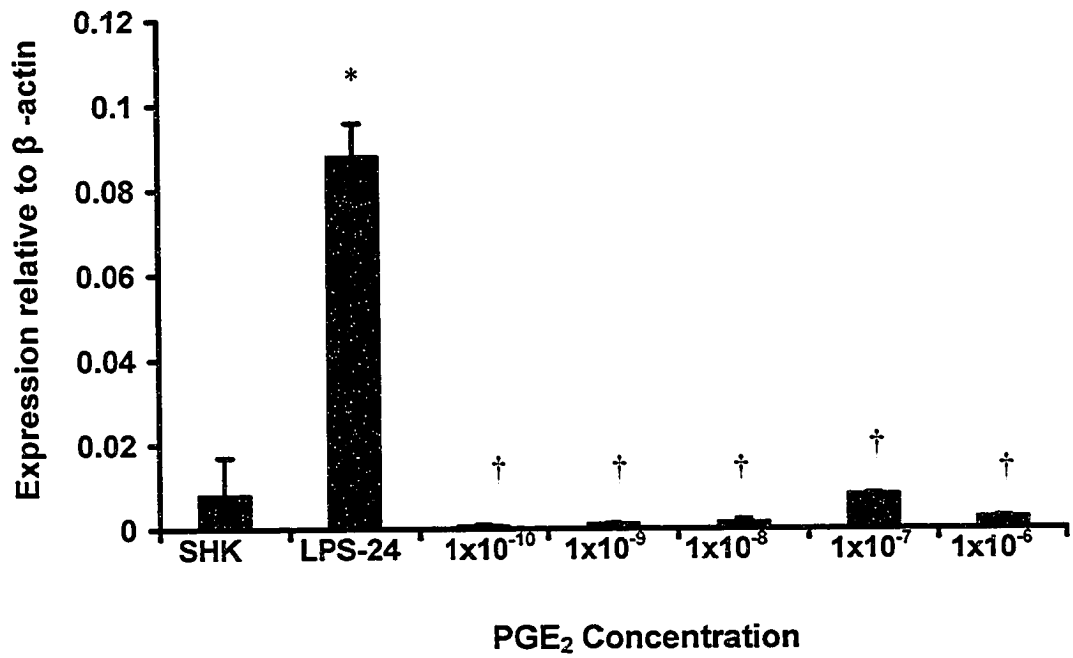
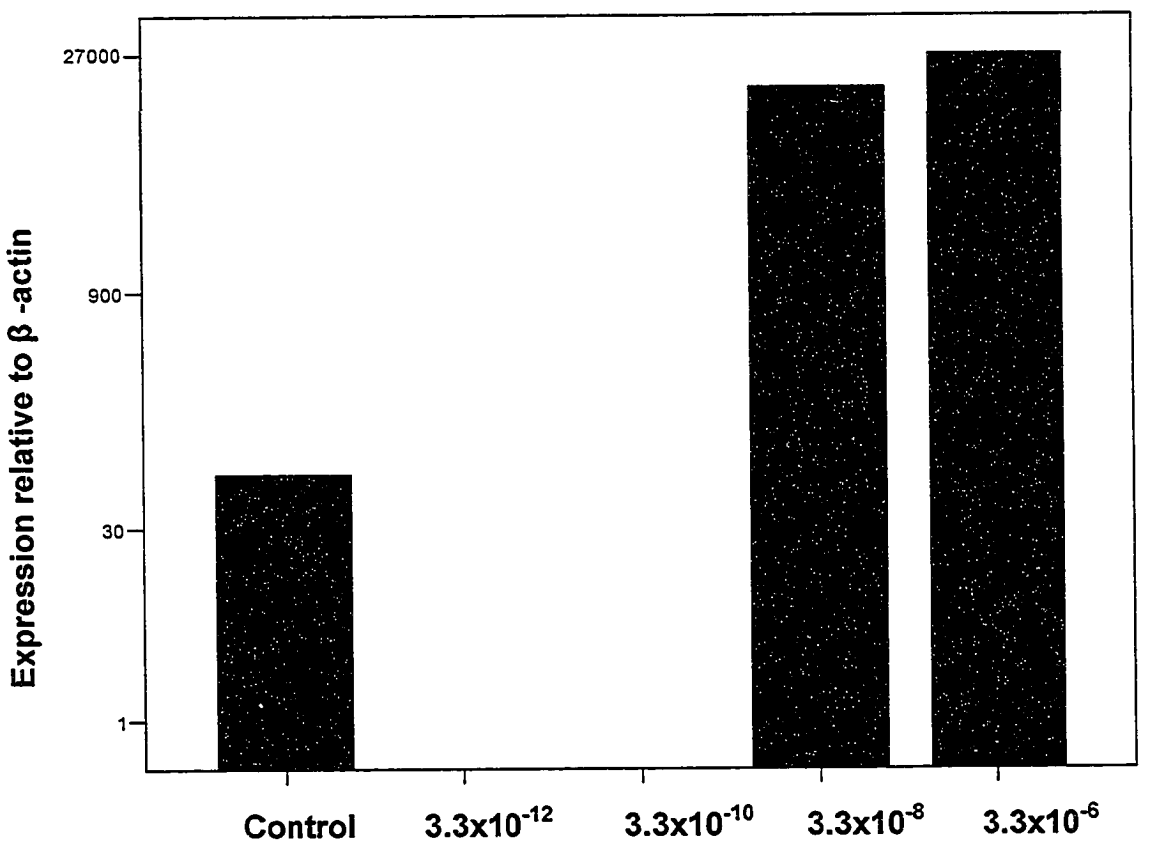


Figure 3.9: Real-time PCR analysis of COX-2 expression in SHK cells stimulated for 4 h with LPS (1.7 µg/ml) under various concentrations of prostaglandin E_2. Total RNA(1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. Values are expressed on a Logarithmic (base 30) scale (n=6).



and $1.6x10^4$ ERB, respectively (Fig. 3.9). After 24-h stimulation with LPS, COX-2 expression was significantly lower than that seen in cells that were stimulated for 4-h and no different from the expression levels of the non-stimulated controls (Fig. 3.10). Co-incubation with all PGE_2 concentrations ($1.0x10^{-6}$ to $1.0x10^{-10}$ M) inhibited COX-2 expression to nearly undetectable levels (Fig. 3.10).

3.5.5 TNFα-like gene

The SHK-1 cell line did not constitutively express the TNFα-like gene, nor was any expression observed following 4-h stimulation with 1.7 µg/mL LPS (Fig. 3.11). After 24-h co-incubation with 1.7 µg/mL LPS expression of TNFα-like gene was observed at very low levels. Co-incubation with PGE_2 at concentrations of $1X10^{-10}$ to $1X10^{-6}$ M stimulated expression of TNFα-like gene. The highest expression was observed at a PGE_2 concentration of $1.0x10^{-7}$ M (Fig. 3.11).

3.5.6 TGF $\beta_{1/5}$-like gene

Constitutive expression of the TGF$\beta_{1/5}$-like gene was very low (near the detection limits); less than 1% of β-actin. No effect was observed following stimulation with LPS for either 4 or 24-h. Co-incubation with PGE_2 had no effect on TGF$\beta_{1/5}$-like gene expression (data not shown).

Figure 3.10: Real-time PCR analysis of COX-2 expression in SHK cells stimulated for 24 h with LPS (1.7 µg/ml) under various concentrations ($1x10^{-6}$ to $1x\ 10^{-10}$ M) of prostaglandin E_2. (SHK) are controls without LPS stimulation, and (LPS-24) are control cells stimulated for 24 h. All other incubations included PGE_2. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$), † denotes significantly lower than LPS stimulated controls ($p<0.05$, n=3 for 24 h, n=6 for 4 h).

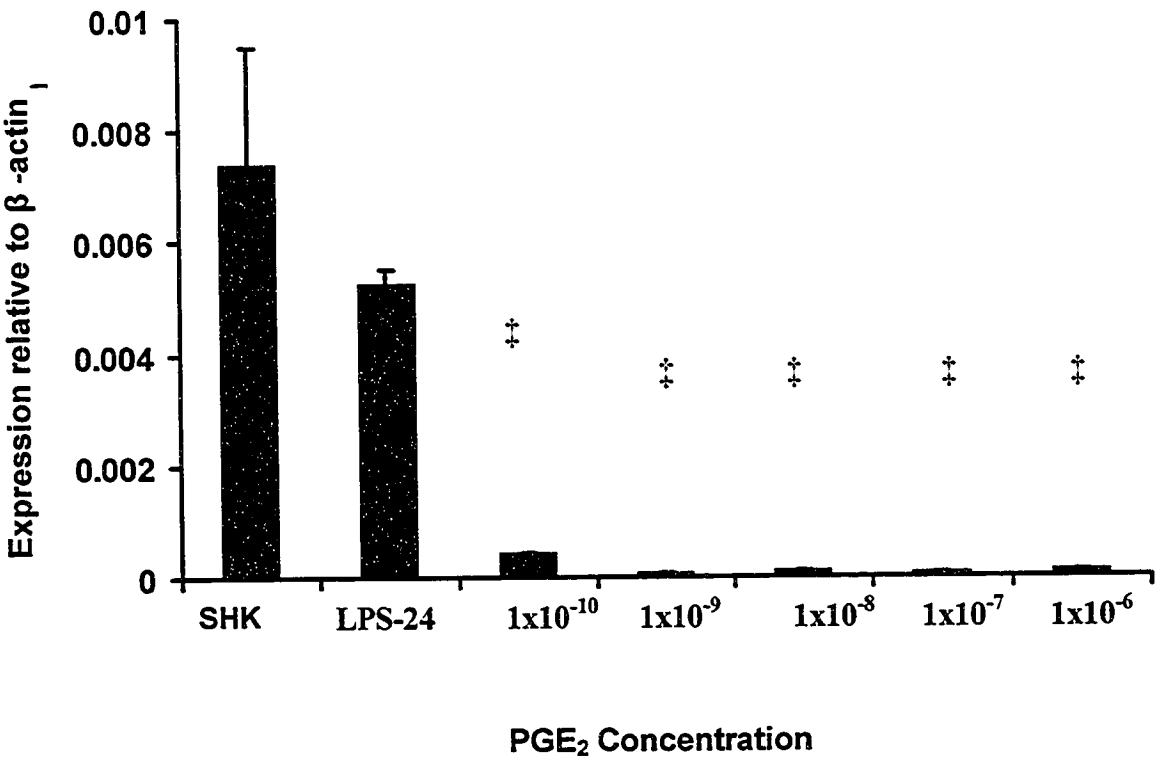
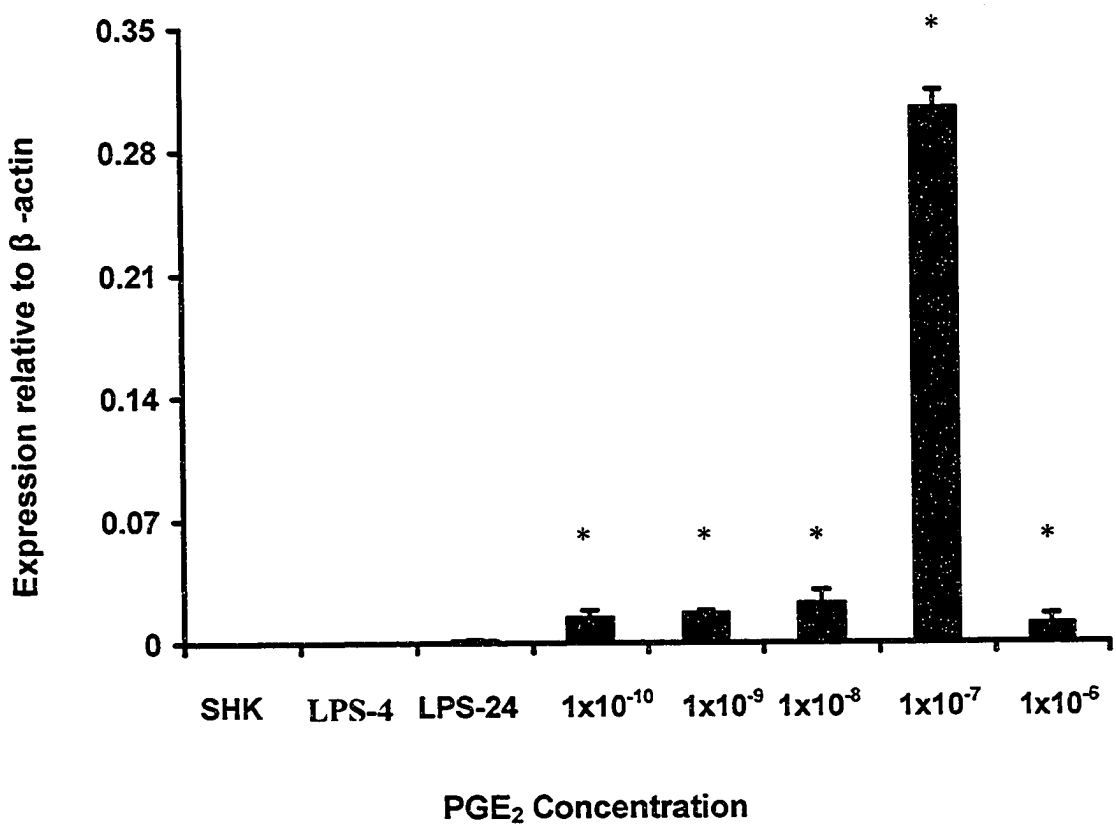


Figure 3.11: Real-time PCR analysis of TNFα-like gene expression in SHK cells stimulated for 24 h with LPS (1.7 µg/ml) under various concentrations ($1x10^{-6}$ to $1x\ 10^{-10}$ M) of prostaglandin E_2. (SHK) are controls without LPS stimulation, and (LPS-24) are control cells stimulated for 24 h. All other incubations included PGE_2. Total RNA (1 µg) extracted from SHK cells was used in each reverse transcription prior to real-time PCR analysis. All results are relative to β-actin control gene. * denotes significantly higher than unstimulated controls ($p<0.05$, n=3 for 24 h, n=6 for 4 h).



3.6 Discussion

In this study we used real-time quantitative PCR to determine the effects of LPS stimulation and PGE_2 administration on gene expression in the SHK-1 cell line. The concentrations of PGE_2 and LPS that were used did not affect cell viability, morphology or adherence properties. Similar findings are reported for mammalian macrophages incubated with PGE_2 at equivalent concentrations (Snyder et al., 1982). Our goal was to better classify this cell line, as well as to obtain information on the role that PGE_2 plays in immune gene regulation in fish. In mammals the lipid mediator, PGE_2 is found at physiological concentrations that range from $1.0x10^{-11}$ M at basal levels to $1x10^{-7}$ M in inflammatory reactions (Higgs and Salmon, 1979; Samuelsson, 1973). To the authors' knowledge there are no data on basal levels of PGE_2 in fish tissues. However, Rowley et al. (1995) observed a concentration of 5.33×10^{-11} M PGE_2 in rainbow trout head kidney leucocytes following calcium ionophore stimulation. Thus, in addition to the numerous known similarities between fish and mammalian leucocytes, they appear to have comparable physiological processes regarding PGE_2.

In mammalian models, PGE_2 inhibits: proliferation of peripheral blood mononuclear leucocytes (PBML) at concentrations of 1.4×10^{-6} M, peritoneal macrophage spreading at $1.0x10^{-8}$ M, B cell responsiveness at concentrations ranging from $1.0x10^{-9}$ to $1.0x10^{-7}$ M and IgM production at concentrations ranging from $1.0x10^{-8}$ to $1.0x10^{-5}$ M (Haynes et al., 1992; He et al., 2002; Papadogiannakis and Johnsen, 1987; Simkin et al., 1987). Several studies have

investigated the effects of PGE_2 on fish leucocytes. Significant decreases in plaque forming cells were reported at PGE_2 concentrations of $1.0x10^{-8}$ and $1.0x10^{-7}$ M (Rowley et al., 1995). Inhibited macrophage respiratory burst was reported at $2.6x10^{-6}$ M $diMePGE_2$ (but not < $8.5x10^{-7}$) and inhibited head kidney leucocyte responses to the mitogen phytohaemagglutinin-p at concentrations of $1.0x10^{-7}$, $1.0x10^{-6}$ and $1.0x10^{-5}$ M PGE_2 (Novoa et al., 1996; Secombes et al., 1994).

With respect to gene expression and transcription an increase in vascular endothelial growth factor mRNA and protein levels were reported in murine macrophages treated with PGE_2 at a concentration of $1.0x10^{-7}$ M (Mukutmoni et al., 2001). Administration of PGE_2 at lower basal physiological levels of $1.0x10^{-9}$ to $1.0x10^{-11}$ M had no effect on vascular endothelial growth factor mRNA expression. Prostaglandin E_2 has inhibitory effects on the expression of MH genes in mammalian systems. Snyder et al. (1982) showed significant reductions in the expression of MH class I in murine macrophages following PGE_2 treatment at concentrations as low as $1.0x10^{-11}$ M. Bone-marrow-derived dendritic cells from mice exhibited a PGE_2-dependent inhibition of MH class II gene expression at a concentration of $1.0x10^{-6}$ M PGE_2 (Harizi and Gualde, 2002). At this point, there are no studies that have directly examined the effects of PGE_2 on gene expression in fish.

In the present study, increased levels of MH class I gene expression were seen after 4 and 24-h stimulation with LPS, whereas MH class II gene expression was significantly reduced in 24-h cultures when compared to those stimulated for

4-h. Co-incubation with LPS and PGE_2 generally resulted in lower levels of MH class I and II gene expression when compared to LPS alone. Constitutive expression of MH class II was reported in the macrophage-like rainbow trout cell line RTS11 (Brubacher et al., 2000). However, stimulation of this cell line with 10 µg/ml LPS for 4-h had no apparent effects on MH class II gene expression. Knight et al. (1998) found little to no effect of LPS stimulation on MH molecule expression using primary isolates of rainbow trout head kidney macrophages.

Using reverse transcriptase-PCR (RT-PCR), Koppang et al. (1999) examined the effects of LPS stimulation on MH class I and II gene expression in the SHK-1 cell line but did not examine the effects of PGE_2. In their study, as in ours, constitutive expression of MH class I and II was reported, as well as a reduction in MH class II gene expression after stimulation with LPS (10 µg/ml) for 24-h (Koppang et al., 1999). However, their results differ from those reported here in that they observed a decrease in MH class I gene expression after stimulation with 10 µg/ml LPS for 24-h and only a slight stimulatory effect in some instances after stimulation for 72-h. The differences reported between our study and the study of Koppang et al. (1999) may be related to succession in cell populations being present within the SHK-1 cultures over time (Dannevig et al., 1997). In this study we use cells that had undergone 58 - 60 passages whereas Koppang et al. (1999) used cells that underwent between 30 and 60 passages. Koppang et al. (1999) did not compare MH gene expression to a housekeeping gene (i.e. β actin) and only compared gene expression relative to un-stimulated controls. Without an endogenous gene as a control, any manipulation induced

effects following RNA quantification (ie. non-robust cDNA synthesis) cannot be measured and may explain the differences between the results of our studies. Furthermore, RT-PCR is not fully quantitative, as real-time PCR is, and is less sensitive.

Prostaglandin E_2 has been identified in the saliva and other secretions of a variety of parasites and may assist in host immune evasion (Fast et al., 2004; Inokuma et al., 1994; Ribeiro et al., 1992). As MH class I and II are important in stimulating adaptive immune responses, their regulation by PGE_2 during parasitic challenge has been studied. Inhibition of MH class I and II gene expression by PGE_2 has been observed in *Leishmania donovani* infections of murine macrophage cell line P388D1 and *Entamoeba histolytica* infections of BALB/c mice bone marrow-derived macrophages (Kwan et al., 1992; Wang and Chadee, 1995). However, the effects of PGE_2 on MH class I and II gene expression in BALB/c mice bone marrow macrophages infected with *Toxoplasma gondii* and bovine endothelial cells infected with *Cowdria ruminantium* were insignificant (Luder et al., 1998; Vachiery et al., 1998).

The low constitutive expression of IL-1β in SHK-1 cells is similar to that reported for rainbow trout primary macrophage cultures and the RTS-11 cell line. Both of these showed very low levels of constitutive expression of IL-1β, which increased after LPS stimulation (Brubacher et al., 2000; Zou et al., 2000). In this study, we reported higher levels of IL-1β expression in cells that had been stimulated with LPS for 4 h when compared to a 24-h period of stimulation. This result is similar to that found in rainbow trout and higher vertebrates (Brubacher

et al., 2000; Dinarello, 1991; Zou et al., 2000). The decline in these other species is attributed to unstable mRNA motifs in the 3' untranslated region of certain cytokines (Kern et al., 1997; Shaw and Kamen, 1986). The mechanism behind the 30-fold increase in IL-1β expression after 4-hr co-incubation with LPS and $1.0x10^{-6}$ M PGE_2 is unknown. As IL-1β upregulates COX-2 expression, this may be the indirect mechanism by which PGE_2 at higher concentrations causes increased COX-2 expression. In both cases, the synergistic effect of LPS and PGE_2 at this higher concentration is completely reversed after 4 h. Since IL-1β mRNA changes 2-4 h following LPS stimulation, this time point may be more conducive to variability, but may result from insufficient PGE_2 dispersal through the culture at this time point (Windle et al., 1984).

Interleukin-1β is a pro-inflammatory cytokine that activates macrophages and neutrophils and stimulates T-cells (Titus et al., 1991). Interleukin-1β derived peptide stimulates phagocytosis and bactericidal activity in head kidney macrophages of rainbow trout (Peddie et al., 2002). Interleukin-1β expression increases in the skin of rainbow trout infected with the ectoparasite *Gyrodactylus derjavini* (Lindenstrom et al., 2003). As in other species, PGE_2 inhibits IL-1β expression at all concentrations (except $1.0x10^{-6}$ M) and at both time points (Kunkel et al., 1986). These results are in agreement with the role of PGE_2 in a natural negative feedback mechanism to control inflammation. Importantly, these results support the view that exogenous PGE_2 can be used as an effective means of modulating site-specific inflammation (Inokuma et al., 1994).

In higher vertebrates, stimuli such as LPS, the cytokines IL-1β and TNFα, and growth factors increase levels of COX-2 expression in a variety of tissues (Fu et al., 1990; Niiro et al., 1995). In fish, LPS stimulation for 12 to 48 h increases levels of PGE_2 in primary cultures of rainbow trout macrophages but the role that COX-2 plays in this increase is not determined (Rowley et al., 1995). Brubacher et al. (2000) reported that the macrophage-like RTS-11 cell line constitutively expresses COX-2 and that the level of expression is markedly increased upon a 4-h stimulation of the cells with 5 μg/ml LPS. We cannot attribute the massive increase in COX-2 expression solely to direct LPS stimulation, as we find increased IL-1β expression under the same conditions.

Pang and Hoult (1997) reported that pre-treatment of mouse macrophage cells with PGE_2 prior to LPS stimulation resulted in a dose dependent reduction in the expression of COX-2 protein. This result was taken as evidence for a feedback regulatory suppression of COX-2 induction by a prostaglandin driven cAMP mediated process. Such a proposed regulatory mechanism may act to reduce or halt the inflammatory response (Beltinger et al., 1999). A similar mechanism may exist in fish. At physiological levels of PGE_2, inhibition of COX-2 and IL-1β expression was observed in the SHK-1 cell line.

The absence of constitutive expression of the TNFα-like gene in SHK-1 cells was not surprising as this cytokine is usually associated with activated (via LPS) macrophages (Aggarwal, 2003). Lipopolysaccharide is a powerful stimulator of TNFα expression in mammalian systems (Dumitru et al., 2000). In this study exposure to LPS failed to elicit an increase in TNFα-like gene

expression and it was only after 24 h co-incubation with PGE_2 that increased expression was observed. This may be due to a unique fish melanomacrophage trait or the TNFα-like gene functions through a different pathway than mammalian TNFα. Work on rainbow trout TNFα revealed two different isoforms of the gene, with one exhibiting much higher LPS-induced expression (Zou et al., 2002). Interestingly, the TNFα -like gene studied here is more like rainbow trout TNF1, or the less inducible form, and may explain why LPS stimulation did not increase expression. Synergistic effects of PGE_2 and LPS after 24 h incubation were observed in rat macrophages but only at the lowest PGE_2 concentrations ($2.9x10^{-9}$) tested, whereas in this study the greatest synergy occurred at relatively high PGE_2 concentrations ($1.0x10^{-7}$ M) (Renz et al., 1988). While PGE_2 alone was not observed to stimulate TNFα-like gene expression in SHK-1 cells after 4 h, this was not examined after 24 h stimulation, when TNFα-like gene expression was observed.

The low level of constitutive expression of the anti-inflammatory cytokine $TGF\beta_{1/5}$-like gene in the SHK-1 cell line is similar to the low level of constitutive expression reported for RTS-11 (Brubacher et al., 2000). In this study as well as in the study of Brubacher et al. (2000) LPS stimulation had no effect on $TGF\beta_{1/5}$-like gene expression.

For some genes, only a small increase in the level of expression was observed following LPS stimulation (i.e. TNFα). While changes in expression do not necessarily result in corresponding changes in translation or concentration of expressed proteins, in the case of the cytokines, TNFα, IL-1β, and TGFβ, large

changes in protein concentration are not required to create wide scale immunological changes. Many cytokines initialize and can saturate their receptors signalling at very low concentrations ($1.0x10^{-9}$ to $1.0x10^{-12}$ M).

The SHK-1 cell line has been described as macrophage-like in its ability to phagocytose bacterial cells (Dannevig et al., 1997). Our study supports this description as these cells show similar responses to rainbow trout and mammalian macrophage cells with respect to MH class I, MH class II, COX-2 and IL-1β gene expression following LPS and PGE_2 exposure. This cell line has potential for use as a model system to study the effects a variety of conditions and compounds on the immune system of fish.

Chapter 4.0 *Lepeophtheirus salmonis* secretory components and their effects on salmonid immune gene regulation.

4.1 Abstract

The inability of Atlantic salmon to mount a significant inflammatory response against *Lepeophtheirus salmonis* infection has led to the belief that immunomodulatory compounds exist in this parasite's secretions. Following dopamine stimulation of *Lepeophtheirus salmonis*, parasitic secretions/excretions (SEPs) were collected, concentrated and fractionated using size exclusion chromatography. Several peptides contained within the fractions showed homology with *L. salmonis* ESTs. Four of these *L. salmonis* ESTs were differentially expressed over the life cycle of the parasite. Most noticeably, SL-0858, SL-0903 and SL-1469 exhibited significant upregulation following host infection. To determine biological significance of each fraction, they were incubated with lipopolysaccharide (LPS) in both macrophage-enriched isolates of Atlantic salmon head kidneys as well as the Atlantic salmon head kidney cell line (SHK-1). Results from the macrophage-enriched isolate incubations showed that PGE_2 was inhibitory, in some cases synergistically with unfractionated SEPs, towards interleukin-1β (IL-1β) and major histocompatability class I gene expression. Inhibition of LPS-induced IL-1β expression in the SHK-1 cell line was observed within all three pools of the SEP fractions. Further isolation of fractions within these pools revealed that fraction 1-2 could fully account for the inhibition of IL-1β expression in SHK-1 cells observed in pooled fraction 1. This chapter provides evidence for the presence of immunomodulatory compounds, not related to PGE_2, in the secretions of *L. salmonis*, which suppress expression of Atlantic salmon immune-related genes *in vitro*.

4.2 Introduction

The emergence of arthropod parasites as economically significant organisms that hamper animal production in both terrestrial and aquatic systems, has initiated study into their interactions with their hosts. *Lepeophtheirus salmonis* is a marine copepod, which is ectoparasitic on salmonid hosts. Despite its economic importance, gaps of knowledge exist with respect to *L. salmonis* biology, physiology and host-parasite interactions. In particular, the means by which *L. salmonis* limit host immune responses in species such as Atlantic salmon (*Salmo salar*), which are highly susceptible to infection, are poorly understood. Furthermore, the reason for their apparent inability to limit host responses in relatively resistant species, such as coho salmon (*Oncorhynchus kisutch*) is poorly understood (Fast et al., 2002; Johnson and Albright, 1992a). Improvements in our understanding in these areas is critical in the development of more effective treatment and control measures. The development of new molecular and biochemical technologies in the areas of genomics and proteomics provide the tools necessary to improve understanding in these areas.

There is growing literature concerning compounds produced by arthropod ectoparasites and their possible host immunomodulatory capabilities. Proteases, phosphatases and prostaglandins are major salivary constituents of numerous arthropod parasites (Wikel, 1996b). In addition to these there are macrophage migration inhibitory factors, apyrases, peroxidases, and many other as yet unidentified compounds in the secretions of these parasites (Jaworski et al., 2001; Wikel, 1996b).

Trypsin-like enzymes and prostaglandin E_2 (PGE_2) have been identified in the secretions of several arthropod parasites including *L. salmonis* (Chapter 2.0; Fast et al., 2002; 2003; 2004; Firth et al., 2000; Gillespie et al., 2000; Gwakiska et al., 2001; Kerlin and Hughes, 1992; Rosenfeld and Vanderberg, 1998; Ross et al., 2000; Schoeler and Wikel, 2001). It is thought that the trypsin-like proteases play important roles in *L. salmonis* infections, such as invasion of host tissues and evasion of host immune responses, as they do in a variety of other parasitic diseases. They have been well studied for numerous parasitic arthropods including: the cattle tick (*Boophilus microplus*), the mosquito (*Aedes aegypti*), the sheep blowfly (*Lucilia cuprina*), the buffalo fly (*Haematobia irritans exigua*), the warble-fly (*Hypoderma lineatum*) and the cat flea (*Ctenocephalides felis*) (Casu et al. 1994; 1996; Gaines et al., 1999; Kerlin and Hughes, 1992; Moiré et al., 1994).

In the host-parasite relationship, prostaglandins, such as PGE_2, are thought to regulate vasodilation, anti-coagulation and T-lymphocyte regulation (Betz and Fox, 1991; Williams and Higgs, 1988). In Chapter 2.0 of my thesis I report the recent discovery of PGE_2 in the secretions of *L. salmonis* (Fast et al., 2004). The presence of PGE_2 is viewed as a mechanism by which *L. salmonis* evades host immune responses, through its anti-inflammatory effects (Chapter 2.0, published in Fast et al., 2004; Chapter 3.0).

Compounds other than trypsin-like enzymes and PGE_2 have been identified in the secretions of arthropod parasites. The goal of this Chapter was to identify other compounds present in the secretions and assess their biological

significance with respect to the maintenance of *L. salmonis* on Atlantic salmon. This was done using a combination of proteomic, biochemical and molecular techniques.

4.3 Materials and Methods

4.3.1 Lepeophtheirus salmonis collection

Adult *L. salmonis* were collected from farmed Atlantic salmon held at various seawater net cage sites in the Bay of Fundy, Canada, as well as wild Pacific salmon caught off the coast of Vancouver Island (August 2004). Collected animals were washed with sterile seawater and maintained off hosts in sterile seawater for 24 h prior to incubation with dopamine.

In addition to copepodids that had never been on hosts, other developmental stages of *L. salmonis* (Chalimus I-II, Chalimus III-IV, pre-adult and adult) were collected from Atlantic salmon that were infected in our laboratory. Rearing of the infectious copepodid stage and methods of fish infection are given in Chapters 5.0 and 6.0. Copepods, at these various stages, were placed immediately in RNAlater (Ambion) and stored at 4°C. Copepodids that had never been on a host were collected to serve as a pre-infection control for RT-PCR.

Pre-adult *L. salmonis* stored in RNAlater were dissected so that RT-PCR could be carried out on different body regions. The body regions were divided into the anterior cephalothorax, posterior cephalothrorax and the genital complex/abdomen. The division into the anterior and posterior cephalothorax was made at the midpoint between the maxillipeds and the first thoracic legs.

4.3.2 *Lepeophtheirus salmonis incubation*

Secretory/excretory products (SEPs) were collected from approximately 1200 pre-adult and adult *L. salmonis* collected in New Brunswick and British Columbia using two methods as previously described (Chapter 2.0, published in Fast et al., 2004). Briefly, live *L. salmonis* were washed in sterile seawater (SSW) and then incubated at 10-15°C in SSW with 1 mM dopamine (DA) for 45 min. Anywhere from 80-100 *L. salmonis* were incubated (per tube) at 2 lice/ml. *Lepeophtheirus salmonis* were then removed and the samples spun through 3,000 Da molecular weight cut off columns to concentrate constituents and remove SW and DA.

In the second method, live *L. salmonis* were washed with SSW and then placed on parafilm wax and oriented so that the ventral surface of their cephalothorax covered a 20 µl droplet of 1 mM DA in SSW. *Lepeophtheirus salmonis* were incubated for 45 min inside a humidified chamber (15°C). After 45 min each droplet, as well as any solution that had been retained within the convex of the cephalothorax was collected with a pipette. Solutions were then centrifuged (3500 • *g*) for 5 min, and stored at –80°C. This second method was used to minimize the SSW + DA volume, as well as to lessen the possible contribution of *L. salmonis* excretory products to the solution. These solutions are referred to as SPs.

The same manipulations were carried out on samples of DA + SSW and SSW in the absence of *L. salmonis*. These samples were used as controls in case DA removal was incomplete. After centrifugation all samples were then re-

suspended in dd H_2O and were stored at –80°C. Samples were lyophilized prior to size exclusion chromatography.

4.3.3 Size exclusion chromatography and protein determination

Lyophilized SEPs were reconstituted with 1.0 M ammonium acetate (AMA) (pH 6.0). An Agilent 1100 HPLC equipped with a diode array detector (monitoring at 230 and 256 nm) and a Taso Haas (G3000PWX2, 6 µm d_p (7.8 mm x 300 mm)) column were used to separate proteins/peptides in the secretions. Fractions were collected using a Waters Fraction collector according to time intervals shown in Table 4.1, for 6 separate HPLC runs and pooled for each time interval. These samples were freeze dried (-80°C) prior to protein determination. The column was kept at room temperature and eluted isocratically with 98:2 AMA: acetonitrile (ACN) for 30 minutes at 0.2 ml min^{-1}. Standard solutions of bovine serum albumin (BSA) (20 µg, 2.0 µg, and 0.2 µg), SW + DA ($1x10^{-4}$M), and bovine trypsin (40 µg) were run as controls for peak comparison with SEPs.

Protein concentrations of *L. salmonis* secretory fractions were determined using a dye binding method with bovine γ-globulin as a standard (Bradford, 1976). All assays were run on a Thermomax Microplate Reader (Molecular Devices). Samples were reconstituted in ddH_2O and, following protein determination, were split equally between cell-based functional assays and proteomic analysis.

4.3.4 *Proteomic analysis*

Prior to MS-MS analysis, 10% of each fraction (by volume) was analyzed using SDS-PAGE as previously described by Fast (2001). Briefly, samples were diluted 1:1 with SDS-PAGE sample buffer containing DL-dithiothreitol (5%) and heated prior to loading. Proteins were electrophoresed in 12% acrylamide gels at 100 V and silver stained as described previously (Laemmli, 1970; Swain and Ross, 1995).

4.3.5 *Protein digestion*

The proteins were resistant to digestion using trypsin alone, therefore, digestion was performed using cyanogen bromide cleavage followed by trypsin. Cyanogen bromide digestion was conducted following Crimmins et al. (2000). Briefly, 88% formic acid (80 µl) was added to distilled deionized water (15 µl of ddH_2O) and CNBr in acetonitrile (5 µl of 0.53 $g \cdot ml^{-1}$) and incubated with the sample (0.05-10 µg protein) in an opaque container at room temperature for 19 h. The protein was vortexed to assure complete solubilization with an opaque container used to minimize side reactions with other amino acid side chains. Following incubation, 10 volumes of ddH_2O were added to each reaction and lyophilized for 19 h. Samples were resolubilized in 0.1 M ammonium bicarbonate (ABC) (50 µl) containing trypsin (at 1:50 final enzyme:substrate concentration). The tryptic digestion was carried out for 19 h at 37°C and the reaction stopped by

Table 4.1 Fraction interval times for size exclusion chromatography of *Lepeophtheirus salmonis* secretory/excretory products. Protein concentrations are expressed as total protein obtained for each fraction following 6-pooled runs.

Fraction number *(SHK-1-Trial 2)*	**Fraction Collection start**	**Protein Concentration (ng/ul)**	**Fraction Collection finish**	**Pooled Fraction grouping** *(SHK-1-Trial 1)*
Fraction 1-1	6	50	9.5	1
Fraction 1-2	9.51	149	12	1
Fraction 2-3	12.01	69	15.5	2
Fraction 2-4	15.51	13	17.5	2
Fraction 2-5	17.51	17	19.5	2
Fraction 2-6	19.51	11	21.5	2
Fraction 3-7	21.51	31	24.0	3
Fraction 3-8	24.01	18	26.0	3
Fraction 3-9	26.01	7	28.0	3
Fraction 3-10	28.01	0	29.0	3

the addition of trifloroacetic acid (2%). The reaction mixture was speed vacuumed to obtain a final volume of 10 µl for mass spectrometric analysis.

4.3.6 Mass spectrometry

Both fractionated and non-fractionated SEPs were analyzed and compared against SW and SW+DA controls using LC-MS/MS analysis to obtain partial sequence data. All samples were analyzed by LC-MS/MS using an LC Packings HPLC system equipped with a 5 cm x 300 µm PepMap C_{18} column. The separation was carried out using a linear gradient from 10% to 50% B over 20 minutes (A: 5% ACN, 0.5% formic acid, B: 90% ACN, 0.5% formic acid) at 5 µl/min. The HPLC was interfaced to an Applied Biosystems MDS SCIEX QStar Pulsar *i* mass spectrometer via a nanoflow source. Data were acquired in the information dependent acquisition mode, i.e. the m/z values of the tryptic peptides were measured using a TOF-MS scan and this scan was used to generate a peak list of peptides for tandem MS analysis. The tandem MS spectra were submitted to the database search program MASCOT (Matrix Science Ltd., England) in order to identify the proteins. The databases searched against were an *L. salmonis* EST database constructed at the National Research Council of Canada's Institute for Marine Biosciences, as well as the NCBI nucleotide database.

4.3.7 *In situ hybridization*

Lepeophtheirus salmonis (Chalimus III-IV and pre-adults) were sampled from experimentally infected Atlantic salmon and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 6-8 h at 4°C. Lice were transferred to 50% ethanol for 15 min before storing in 70% ethanol at 4°C (Johnson et al., 2002; Murray 2000). Lice were processed for embedding by conducting a series of washes: fresh 70% ethanol (1x), 95% ethanol (1x), 100% ethanol (2x), xylene (2x) and paraffin kept at 60°C under vacuum (2x) for 30 min each. Following the series of washes, *L. salmonis* were embedded in paraffin and stored at 4°C until sectioning. Serial sections of 7 µm were cut, and baked over night at 60°C on glass slides, previously treated with 3-aminopropyl triethoxysilane (Johnson et al., 2002; Murray, 2000). Probes were produced using *in vitro* transcription and a digoxigenin UTP labelling kit (Roche). The *in situ* hybridization (ISH) protocol followed the procedure given by Murray (2000). Probes were generated and ISH attempted for SL-0547, SL-0903, SL-1469.

4.3.8 *Macrophage isolation and manipulation*

Macrophages were isolated from 10 Atlantic salmon anterior kidneys (Ottinger et al., 1999). Anterior kidneys were removed aseptically and placed immediately into 5 ml L-15 media supplemented with 2% fetal bovine serum (FBS), 100 units ml^{-1} penicillin/streptomycin (P/S) and 10 units ml^{-1} heparin. Tissues were stored on ice until further processing. Individual head kidneys from each fish were dissociated by repeated passage through a 3-ml syringe and

fragments allowed to settle for 10 min prior to removal of suspended cells. Cell suspensions were pelleted (500 x *g* for 10 min at 4^0C) and washed twice prior to layering on Percoll gradients (34/51%). Cells were centrifuged at 400 x *g* for 20 min at 4^0C and the macrophage enriched fraction collected at the 34/51% interface. Cells were re-suspended in 10 ml L-15/2% FBS, pelleted by centrifugation at 500 x g for 10 min at 4^0C, washed with 10 ml of L-15/2% FBS and then resuspended in L-15/5% FBS with 100 units ml^{-1} P/S. Viable cells were counted using the trypan blue exclusion method and cell density adjusted to $1x10^7$ cells ml^{-1} in L-15/0.1% FBS. Cells were plated at 100 µl per well on 96 well plates and incubated at 18^0C for 2 h. After 2 h media and non-adherant cells were removed and an equal volume of L-15/5% FBS added. Cells were maintained for 1.5 days at 18^0C prior to manipulation, then media was removed and 100 µl of fresh L-15/5% with or without (control) 5 µg/ml lipopolysaccharide (LPS), 5 µg/ml LPS + 1 $x10^{-8}$ M prostaglandin E_2 (PGE_2), 5 µg/ml LPS + 660 ng/ml SEPs, 5 µg/ml LPS + 3% DA+SW, and 5 µg/ml LPS + 1 $x10^{-8}$ M PGE_2 + 660 ng/ml SEPs was added. Secretory/excretory products used in these incubations did not undergo size exclusion fractionation but were used immediately following concentration, removal of small molecules (DA, etc.) and protein concentration. Stimulation of cells was carried out for 4 h at 18^0C before media was removed and the cells were stored in RNAlater at -80^0C until RNA extraction.

Throughout this study, none of the conditions tested affected cell viability, morphology or adherence properties. This is consistent with reports on

mammalian macrophages incubated with PGE_2 at similar concentrations (Snyder et al., 1982).

4.3.9 SHK Cell Culture

SHK-1 cells were cultured following methods previously described (Chapter 3.0, published in Fast et al., *in press*). Briefly, SHK-1 cells were cultured at 18°C in 75 cm^2 tissue-culture-treated flasks (Costar), in L-15 medium (with 300 mg/L L-glutamine) supplemented with 500 µl gentamicin sulphate (50 mg/mL distilled in water), 365 µl 2-mercaptoethanol (55 mM in D-PBS) and 5% FBS. All media components were purchased from Gibco. Confluent flasks were passaged weekly by dividing cells and medium evenly between two flasks and adding an equal volume of new media to each flask. Cells used in this study were passaged between 64 and 68 times.

SHK-1 cells were seeded at approximately 4 x 10^6 cells/flask in L-15 medium supplemented as described above. Cell stimulation followed the same procedure as given in Chapter 3.0 (Fast et al., *in press*). Briefly, following a 48 h period, to allow any manipulation-induced gene expression to return to constitutive levels, media was removed and 20 ml fresh media was added. Lipopolysaccharide was added to all flasks, except the controls, to obtain a final concentration of 5 µg/mL.

In the first experiment, SEP fractions, obtained from size exclusion HPLC, were pooled into 3 groups (Table 4.1), each containing equal time ranges (10 min) and volumes from the size exclusion chromatography. This resulted in 13

µg/ml (pooled fraction 1), 8.0 µg/ml (pooled fraction 2) and <1.0 µg/ml (pooled fraction 3) being added to each flask. These incubations were carried out for 4 h at 18^{0}C after which the media was removed, the cells transferred into RNAlater and stored at –80^{0}C until RNA extraction. This experiment was repeated twice with triplicate flasks for each condition.

In the second experiment, SEP fractions 1-1 and 1-2 (Table 4.1) were added at 1.0 and 1.4 µg/ml, respectively. These concentrations were attained after concentrating 4 size exclusion HPLC runs for each fraction. To test the effect of residual solvent on the cell-based assay, 4 blank runs of AMA underwent the same treatment and were included in the experiment as controls. The non-fractionated SEPs (660 ng/ml) were incubated as a positive control. These incubations were carried out in triplicate and followed the same procedure as the first experiment.

4.3.10 Isolation of RNA and cDNA synthesis

Total RNA was isolated from different life cycle stages and body regions of *L. salmonis*, SHK-1 cells and macrophage-enriched cell cultures using the Nucleospin RNA II kit (Clontech). Concentrations of total RNA were determined spectrophotometrically. For macrophage-enriched cell cultures, multiple wells containing cells from individual fish under a single incubation condition were pooled. RNA samples underwent PCR to verify the lack of DNA contamination. For reverse transcription 1.0 µg of total RNA from each sample was dissolved in molecular biological grade water. Reverse transcription-PCR was carried out for

L. salmonis genes, in different developmental stages and body regions, using the Retroscript (Ambion) kit, with random hexamers, as per supplier's instructions. The PCR products were resolved in 1% (w/v) agarose gels and stained (10 min) with ethidium bromide (0.5 $mg \cdot ml^{-1}$ in Tris acetate buffer) and destained (10 min) with water. Quantification of each band was carried out using Multi-Analyst Version 1.0.2 densitometry (BioRad). All product abundances for *L. salmonis* genes were quantified relative to known concentrations of the β-actin control gene.

Sequences for PCR primers were designed using Primer 3 software (Rosen and Skaletsky 2000) and Dr. Michael Zuker's (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) mfold server. *Lepeophtheirus salmonis* primers were developed from unpublished *L. salmonis* EST databases. Reverse Transcription – PCR products for β actin, SL-1469, SL-0547, SL-0858 and SL-0903 were all cloned into a TA-cloning vector (pCR 4-TOPO Invitrogen) and sequenced to confirm amplification of the correct sequences.

4.3.11 Real-Time PCR of Atlantic salmon genes

Real-time PCR primers were designed, tested and their products sequenced as previously described in Chapter 3.0 (Fast et al., *in press*) (Table 3.1). Real-time quantitative PCR was performed using an iCycler iQ™ Real-Time detection system and SYBR green kits (Bio-Rad) also described in Chapter 3.0 (Fast et al., *in press*). To ensure no genomic DNA contamination added to

the quantified cDNA, non-RT controls for each RNA isolation were run under PCR and observed by 2.5% agarose gel electrophoresis.

The PCR profile was as follows: an initial 3 min denaturation step at 95°C, followed by 40 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C) and extension (30 s at 72°C), and finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products such as primer dimers, indiscriminately detected by SYBR green (ie. SYBR green binds to all double stranded DNA), were evaluated by amplifying 10 fold dilutions of the clones (10^{-2} to 10^{-8} ng) and duplicate samples as well as by performing a blank without cDNA with each run. The relationship between the threshold cycle (Ct) and the log (RNA) was linear (-3.6<slope<-3.3) for all reactions. Copy numbers were estimated based on the molecular weight of clones and OD 260, described in Chapter 3.0 (Fast et al., *in press*).

Single product amplification was further verified by melt curve analysis. Melting curves were obtained following 40 cycles of amplification on the Lightcycler by integrating the signal every 0.1 s during a linear temperature transition from 95°C to 70°C. Fluorescence data was converted by iCycler software in which background fluorescence and the effect of temperature on fluorescence were removed.

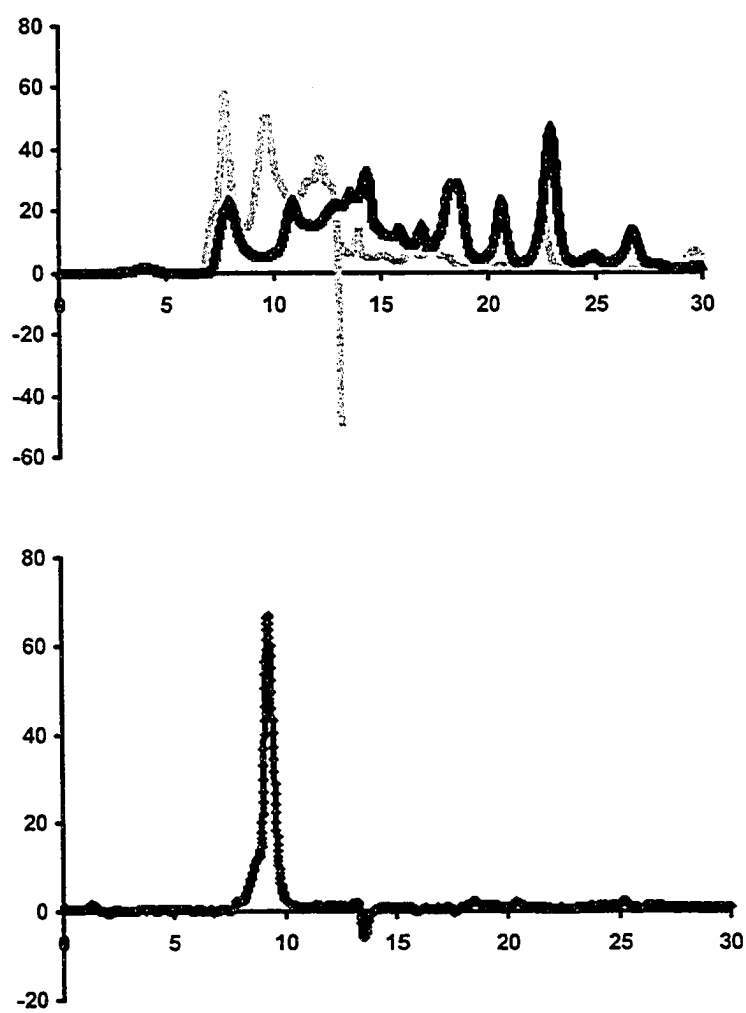
4.4 Statistical analysis

All gene expression is presented as expression changes relative to β- actin. Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All non-normal data were transformed (ie. Log_{10}) prior to analysis. All values shown are means of trials ± SEM. The statistical significance of differences was assessed using one-way ANOVA ($p<0.05$).

4.5 Results

Samples of *L. salmonis* SEPs and SPs were run under size exclusion HPLC. Several peaks were common to both types of samples, most notably those at ca. 7.5, 14, 16.5, and 22.5 min elution times (Fig. 4.1a). However, there were noticeable differences with three peaks at 9.5, 12.1, 17.3 min elution times exclusive to SEPs, and five peaks at 10.9, 15.8, 18, 20.6 and 26 min elution times exclusive to SPs (Fig. 4.1a). Based on these profiles *L. salmonis* SEPs appeared to contain more large compounds (<10 min retention similar to BSA, Fig. 4.1b) when compared to SP isolations. A large negative peak that corresponded to ca. 13 min, seen in the SW + DA control, was only observed in *L. salmonis* SEPs (Fig. 4.1a, c). Despite concentration of numerous *L. salmonis* + DA/SSW incubations to obtain reasonable SEP protein concentrations, DA concentration was still much lower in SEPs than the DA + SSW control (Fig. 4.1a, c). The SPs on the other hand produced three peaks that corresponded with the three major peaks observed in trypsin (ca. 10.8, 20.5 and 22 min elution times) (Fig. 4.1d).

Figure 4.1: Size exclusion HPLC chromatogram (254 nm) of *Lepeophtheirus salmonis* secretory/excretory products (SEPs) (—) and secretory products (SPs) (—) (a), bovine serum albumin (b), sterile seawater and dopamine (1.0×10^{-4}M) (c), porcine trypsin (d). The column was kept at room temperature and eluted isocratically with 98: 2 ammonium acetate:acetonitrile for 30 min at a flow rate of 0.2 ml min^{-1}. Injections of 20 µl were made of samples and standards dissolved in 1.0 M ammonium acetate.



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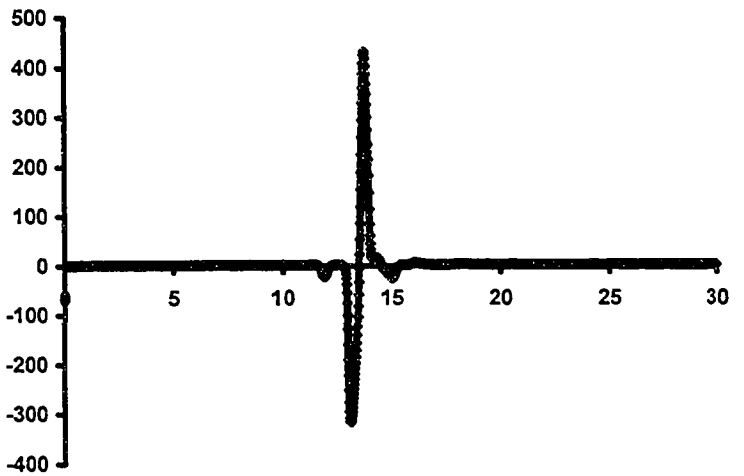
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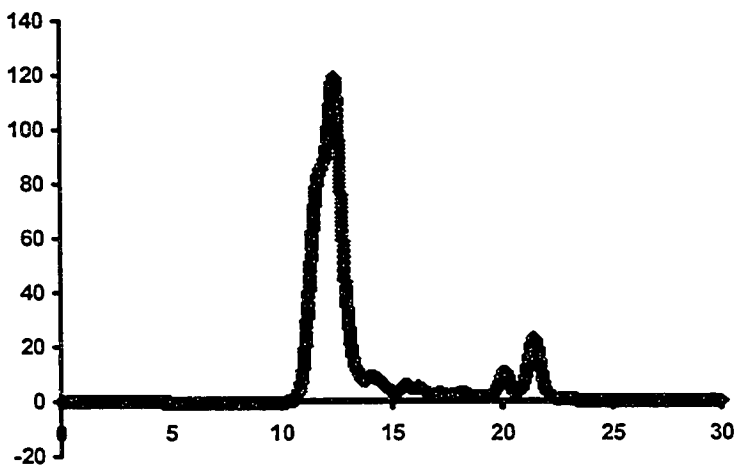
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4.5.1 *Proteomic analysis*

Initial attempts at analyzing unfractionated SEPs using 2-D SDS-PAGE and tryptic digestion of separated proteins were unsuccessful (data not shown).

Protein concentrations of the SEP fractions were relatively low in all samples (Table 4.1). However, silver stained SDS-PAGE revealed protein bands in fractions 1-1, 1-2, 2-3 and 3-7 (Fig. 4.2). Fraction 1-2 was enriched in 2 proteins at ca. 40 kDa, whereas fraction 1-1 exhibited numerous proteins all of equally low concentrations (Fig. 4.2). Protein bands in both fractions 2-3 and 3-7 were extremely faint and difficult to detect. To identify the proteins observed in these and other fractions, as well as unfractionated SEPs, the samples were digested with CNBr followed by trypsin and then subjected to capillary-HPLC MS/MS analysis (Fig. 4.3). Peptide matches were found for 6 individual proteins, and all were contained within the *L. salmonis* EST database (developed from pre-adult stages of both sexes). The ESTs with significant homology to the SEPs were: SL-0547, SL-0858, SL-0903, SL-1469, MB4c11 and *L. salmonis* trypsins type 1-4. In agreement with size exclusion chromatography on porcine trypsin, *L. salmonis* tryptic peptides were observed in SEP fractions 1-2 and 3-6. All other peptide matches occurred within fractions 1-1 and 1-2.

In the case of trypsin and *SL-1469*, the protein was identified from at least two fully sequenced peptides (Table 4.2). All other proteins were identified from only one fully sequenced peptide. However, these peptides were all found in duplicate samples (n=2). All protein identifications were manually verified by

Table 4.2: Summary of *L. salmonis* secreted proteins identified from LC/MS/MS

Sea Lice protein matches	Assoc. Fraction	Parent Ion (m/z)	Mr (Da)	Error (ppm) [a]	Score [b]	Peptide sequence (Start-end) [c]
Sea Lice Trypsin (types 1-4)	1-2	579.80	1157.77	27	46	^{215}FIDWIAEHQ^{223}
	1-1	638.35	1274.69	38	72	^{71}IAVSDITYHEK^{81}
	3-6	920.18	1840.28	13	25	^{115}DQEFIGDVVVSGWGTI SSSGPPSPVLK^{141}
SL-0903	1-1	580.28	1158.48	46	27	Sequence awaiting patent protection
SL-1469	1-1	724.85	1447.66	17	24	Sequence awaiting patent protection
	1-2	879.98	1757.91	29	72	Sequence awaiting patent protection
SL-0547	1-1	604.31	1204.67	19	25	Sequence awaiting patent protection
SL-0858	1-2	1248.71	2495.33	65	35	Sequence awaiting patent protection
MB4c11	1-1	951.51	1900.94	54	37	Sequence awaiting patent protection

[a] Difference (in parts-per-million) between measured mass and mass predicted from the DNA sequence.

[b] Score from MASCOT search, scores above 21 indicate identity or extensive homology ($p<0.05$), for all peptides except MB4c11 (>26).

[c] Cyanogen bromide/tryptic peptide sequence predicted from the DNA sequence.

Figure 4.2: Protein profiles of *Lepeophtheirus salmonis* secretory fractions using SDS-PAGE (12% acrylamide). Molecular masses are along the left hand side of the gel (kDa). (Fr 1) indicates fraction 1-1, (Fr 2) indicates fraction 1-2, (Fr 3) indicates fraction 2-3, and (Fr 7) indicates fraction 3-7.

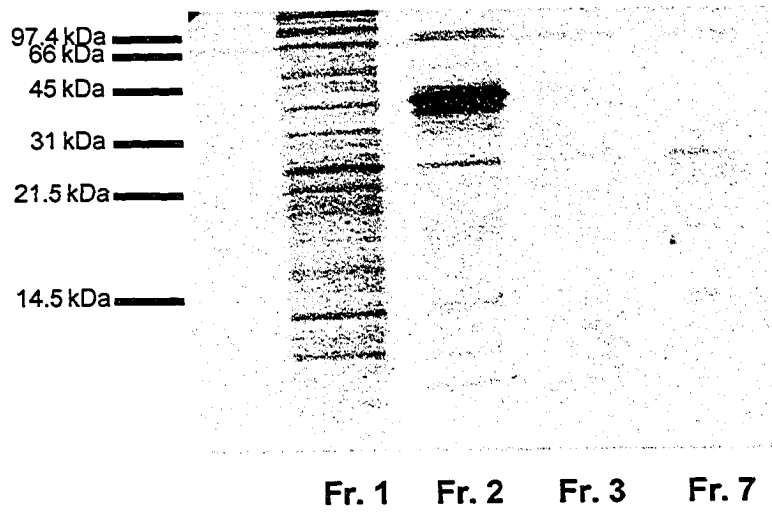
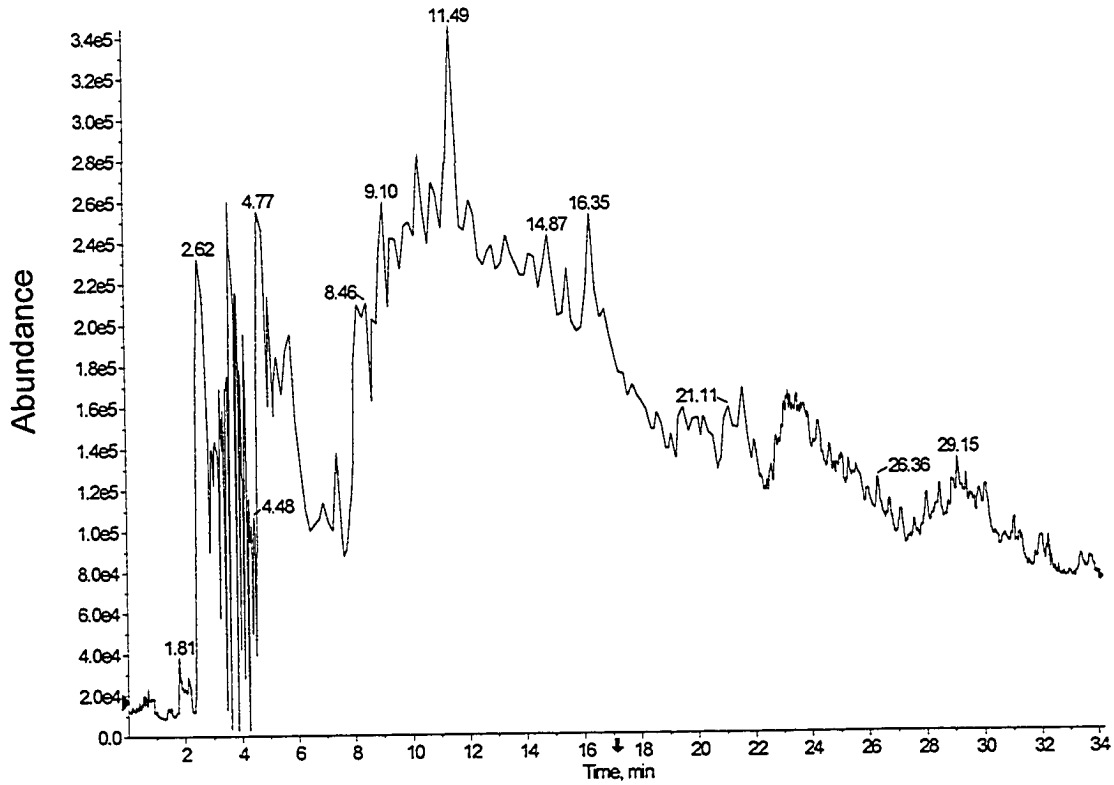


Figure 4.3: The total ion scan of *Lepeophtheirus salmonis* unfractionated SEPs.



comparing the experimentally obtained MS/MS spectrum with the predicted peptide sequence (Figs. 4.4, 4.5; Appendix 1).

4.5.2 Gene expression studies for proteins identified in SEPs

Expression of genes responsible for production of the proteins identified in SEPs was examined by RT-PCR over a range of different *L. salmonis* developmental stages (Figs. 4.6-4.9). The gene SL-0903 was expressed at a relatively low level in the copepodid stage prior to attachment (Fig. 4.6). There was significantly higher expression of this gene in both the early (chalimus I-II) and late (chalimus III-IV) chalimus larvae, when compared to copepodids or pre-adult/adults. The gene, SL-1469, was not expressed in copepodids prior to attachment (Fig. 4.7). There was significantly higher SL-1469 expression in late chalimus and pre-adult/adult stages when compared to early chalimus stages. Expression of SL-0858 was evident in all developmental stages including copepodids prior to attachment (Fig. 4.8). There was significantly higher expression in the late chalimus larvae when compared to the copepodids (Fig. 4.8). Expression of SL-0547 was relatively low in comparison to the other genes studied and there was no evidence of expression of this gene in early chalimus larvae. There was no significant difference in the level of expression of SL-0547 between copepodids collected prior to attachment and pre-adults/adults (Fig. 4.9). Finally, no expression of MB4c11 was observed in any of the developmental stages, using several different primer sets.

Figure 4.4: Time of flight - Mass spectrometry scan of the tryptic peptide (selectedion), FIDWIAEHQ. (a) and TOF-MS 2 of FIDWIAEHQ at 10.49 min (b).

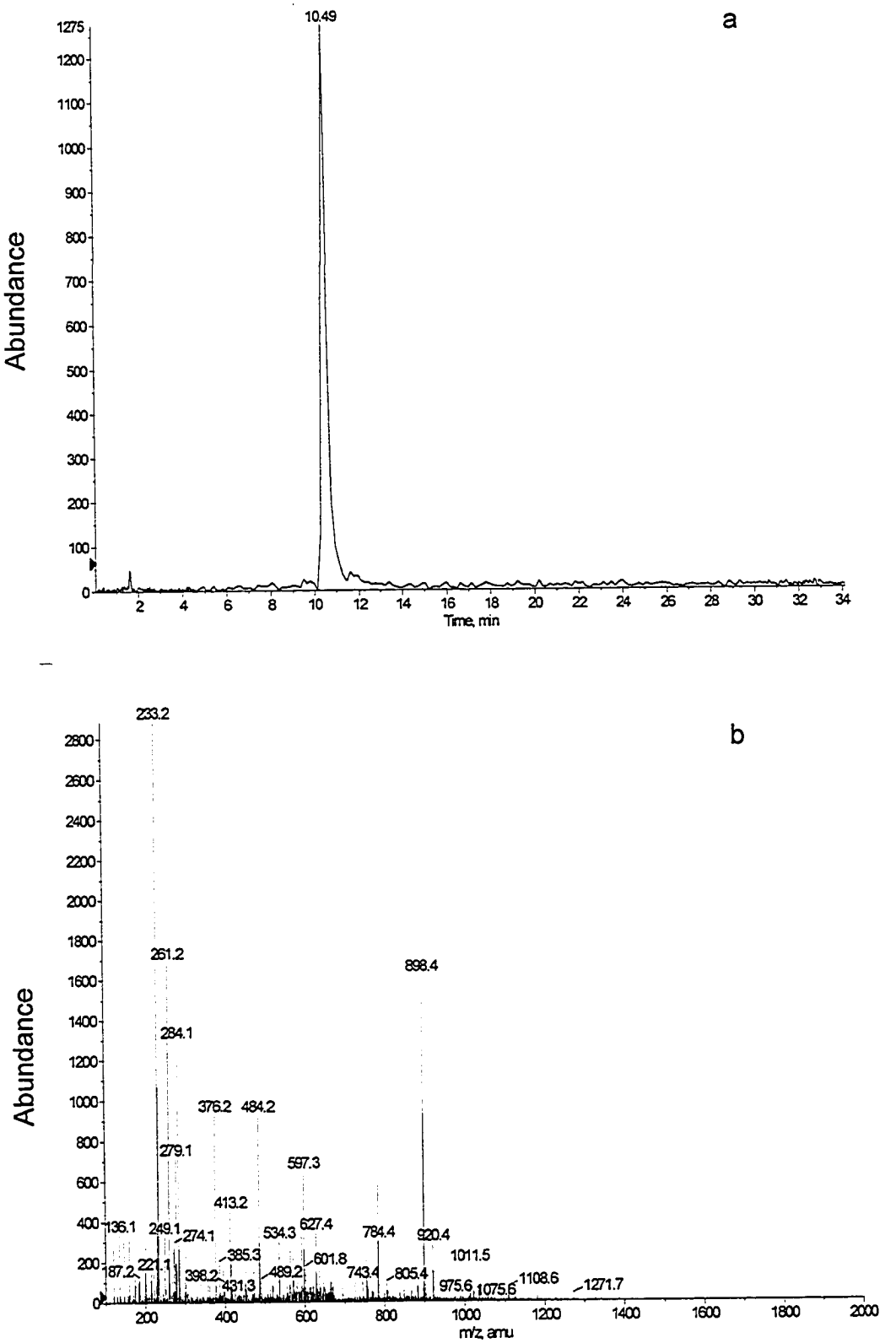


Figure 4.5: Time of flight - Mass spectrometry scan of the SL-1469 peptide (selected ion).

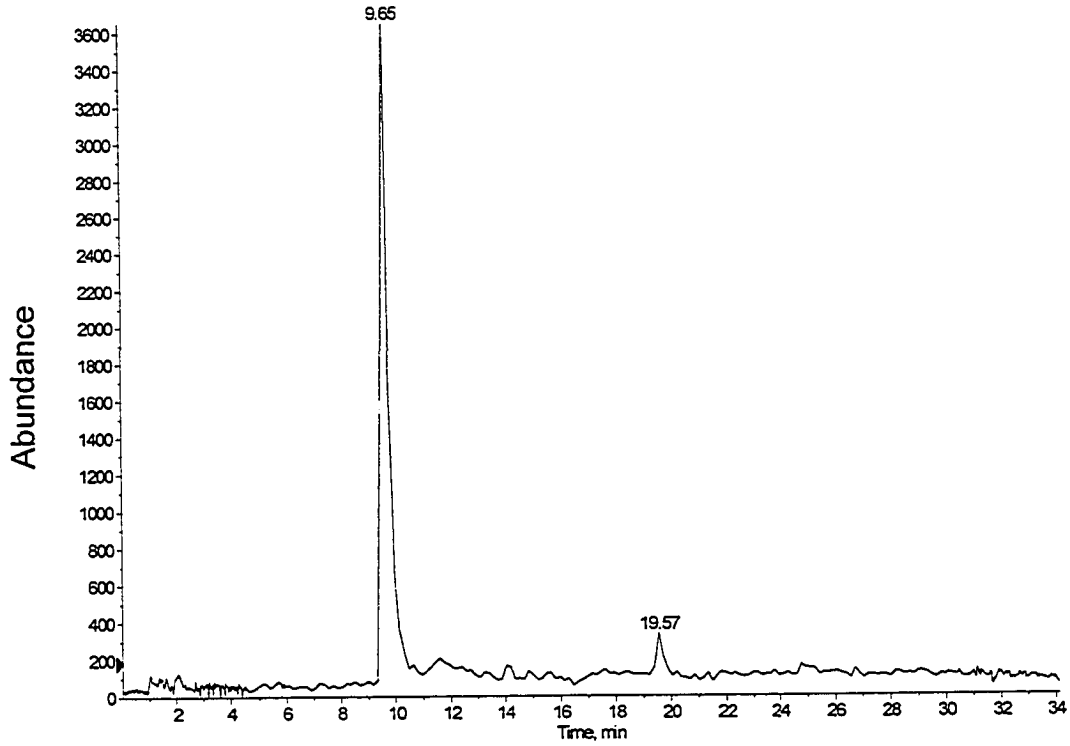
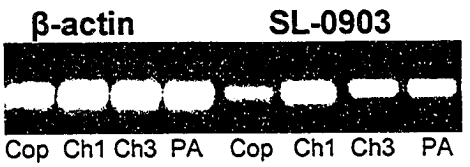


Figure 4.6: Mean (±SEM) RT-PCR expression of the EST, SL-0903, relative to β-actin, over different *Lepeophtheirus salmonis* life stages. Means with different letters are significantly different (n=30 for all stages).



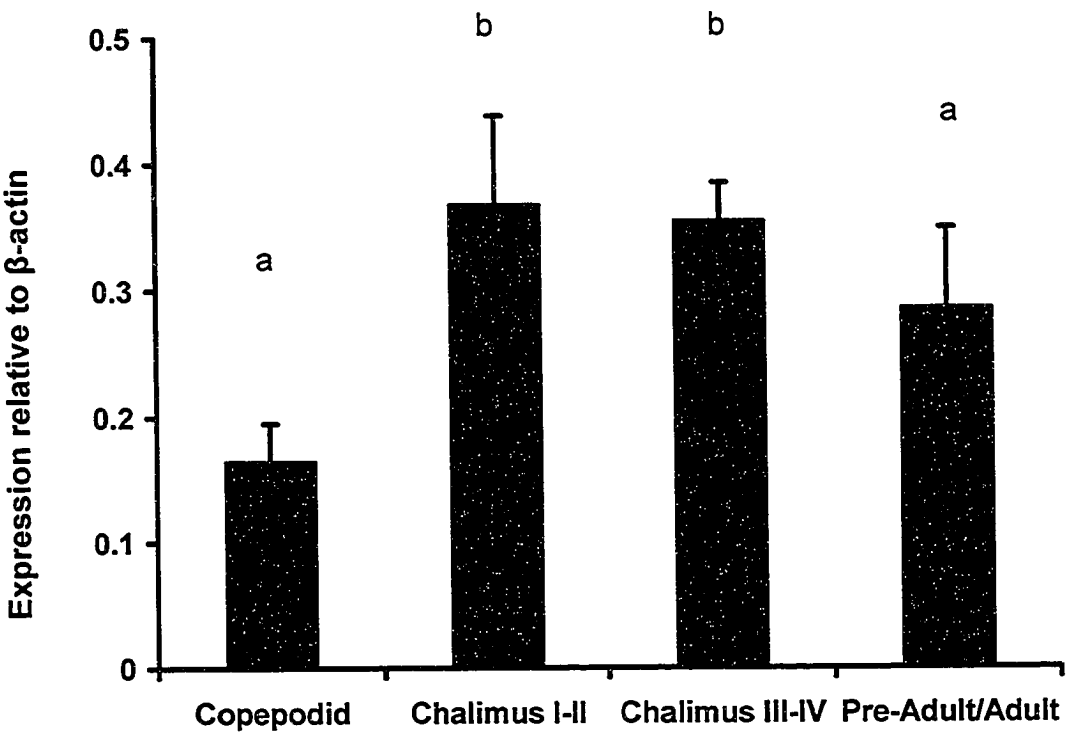


Figure 4.7: Mean (±SEM) RT-PCR expression of the EST, SL-1469, relative to β-actin, over different *Lepeophtheirus salmonis* life stages. Means with different letters are significantly different (n=30 for all stages).

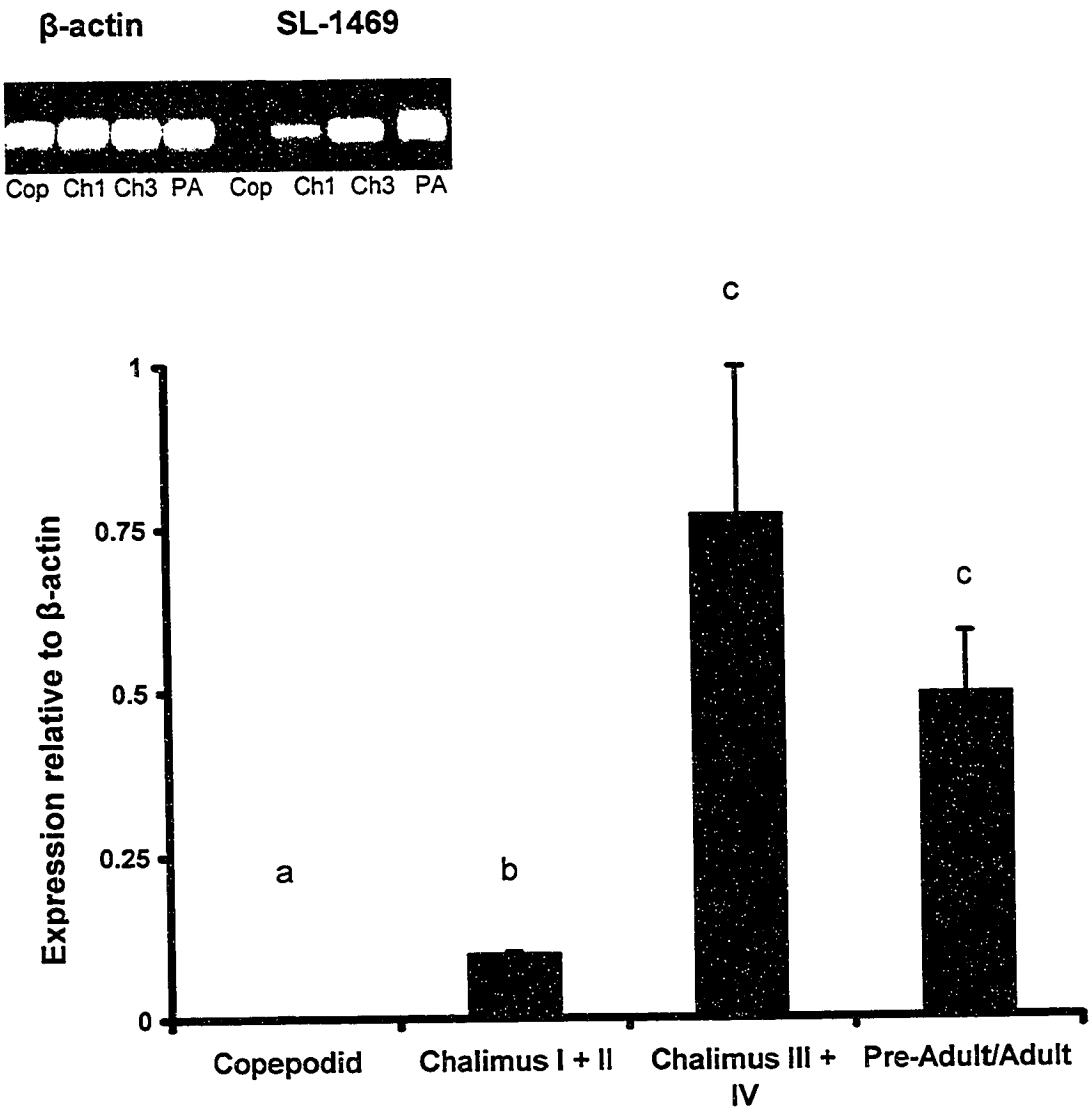
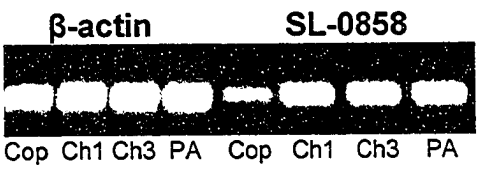


Figure 4.8: Mean (±SEM) RT-PCR expression of the EST, SL-0858, relative to β-actin, over different *Lepeophtheirus salmonis* life stages. Means with different letters are significantly different (n=30 for all stages).



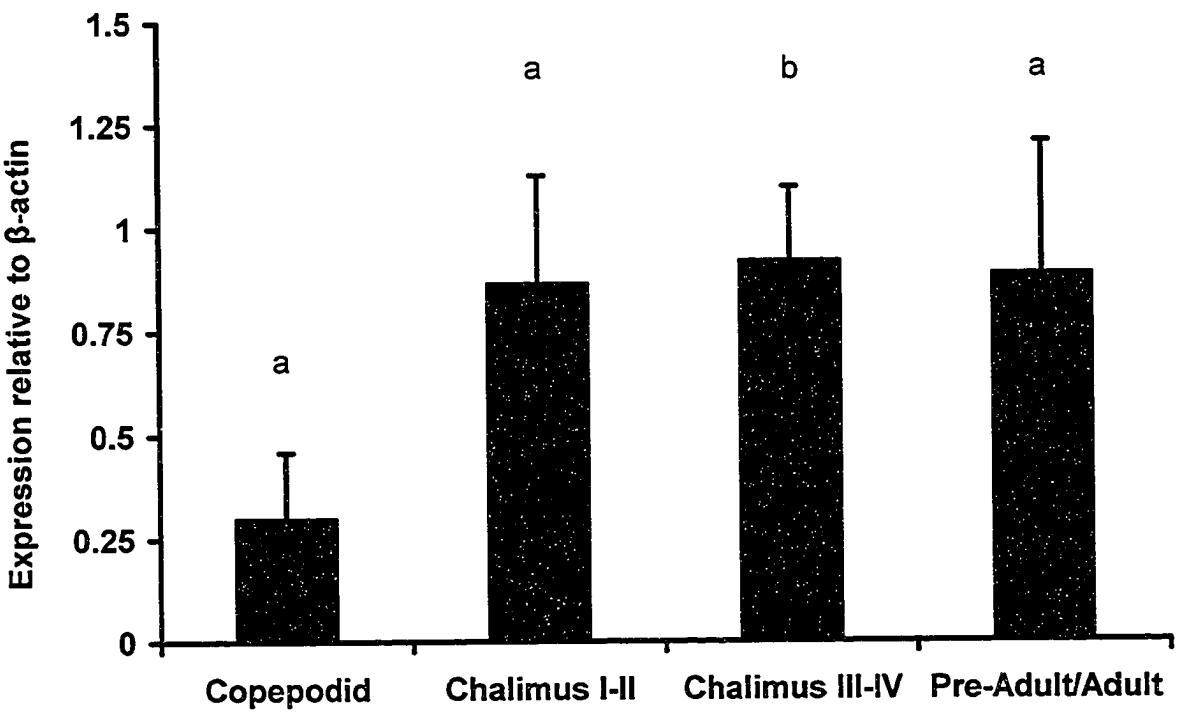
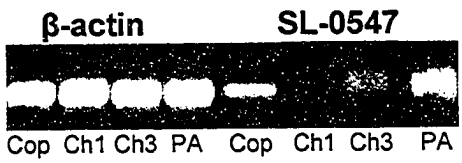
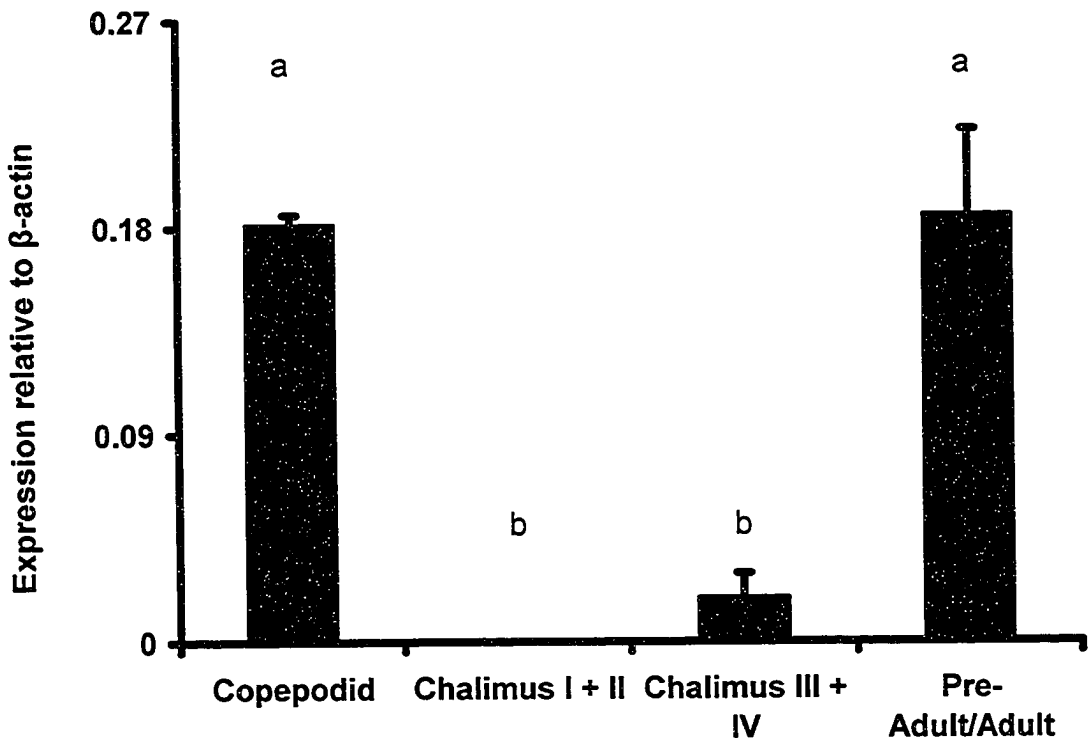


Figure 4.9: Mean (±SEM) RT-PCR expression of the EST, SL-0547, relative to β-actin, over different *Lepeophtheirus salmonis* life stages. Means with different letters are significantly different (n=30 for all stages).





To localize sites of expression of these genes, ISH was attempted using RNA probes constructed from primers designed using EST sequences. For the most part ISH was unsuccessful in obtaining signals for these genes. However, high levels of expression of SL-0547 were observed in a glandular-like structure that ran the length of the cephalothorax in *L. salmonis* (Figure 4.10b). Some non-specific binding to the exoskeleton was also observed, however, no binding of the sense probe was observed to any tissue (Fig. 4.10a). These results were not reproducible. Following the failure to localize the expression of these genes, RT-PCR was carried out for each on different body regions of pre-adult *L. salmonis* (Fig. 4.10c). The gene SL-0547 was expressed in all regions of the body (anterior cephalothorax, posterior cephalothorax and the genital complex/abdomen). The other genes (SL-0903, SL-1469, SL-0858) were also expressed throughout all three body regions (Fig. 4.10c).

4.5.3 Effects of SEPs on immune gene expression in isolated head kidney macrophages

The effects of SEPs on the expression of genes involved in host immunity was studied in Atlantic salmon head kidney macrophages (HKMs) by Real-Time PCR (Figs. 4.11-4.15). With the exception of MH class I, constitutive expression of COX-2, IL-1β, MH class II and TGFβ-like gene in HKMs was relatively low in comparison to the control gene, β –actin (Figs. 4.11-4.15). It was therefore decided to stimulate macrophages with LPS prior to testing of SEPs. As

Figure 4.10: Localization of *Lepeophtheirus salmonis* expression of ESTs throughout the body. The arrow indicates the first thoracic leg, (a) indicates in situ hybridization (ISH) of SL-0547 expression using a sense probe; (b) indicates in situ hybridization (ISH) of SL-0547 expression using an anti-sense probe; (c) indicates expression of ESTs in different body regions. (A) indicates anterior cephalothroax; (P) indicates posterior cephalothorax; (G) indicates abdomen/genital complex; (1) indicates SL-0547; (2) indicates SL-0858; (3) indicates β-actin; (4) indicates SL-0903; (5) indicates SL-1469; (L) indicates 1 Kb plus ladder.

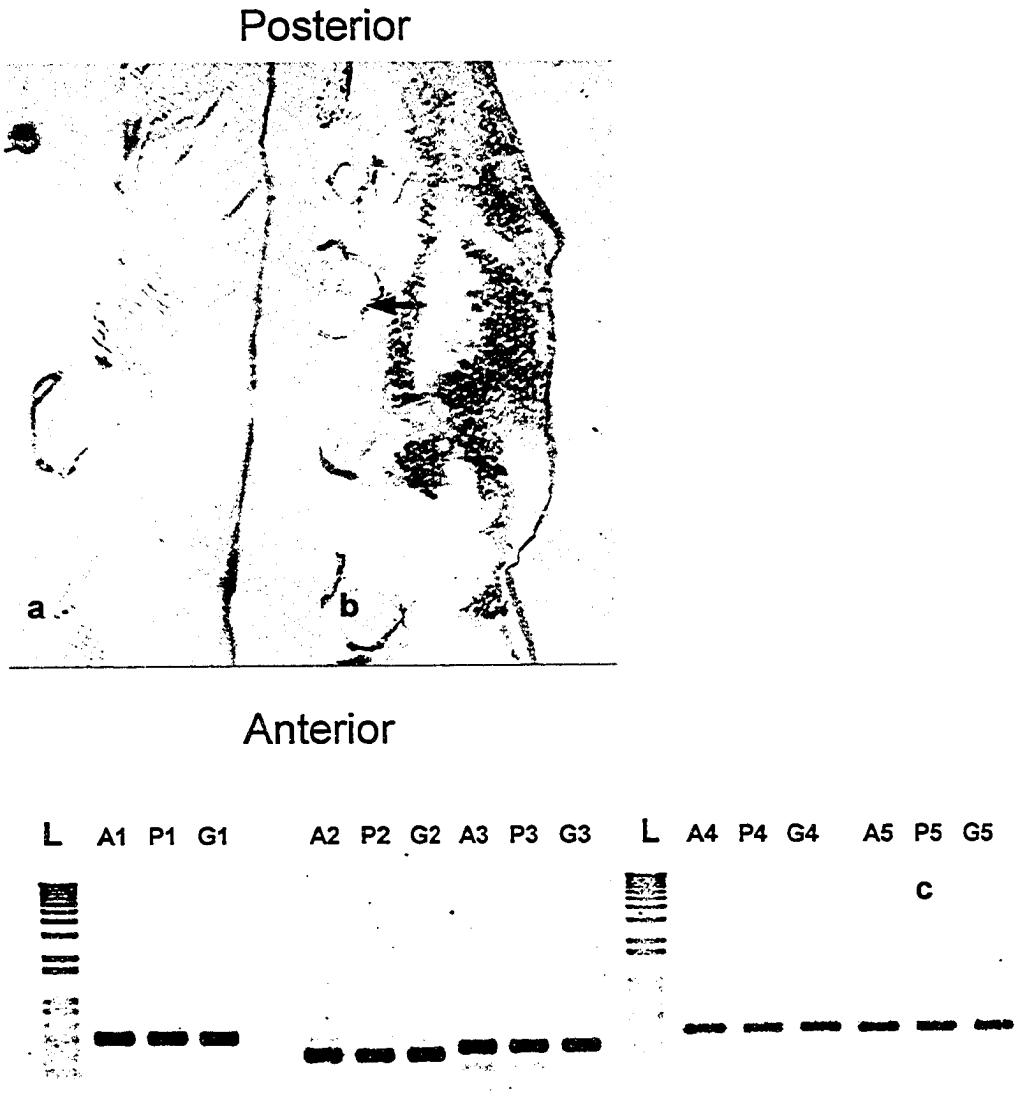


Figure 4.11: Mean (±SEM) Real-Time PCR expression of cyclooxygenase-2 gene, relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). * indicates significant differences from control; † indicates significant differences from LPS; ‡ indicates significant differences from LPS + DA (n=10).

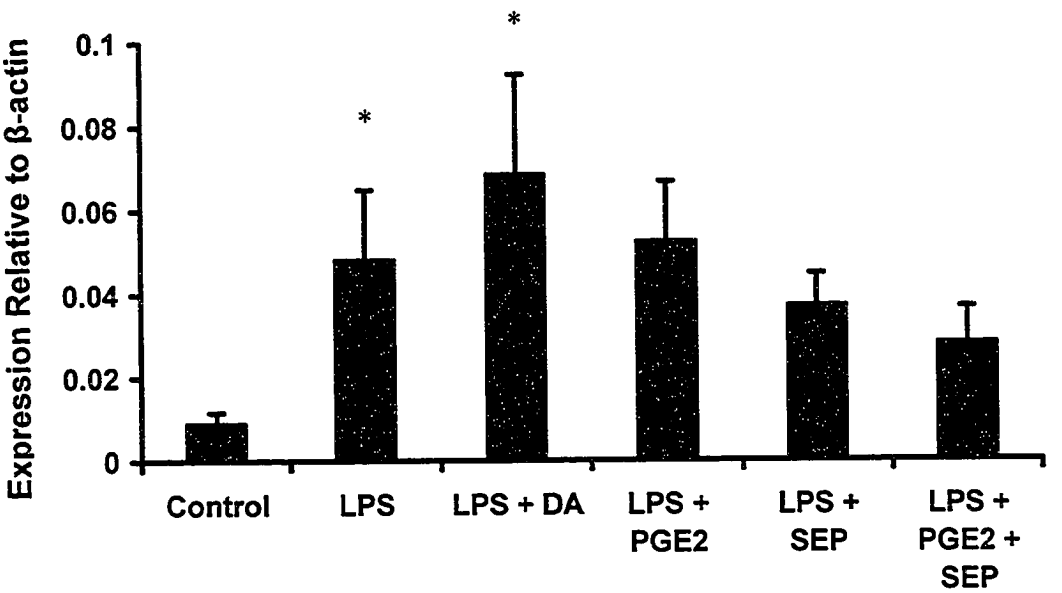


Figure 4.12: Mean (±SEM) Real-Time PCR expression of interleukin-1β gene, relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). * indicates significant differences from control; † indicates significant differences from LPS; ‡ indicates significant differences from LPS + DA (n=10).

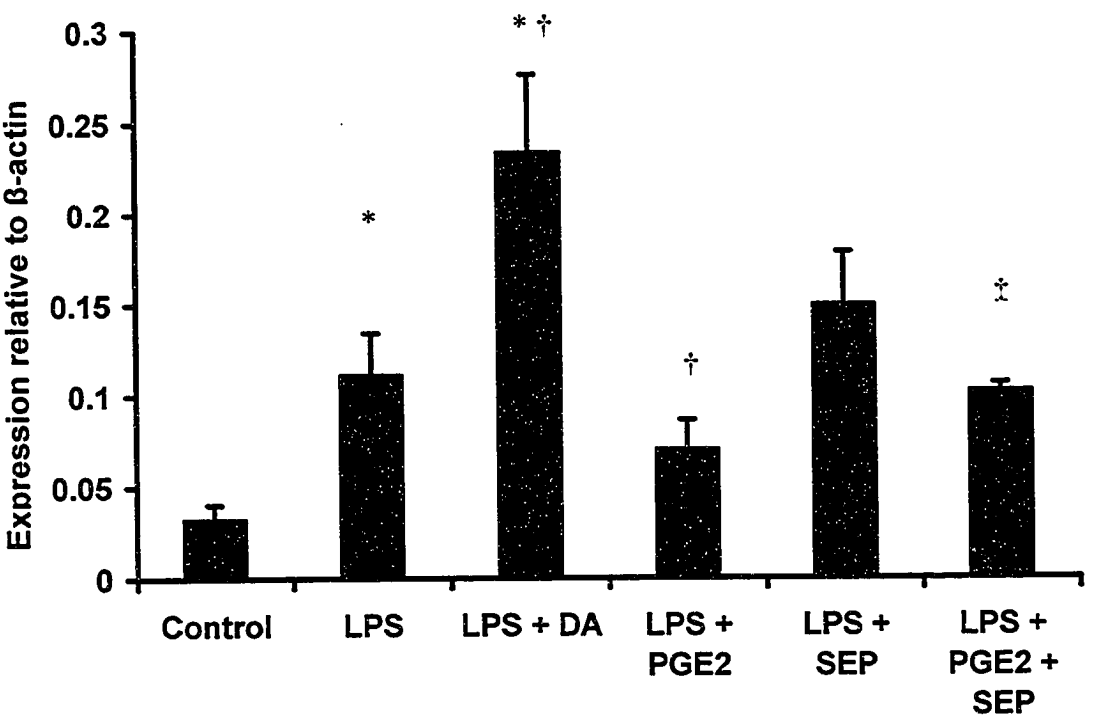


Figure 4.13: Mean (±SEM) Real-Time PCR expression of major histocompatibility class I gene, relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). * indicates significant differences from control; † indicates significant differences from LPS; ‡ indicates significant differences from LPS + DA (n=10).

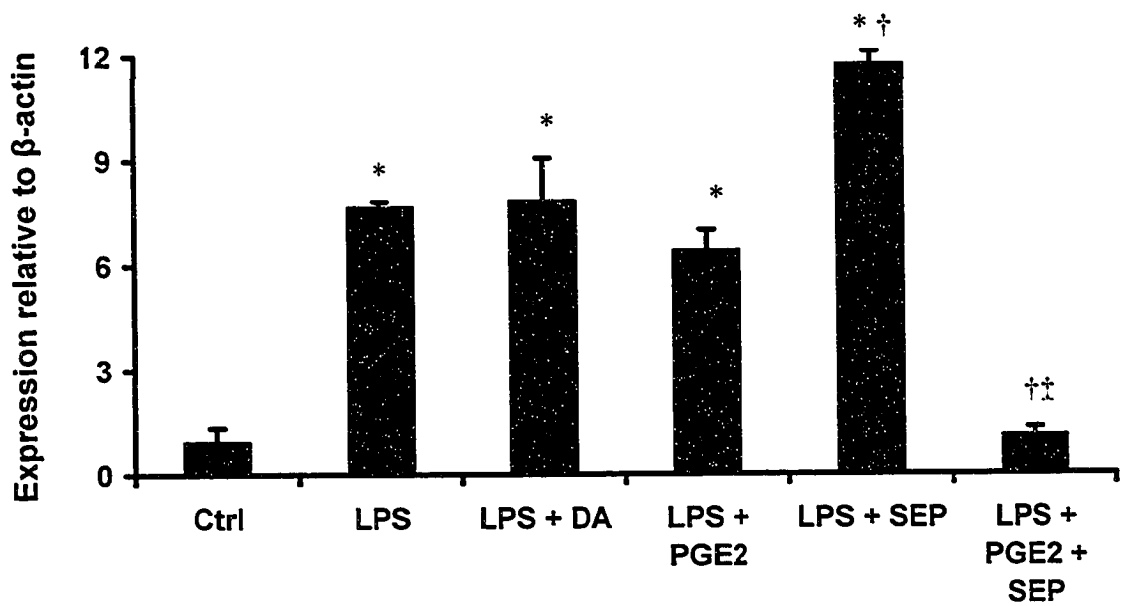


Figure 4.14: Mean (±SEM) Real-Time PCR expression of major histocompatibility class II gene, relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). * indicates significant differences from control; † indicates significant differences from LPS; ‡ indicates significant differences from LPS + DA (n=10).

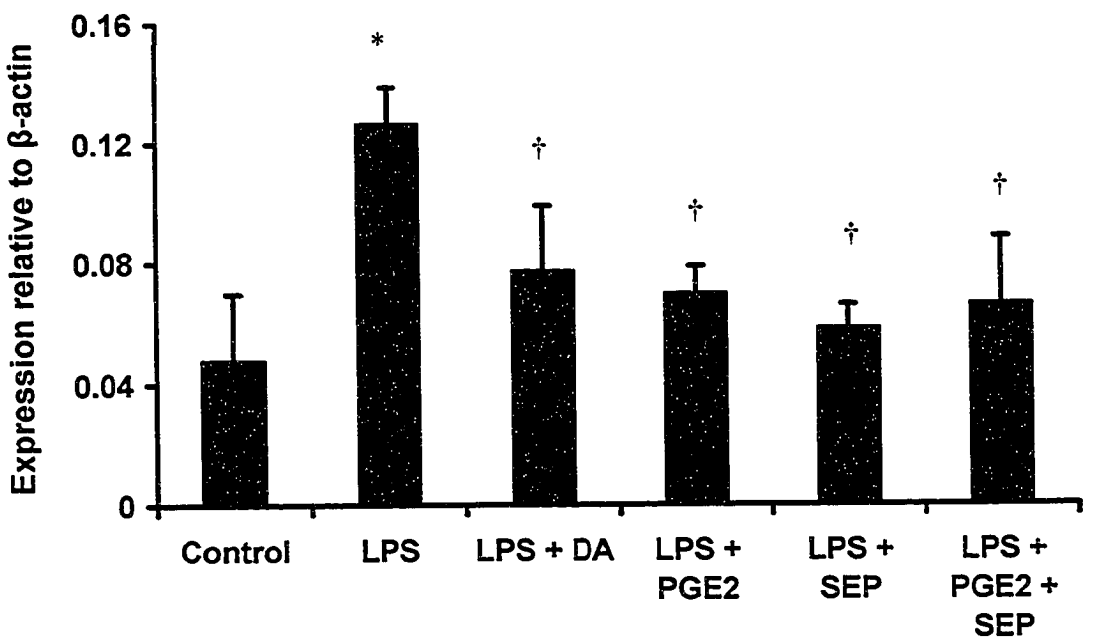
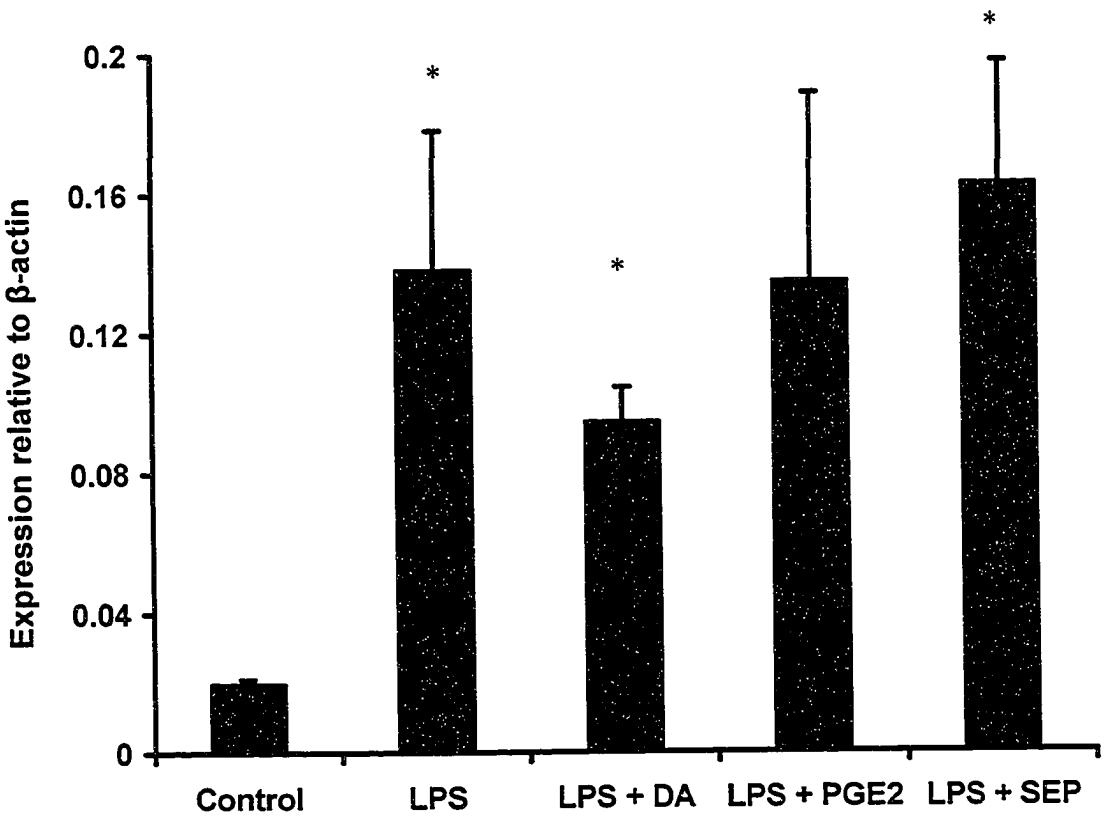


Figure 4.15: Mean (±SEM) Real-Time PCR expression of transforming growth factor β gene, relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). * indicates significant differences from control; † indicates significant differences from LPS; ‡ indicates significant differences from LPS + DA (n=10).



residual amounts of DA were present in SEPs (Fig. 4.1c), a SW + DA control incubation was added to determine whether its presence in SEPs might affect salmon immune gene regulation.

The expression of COX-2 was significantly up-regulated by the addition of LPS, as well as LPS and DA (Fig. 4.11). While incubation of HKMs with PGE_2, SEPs, alone or in combination reduced LPS-induced stimulation of COX-2, this reduction was not significant (Fig. 4.11). Similar to COX-2, IL-1β expression was induced in HKMs following LPS and LPS + DA incubations (Fig. 4.12). However, unlike COX-2, addition of PGE_2 or PGE_2 with SEPs significantly reduced LPS and LPS+DA-induced stimulation of IL-1β, respectively (Fig. 4.12). Following incubation of LPS-stimulated HKMs with SEPs, decreased expression was observed in IL-1β, however this was not significant.

Lipopolysaccharide stimulation also significantly increased MH class I gene expression when compared to the non-stimulated control (Fig. 4.13). Addition of DA or PGE_2, to LPS, resulted in no significant change in expression when compared to HKMs stimulated only with LPS. Incubation of HKMs with both LPS and SEPs appeared to have a synergistic effect, further increasing the expression of MH class I gene. However, incubation with LPS, PGE_2 and SEPs resulted in a significant reduction in MH class I gene expression to a level similar to that seen in unstimulated controls (Fig. 4.13).

The expression of MH class II and TGFβ-like gene were significantly increased, following incubation with LPS, in comparison to controls (Figs. 4.14, 4.15). Incubation with LPS and DA resulted in significantly lower MH class II

gene expression and a slight but not significant decrease in TGFβ-like gene expression. The further addition of PGE_2, SEPs or the combination of both reduced LPS-induced expression of MH class II gene expression but had no effect on the expression of TGFβ-like gene (Figs. 4.14, 4.15).

4.5.4 Effects of SEPs on immune gene expression in SHK-1 cells

To avoid some of the inherent variability that was experienced during use of primary isolates of head kidney macrophages, it was decided to use the Atlantic salmon head kidney cell line (SHK-1) to observe the effects of SEP fractions on the expression of IL-1β. Due to the difficulties in obtaining sufficient SEP fractions, the study was limited to a single gene. As seen in HKMs, LPS induced a significant increase in the expression of IL-1β following 4 h incubation (Fig. 4.16). Following incubation with LPS and pooled SEP fraction 1, there was a significant inhibition of the LPS-stimulated IL-1β expression, yet the expression was still significantly higher than that found in unstimulated control SHK-1 cells (Fig. 4.16). Pooled SEP fractions 2 and 3 not only abrogated LPS-stimulated IL-1β expression, but reduced levels below that of the unstimulated control SHK-1 cells.

To discern whether both Fraction 1-1 and 1-2 contributed to the inhibition observed from pooled fraction 1 as seen above, they were individually incubated with LPS-stimulated SHK-1 cells (Fig. 4.17). While LPS-stimulation induced IL-1β expression at a much lower level than in the pooled trial, the relative change

Figure 4.16: Mean (±SEM) Real-Time PCR expression of interleukin-1β gene, relative to β-actin, in SHK-1 cells incubated with and without (control) lipopolysaccharide (LPS), pooled *Lepeophtheirus salmonis* secretory/excretory product (SEP) fraction 1 (PF1), pooled *Lepeophtheirus salmonis* SEP fraction 2 (PF2), and pooled *Lepeophtheirus salmonis* SEP fraction 3 (PF3). * indicates significant differences from control; † indicates significant differences from LPS.

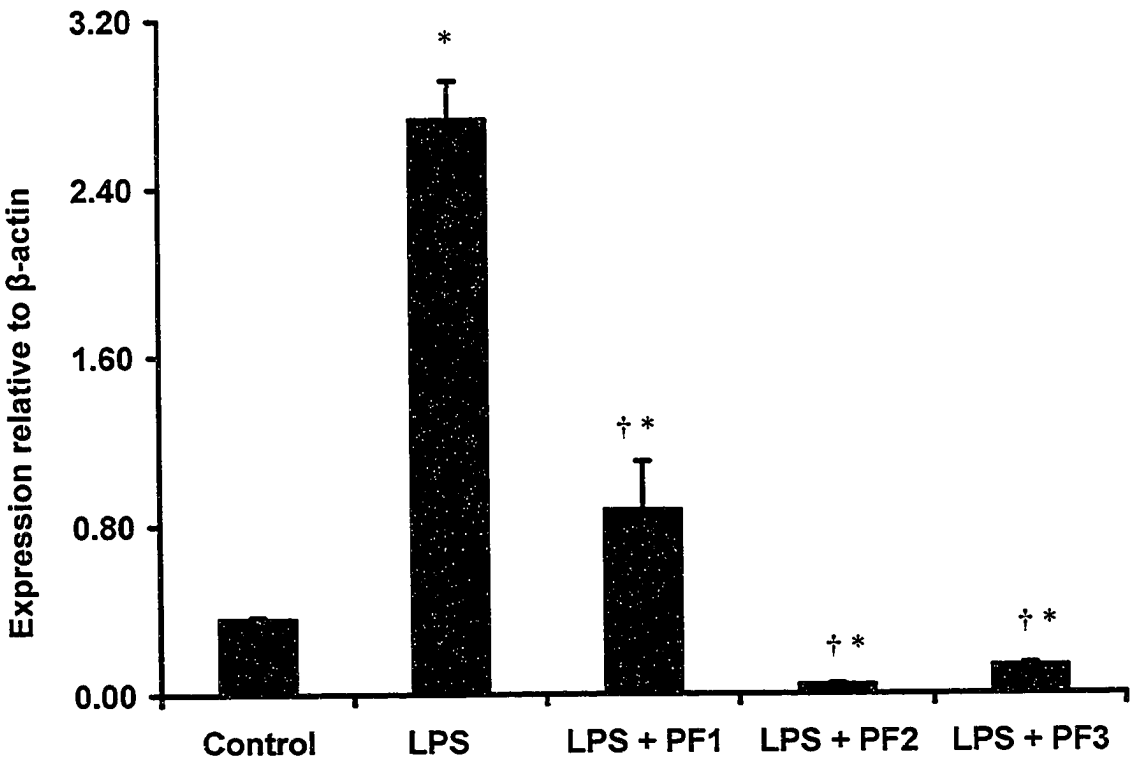
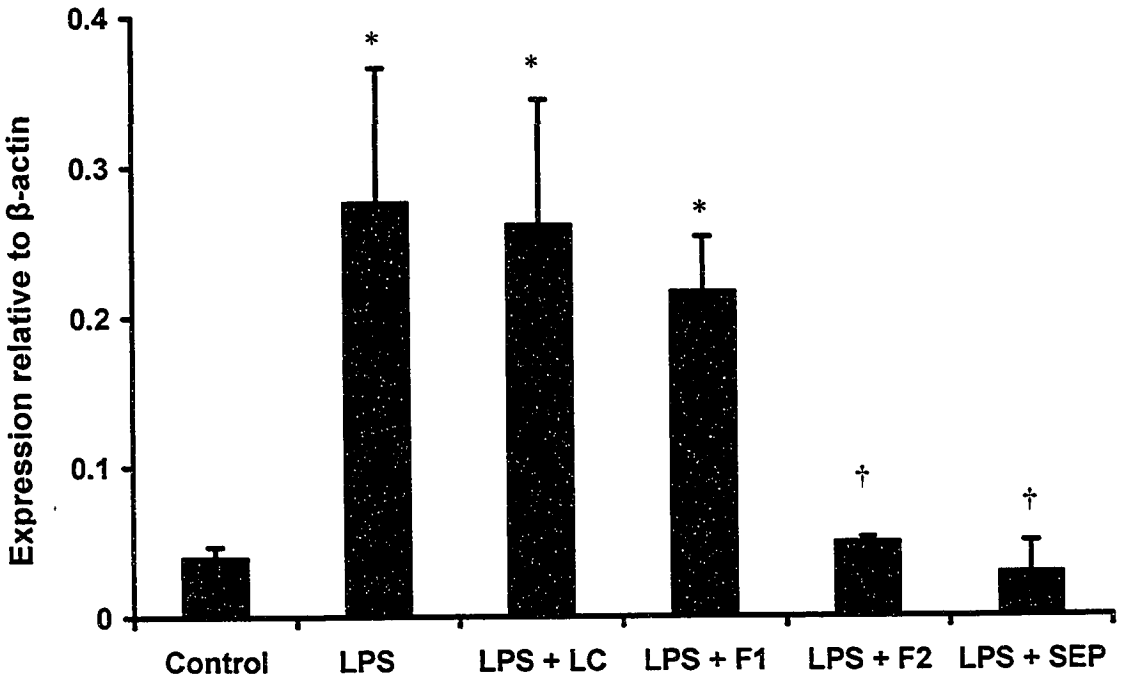


Figure 4.17: Mean (±SEM) Real-Time PCR expression of interleukin-1β gene, relative to β-actin, in SHK-1 cells incubated with and without (control) lipopolysaccharide (LPS), LPS and lyophilized liquid chromatography solvent (LC), *Lepeophtheirus salmonis* secretory/excretory product (SEP) fraction 1-1 (F1), *Lepeophtheirus salmonis* SEP fraction 1-2 (F2), and *Lepeophtheirus salmonis* unfractionated SEP. * indicates significant differences from control; † indicates significant differences from LPS.



(7-fold), with respect to the unstimulated controls was the same (Fig. 4.17). As the possibility remained that residual solvent from the separation procedure adversely affected the SHK-1 cells, a diluted LC solvent control was incubated with LPS-stimulated SHK-1 cells. This LC solvent control showed no significant effect on IL-1β expression when compared to LPS-stimulated cells (Fig. 4.17). Incubation of SHK-1 cells with LPS and fraction 1-1 resulted in no significant difference from LPS-stimulated cells (Fig. 4.17). Incubation with LPS and fraction 1-2, however, significantly reduced LPS-stimulation of IL-1β gene expression to a level similar to that seen in unstimulated cells or those incubated with LPS and the total, unfractionated, SEPs (Fig. 4.17).

4.6 Discussion

In the interests of obtaining a better understanding of the relationship between *L. salmonis* and Atlantic salmon, secretory/excretory products from *L. salmonis* were isolated, partially identified and the biological activity studied. The two collection methods used to produce these products, produced different size exclusion chromatography profiles suggesting that there may be some contamination of secretory products by excretory products in SEPs or due to proteolysis. In Chapter 2.0, it was reported that differences existed between the SP and SEP fractions (Fast et al., 2004). The amount and constituents of the excretory products are unknown.

In arthropod parasites, which take blood meals, the timing of collection of salivary products can greatly affect secretion contents (Lawrie and Nuttal, 2001;

Ramchandra and Wikel, 1992). In this study the time of collection was unlikely to have affected the results. This is because *L. salmonis* spend most of their time feeding on host mucus, tissue, and blood, and all of the collections of SEP and SP were pools from numerous individuals at different stages in their development and moult cycles (Pike and Wadsworth, 1999).

Throughout the study, primarily whole or fractionated SEPs were used. This was due to the fact that SEPs were easier to collect under field conditions. It was reasoned that hosts are exposed to both secreted and excreted compounds at the site of attachment and feeding. Future studies should attempt to resolve differences in the makeup of SP and SEPs.

At relatively high protein concentrations I was unable to obtain peptide information following trypsin digestion of separated proteins and MS analysis. This lead to the hypothesis, that due to the prevalence of trypsin-like enzymes in *L. salmonis* SEPs (Fast et al., 2003), any proteins having biological activity in the SEPs would probably be resistant to tryptic cleavage and would therefore require other means of digestion. Tightly folded proteins, often stabilized by disulphide bonds, can be extremely resistant to proteolytic cleavage and give very few if any small peptides (Slysz and Schriemer, 2003). To unravel these tightly bound proteins, thereby allowing tryptic digestion, it was decided that rather than increasing the ratio of protease:protein and possibly increase protease autolysis products, that cyanogens bromide would be used, as it cleaves at methionine residues (Crimmins et al., 2000). Using this 2-step digestion, I was successful in identifying 9 peptide fragments that had significant homology to sequences in *L.*

salmonis EST databases. In total, 6 ESTs had significant homology to proteins within the SEP.

Of the peptides showing significant homology to ESTs, only one was observed to show homology to a known protein, *L. salmonis* trypsin (Johnson et al., 2002; Kvamme et al., 2004a, b). Trypsin-like serine proteases are dominant digestive proteases found in several insect species (Terra and Ferreira, 1994) and they cleave sheep IgG heavy chain (Tabouret et al., 2003). Trypsin release may lead to an incapacitation of the IgG dependent cytotoxic activity of eosinophils in host sheep (Tabouret et al., 2003). Previously, *L. salmonis* trypsin-like proteases were identified in the mucus of infected hosts (Firth et al., 2000; Fast et al., 2003). *Lepeophtheirus salmonis* produces several different trypsins throughout several cell types lining the midgut (Johnson et al., 2002; Kvamme et al., 2004b). The current study provides the first direct measurement of trypsin within SEPs as evidenced by the presence of multiple trypsin fragments in MS analysis of fractions 1-1,1-2 and 3-6. There have been 5 trypsin-like enzymes identified in *L. salmonis* (Johnson et al., 2002; Kvamme et al., 2004b). It is unknown whether these different enzymes play different roles in *L. salmonis*, however, they all appear to be up-regulated from planktonic copepodid to adult developmental stages (Kvamme et al., 2004b). The data obtained herein do not allow assignment of SEP trypsin fragments to individual genes.

Early developmental stages of *L. salmonis* including the copepodid stage, prior to attachment to the host, are non-feeding and the digestive tract is largely

inactive (Bron et al., 1993b). Furthermore, there is little evidence for the tissues associated with the frontal filament (attachment structure) and a significant number of peroxidase secreting glands, observed in later stages, are absent at these stages (Bell et al., 2000; Gonzalez-Alanis et al., 2001). Although trypsin expression was not studied here, as mentioned above, several *L. salmonis* trypsins up-regulate from copepodid to pre-adult and adult stages (Kvamme et al., 2004a, b). These data taken together suggest that relatively little is going on with respect to the parasite prior to feeding on the host. For this reason it is not surprising that 3 out of the 4 ESTs with significant similarities to *L. salmonis* SEPs, in this study, were significantly up-regulated in actively feeding stages. It may be inferred, therefore, that SL-0903, SL-0858 and SL-1469 are important for *L.salmonis* in feeding and/or maintaining itself on the host. These could be useful as future vaccine targets. The gene SL-0547 was highly expressed in the copepodid stage prior to host attachment and in the preadult and adult stages. The product of this gene may be involved in mobility and initial establishment of the parasite on host surfaces. Primers developed for MB4c11 failed to amplify and detect expression in any of the developmental stages or in the plasmid containing the EST insert. This may have been caused by the presence of poor quality sequence for this EST (only 1x coverage) and/or problems with primer binding.

It was demonstrated in Chapter 1.0, that there are pores within the oral cone of *L. salmonis*. The pores are located on the inner surface of the labrum and are linked to the ventral medial gland, which stains for peroxidases (Bell et al., 2000;

Chapter 1.0). It was attempted in this investigation to identify whether these glands, or other organs of *L. salmonis*, were the site of gene expression for the 4 proteins from the SEP. With exception of SL-0547, ISH procedures were unable to localize sites of gene expression associated with the proteins. With respect to SL-0547, limited expression data indicated that, unlike trypsin, which is expressed in all cell types of the midgut, the gene is expressed in tissues that lie adjacent to the alimentary tract (Johnson et al., 2002).

As the ISH data were inconclusive, RT-PCR was chosen to localize sites of gene expression, associated with SEPs, to gross body regions. In this study the body was divided into three regions: the anterior cephalothorax (anterior to first leg), posterior cephalothorax and the genital-abdominal segments. The anterior cephalothorax contained the ventral medial gland, further discussed above. As these four genes were expressed in all-three body regions studied, confirmation of SEP-associated gene expression in the ventral median gland was not obtained. This does not rule out the possibility of the ventral medial gland being important in the production of other SEPs, not identified in this study. Furthermore, it is possible that the 3 genes, for which no specific localization information was obtained, may be associated with the gut similar to trypsin. Their presence in SEPs may be a result of regurgitation from the parasite due to dopamine stimulation. Currently all 4 ESTs are being fully sequenced, in the hopes of further elucidating their role in the host-parasite relationship. Identification and localization of the ESTs associated with *L. salmonis* SEPs provides an exciting area for future research.

The range of SEP concentrations used in the cell-based assays in this study were established from Bergman et al. (1998), where 20-70% suppression of ConA stimulated T-cell proliferation was observed using 0.5 µg of *Dermacentor andersoni* salivary gland extract and up to 90% inhibition using 4 µg. In this study, incubation with 0.66 µg of SEPs did not show significant inhibition of IL-1β in LPS-stimulated HKMs, but nearly 100% inhibition of LPS-stimulated expression of IL-1β in SHK-1 cells.

Pooled and unpooled fractions of SEPs were tested at higher concentrations. The pooled and single fractions significantly reduced the expression of IL-1β in LPS-stimulated SHK cells often to levels below that seen in unstimulated cells. Salivary gland extract from *D. andersoni* reduces LPS-stimulated production of IL-1β and TNF α by splenic macrophages and peripheral-blood-derived macrophages (Ramachandra and Wikel, 1992; 1995).

The variability between SHK-1 and HKMs may result from inherent differences in the cell types that are present or from slight differences in culture conditions. The SHK-1 cell line is macrophage-like, and similar to a melanomacrophage precursor but over lengthy passaging this cell line may not act identically to freshly isolated HKMs (Chapter 3.0, published in Fast et al. *in press*). Different lots of FBS were another source of variability, since they may contain different ratios of bovine proteins, growth factors and glucocorticoids, all of which have differing effects on cytokine expression in fish cells.

In mammalian systems, while DA effects on T-cells are inhibitory towards cytokine secretions, in macrophages, DA effects are largely stimulatory (Basu

and Dasgupta, 2000; Carr et al., 2003; Ilani et al., 2004). In this study, DA for the most part appeared to have either no effect or acted synergistically with LPS, to increase the expression of HKM genes. Using similar concentrations to this work, Ferreira and Silva (1998), observed no effect of DA incubation on Con-A-induced proliferation of mice splenocytes. Head kidney macrophage LPS-induced expression of the MH class II gene, however, appeared to be inhibited by DA. In this latter case, SEP inhibition of HKM LPS-induced expression of MH class II could not be discerned from the effects of DA.

Due to the potential of residual DA in SEPs, the SEP+LPS incubations were compared with LPS+DA incubations. However, there was still no significant effect of SEPs alone on any of the HKM genes studied. This may be explained by DA immune-stimulation overcoming any possible inhibitory effect of the SEPs. Similar to the results in Chapter 3.0, PGE_2 was inhibitory towards LPS-induced stimulation of IL-1β, MH class II, and in concert with SEPs, inhibitory towards MH class I gene expression. It has been suggested that PGE_2 levels may be elevated in *L. salmonis* infected fish (Pike and Wadsworth, 1999). In BALB/c mice the production of PGE_2 is increased by the presence of a 7-kDa protein (maxadilan) that is found in sandfly (*Lutzomyia longipalpis*) saliva (Bozza et al., 1998 and Soares et al., 1998). Whether any of the proteins found in the SEPs of *L. salmonis* act in the same fashion is unknown, however, if present, such proteins would help to explain the synergistic effects observed between SEPs and PGE_2.

There are mixed reports on whether PGE_2 is the main contributing factor to the immunosuppressive capabilities of other arthropod ectoparasite secretions (Inokuma et al., 1994; Urioste et al., 1994). While PGE_2 concentration was not determined in SEP fractions, it was expected that they would be extremely low to non-existent for several reasons. *Lepeophtheirus salmonis* produce very small amounts of this fatty acid under the incubation conditions used here (Chapter 2.0), and the use of 3,000 NMW filters to concentrate SEPs would remove most if not all of the PGE_2 because it is a small molecule. In this chapter its has been shown that, while PGE_2 can act in an immunosuppressive nature on its own and synergistically with SEPs, the SEP fractions not containing PGE_2 were also able to show 100% inhibition of LPS-induced stimulation of the inflammatory gene IL-1β. It appears then, that *L. salmonis* secretions have immunomodulatory capabilities other than that attributed to PGE_2, again similar to that found for the Rocky Mountain wood tick, *D. andersoni* (Bergman et al., 1995; 1998). The similarities do not end there. Further analysis of fraction 1-2 with SDS-PAGE, showed the existence of two main protein bands at ca. 40 kDa. If this protein is the major contributing compound to immunosuppression it may also be similar to the 36 kDa immunosuppressant protein from the salivary gland of *D. andersoni* (Bergman et al., 1995; 1998; 2000). Further separation and analysis is needed to test this hypothesis.

Both pro- and anti-inflammatory compounds have been isolated from ectoparasitic arthropod secretions (Jaworski et al., 2001; Owhashi et al., 2001). In this study, fraction 1-1 may not only have little to no anti-inflammatory activity

but it may have some pro-inflammatory capabilities. This is evidenced by the observation that when fraction 1-1 and 1-2 were combined into pooled fraction 1, inhibition of LPS-induced IL-1β expression was incomplete unlike fraction 1-2 on its own. It is difficult to say whether this was due to pro-inflammatory action of fraction 1-1 or possibly due to the very high expression of IL-1β, in the pooled experiment, reducing the ability of fraction 1-2 to completely abrogate its stimulation.

This brings us to the interesting point of what biological role these compounds serve in the interactions between the parasite and its host. As already mentioned, the lack of inflammation appears to be correlated with host susceptibility to *L. salmonis* infection. Chalimus stages of the parasite are fixed to one location on the host for several days and yet no tissue response is mounted in susceptible species (Johnson and Albright, 1992a; Jones et al., 1990). Furthermore, histology of host tissue at the site of attachment and feeding of adults shows inflammation to occur only in the periphery of the lesion and not in tissues beneath the cephalothorax (Jonsdottir et al. 1992). This is strong evidence that host immunomodulation is occurring at the site of feeding, and the results of this chapter support this hypothesis.

With respect to relatively resistant host species, although not addressed in this investigation, strong inflammatory responses to *L. salmonis* are not likely to be due to fundamental differences in the immune response of coho salmon. Fast et al. (2003) report that *L. salmonis* does not regularly release SEPs in the presence of coho salmon mucus as evidenced by the lack of trypsin activity.

This lack of SEP release may simply allow the process of inflammation in coho salmon to proceed as expected, which is not the case for susceptible hosts (i.e. Atlantic salmon).

From the parasites vantage point the release of PGE_2, through its vasodilatory action, among possible pro-inflammatory agents should increase blood flow to the feeding site providing a food source for the parasite. However, a negative effect of this would be the increased exposure to antigen presenting cells (APC), antibodies, harmful reactive oxygen species and clotting agents. At the same time, the release of anti-inflammatory compounds can alleviate these negative effects. Prostaglandin E_2, while it may increase blood flow can reduce leucocyte recruitment, and APC capabilities though its inhibition of MH class I and II gene expression. Those compounds found in fraction 1-2 (i.e. 40 kDa protein, trypsin, *SL-1469* and *SL-0858*), also exhibit anti-leucocyte recruitment capabilities through their inhibition of IL-1β. Lawrie and Nuttal (2001) give an excellent example of how parasite secretions change over time, and it is most likely a result of the parasites' evolving a complex milieu of components within their secretions that provide favourable conditions for feeding and survival. These evolutionary traits extend beyond simply terrestrial ectoparasitic arthropods, probably to ectoparasitic copepods, as described here, and other ecto- and endoparasitic organisms. These immunomodulatory secretory/excretory components provide *L. salmonis* with the means to evade and modulate the host's immune response. Their continual release may be the

difference between successful and unsuccessful infections, as seen in relatively susceptible and resistant host species.

Chapter 5.0: Differential gene expression in Atlantic salmon, *Salmo salar*, infected with *Lepeophtheirus salmonis* (Copepoda: Caligidae).

5.1 Abstract

Lepeophtheirus salmonis is an ectoparasitic copepod that causes serious disease outbreaks in salmonids. Atlantic salmon (*Salmo salar*) show very little if any tissue response to infection, whereas less susceptible host species exhibit strong inflammatory responses. As arthropod parasites modulate host immune responses, it was examined whether a low-level *L. salmonis* infection in the absence of a stress response would have an effect on expression of Atlantic salmon immune-related genes. The effects of low-level (ca. 8-11 lice/fish) infection on kidney tissue and isolated head kidney macrophage immune-related gene expression was studied at two time points post-infection. At this level of infection there was no evidence of a cortisol stress response. Using Real Time PCR, constitutive expression of the major histocompatability class (MH) I gene was shown to be depressed 2-10 fold in infected Atlantic salmon by 21 days post infection (dpi) when compared to uninfected fish held under the same conditions. Conversely, by 14 and 21 dpi, constitutive MH class II expression was significantly increased (>10 fold) in infected fish. Constitutive expression of interleukin-1β (IL-1β) increased 3-fold in infected fish by 21 dpi; however, no differences were observed in cyclooxygenase-2 (COX-2) expression. Head kidney macrophage enriched cultures from control Atlantic salmon showed an increase in MH class I and COX-2 expression, upon 3h lipopolysaccharide (LPS) stimulation, for all three-time points collected. Stimulation with LPS of macrophages from infected fish did not further increase levels of MH class I and COX-2 expression. This is the first report on host gene expression during an ectoparasitic copepod infection. The implications of these expression changes will be discussed with respect to the host-parasite relationship.

5.2 Introduction

Lepeophtheirus salmonis is an ectoparasitic copepod with a direct life cycle consisting of 10 stages (Johnson and Albright, 1991). The host species range of this parasite is limited to salmonid fish of the genera *Salmo*, *Oncorhynchus* and *Salvelinus*. Susceptibility to infection is variable among host species and has been linked to the host's inability to mount a significant inflammatory response (Fast et al., 2002; Johnson and Albright, 1992a). Host species such as coho salmon (*Oncorhynchus kisutch*) with a well-developed inflammatory response to the ectoparasite are able to easily resolve laboratory infections within ca. 140-degree days (Fast et al., 2002; Johnson and Albright, 1992a). Although the course of *L. salmonis* infections are well documented in the literature, studies have generally concentrated on host physiological responses, while intricacies of the host-parasite relationship have largely been unexplored (Bjorn and Finstad, 1997; Bowers et al., 2000; Fast et al., 2002; Grimnes and Jakobsen, 1996; Johnson and Albright, 1992a; Nolan et al., 1999; Ross et al., 2000). In many of these studies fish have been infected with relatively high-levels of *L. salmonis*, similar to levels reported to cause disease (Johnson and Fast, 2004). These levels of infection are known to cause a generalized stress response that may ultimately result in reduced immune functions (Mustafa et al., 2000a). What is unclear is whether low-levels of infection can affect immune functions in the absence of a generalized stress response.

Inflammation is a process by which tissues respond to injury, infection or irritation. In the case of ectoparasitic infection, this involves closure of the

epithelial breach and elimination of intruding foreign bodies. To initiate these processes, pro-inflammatory cytokines such as interleukin-1 β (IL-1β) and tumour necrosis factor α (TNF α), are produced by macrophages and monocytes at the site of injury and participate in leucocyte recruitment, lymphocyte activation and the acute phase response (Dinarello, 1994). Following phagocytosis of foreign particles, macrophages express antigens on their cell surface in association with major histocompatibility (MH) class I and II molecules. Antigen presentation is a necessary step in the activation of T cells and the development of an acquired immune response. The mechanism(s) by which *L. salmonis* avoids these responses on Atlantic salmon has not been determined. *Lepeophtheirus salmonis* infection has, however, been observed to decrease macrophage phagocytic capacity and respiratory burst in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in the absence of a cortisol response (Fast et al., 2002).

Lepeophtheirus salmonis secretions contain prostaglandin E_2 (PGE_2) and trypsin (Fast et al., 2004; Firth et al., 2000). Furthermore, it is likely, that other, as yet unidentified, compounds with pro- and anti-inflammatory effects are also present (Fast *et al.*, 2004). Prostaglandin E_2 down-regulates IL-1β and Th1 lymphokines (Chapter 3.0; Betz and Fox, 1991; Demeure et al., 1997; Fast et al., *in press*). Prostaglandin E_2 is a 20-carbon fatty acid product released from arachidonic acid by cyclooxygenase (COX). Up-regulation of the inducible form of cyclooxygenase (COX-2) is linked with many inflammatory diseases, such as

adjuvant arthritis and the enzyme is further up-regulated by production of IL-1β and PGE_2 in mammals (Hinz et al., 2000; Mancini et al., 2001; Raz et al., 1988).

The goal of this study was to determine whether low-level infection of *L. salmonis* affects immune gene expression within lymphatic tissues of Atlantic salmon using Real Time PCR. To achieve this we infected Atlantic salmon with between 0.05 and 0.12 lice/g of body weight, a level below which cortisol and glucose stress responses occur, and monitored constitutive and LPS-inducable expression of immune genes in tissues from infected and uninfected fish (Johnson and Fast, 2004).

5.3 Materials and Methods

5.3.1 Fish

Two infection trials were carried out using a single stock of post-smolt Atlantic salmon (Saint John River strain). Fish were maintained in 2500 L tanks supplied with flow-through 10-13^{0}C seawater and a 12-h light: 12-h dark photoperiod. In Trial 1, fish were separated into two tanks (1 control, 1 test) with 40 fish each 2 wk prior to infection. The weights (mean ± standard error) of the fish from 0, 12 and 21 days post infection (dpi) were 68.5± 8.83 g, 80.5 ± 6.99 g and 83.5 ± 7.07 g, respectively.

This trial was repeated on post-smolt Atlantic salmon from the original stock of fish 1 month later. Fish were maintained under the same conditions with the following exceptions. Control and infected tanks were stocked with higher densities of fish (>200 fish/ 2500 L tank). The weights of fish in the study from 0,

15 and 22 dpi were 104.0 ± 18.5 g, 100.6 ± 16.0 g and 163.6 ± 22.2 g, respectively.

5.3.2 *Infection trials*

Infective copepodids were grown in the laboratory and fish were infected following the methods described by Mustafa et al. (2000a). Approximately 100 infective copepodids/fish were added to the designated test tank under conditions of reduced water volume (15 kg m^{-3}) and flow for 12 h. Dissolved oxygen was maintained between 80-100% saturation (8-9.5 ppm), and 100 µm mesh was placed over outflows to prevent loss of copepodids. The control tank was submitted to the same reduced volume and flow as the test tanks without addition of copepodids.

5.3.3 *Sampling*

In both trials, 6 fish from the control and test tanks were sampled at each time point. Fish were kept off-feed for 24 h prior to sampling. Fish were killed by an overdose of tricaine methanesulfonate, bled and their head kidneys removed aseptically. Blood samples were centrifuged for 5 min at 4000 x *g*, after which the serum was decanted and stored at -80^{0}C until used. Head kidneys collected from the first infection trial were split into two samples: one was used for cell culture (see below) while the other was transferred into RNA later (Ambion) and stored at -80^{0}C prior to RNA extraction for single time point gene expression studies. During the second infection trial, head kidneys were only stored in

RNAlater (-80^{0}C). After tissue removal all fish were individually examined under a microscope and *L. salmonis* counted on all body surfaces.

5.3.4 *Analysis of serum samples*

An ELISA kit (NEOGEN) was used to determine serum cortisol levels for each fish in both trials. The protocol followed the manufacturer's instructions. Briefly, serum samples were thawed on ice and diluted between 1:30 and 1:50 with the provided extraction buffer. Conjugate (120 µl/per plate) was mixed with EIA buffer (6.0 ml) and then added 1:1 (50 µl:50 µl) with standards (ranging from 0.4–10 ng/ml) and diluted samples in the provided 96-well plate. All standards and samples were run in duplicate. The plate was shaken gently, covered and incubated at room temperature (20^{0}C) for 1 h. Enzyme conjugate was removed and each well washed 3X with 250 µl of diluted wash buffer (20 ml wash buffer and 180 ml ddH_2O). After removal of wash buffer, 150 µl of K-Blue substrate was added to each well and the plate shaken gently. The plate was incubated at room temperature (20^{0}C) for 30 min, shaken gently and the absorbance at 650 nm measured using a Thermomax microplate reader (Molecular Devices).

Serum glucose was determined for each fish in trial 1 using a colorimetric assay modified from Trinder (1969). Briefly, thawed serum samples and glucose standards (ranging from 10-90 mg/dl) were added (10 µl) to a 96-well plate in duplicate. Glucose (Trinder) reagent was also added to each well (190 µl) and the plate incubated for 10 min at room temperature (20^{0}C). Absorbance at 505 nm was determined using the same microplate reader and software, as described above.

5.3.5 *Macrophage isolation for cell culture*

Macrophages were isolated from the anterior kidney following a modification of previously described methods (Ottinger et al., 1999). Anterior kidneys were removed aseptically from fish and placed immediately into 5 ml L-15 media supplemented with 2% fetal bovine serum (FBS), 100 units ml^{-1} penicillin/streptomycin (P/S) and 10 units ml^{-1} heparin. Tissues were stored on ice until further processing. Tissues were dissociated by repeated passage through a 3-ml syringe and fragments allowed to settle for 10 min prior to removal of suspended cells. Cells were pelleted (500 x *g* for 10 min at 4^0C) and washed twice prior to layering on Percoll gradients (34/51%). Cells were centrifuged at 400 x *g* for 20 min at 4^0C and the macrophage enriched fraction collected at the 34/51% interface. Cells were re-suspended in 10 ml L-15/2% FBS, pelleted by centrifugation at 500 x *g* for 10 min at 4^0C, washed with 10 ml of L-15/2% FBS and then resuspended in L-15/5% FBS with 100 units ml^{-1} P/S. Viable cells were counted using the trypan blue exclusion method and cell density adjusted to $1x10^7$ cells ml^{-1} in L-15/0.1% FBS. Cells were plated at 100 µl per well in 96 well plates and incubated at 18^0C for 2 h. After 2 h media and non-adherant cells were removed and an equal volume of L-15/5% FBS added. Cells were maintained for 1.5 days at 18^0C prior to manipulation. After this period elapsed, media was removed and 100 µl of fresh L-15/5% with or without 5 µg/ml *Escherichia coli* lipopolysaccharide (LPS) was added. Stimulation of cells was carried out for 3h at 18^0C, at which time the media was removed, the cells

transferred into RNA later and stored at –80^{0}C until RNA extraction. Addition of LPS had no effects on cell viability, morphology or adherence properties in this study (data not shown).

5.3.6 Isolation of RNA and cDNA synthesis

For the first infection trial, total RNA was isolated using 50 mg of head kidney tissue from each fish. In the second trial, head kidney tissue (10 mg per fish) was pooled for each treatment on each day. For isolated macrophage incubations, cells were pooled for individual fish under a single incubation condition. Total RNA was isolated with the Nucleospin RNA II kit (Clontech) and concentration measured by a spectrophotometer. RNA samples underwent PCR to verify the lack of DNA contamination. For reverse transcription 1.0 µg of total RNA from each sample was dissolved in molecular biological grade water. Reverse transcription was carried out using the Enhanced Avian HS RT-PCR (SIGMA) kit, with random nonamers, as per supplier's instructions. cDNA was stored at –20°C until use in Real-Time PCR assays.

5.3.7 Real-Time PCR

Sequences for Real-time PCR primers were designed using Primer 3 software (Rosen and Skaletsky, 2000) and Dr. Michael Zuker's mfold server (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi). Primers were generated from available Atlantic salmon (*Salmo salar*) genome (β-actin: AF012125, MHC I: I51348 and MHC II: X70166) and EST databases (COX-2 and IL-1β) (Table 5.1) (Chapter 3.0, published in Fast *et al.*, *in press*). The TNFα-like gene primers

were designed from comparisons of the highly conserved regions of those genes in rainbow trout and plaice (*Pleuronectes platessa*) (Chapter 3.0; Laing et al., 2001; Fast et al., *in press*). Using these primers I obtained from Atlantic salmon a product with a 99% nucleotide homology to rainbow trout (TNFα 1). All primers used in this study are listed in Table 5.1. All primer sets were tested on head kidney cells isolated from Atlantic salmon to confirm single amplification products. PCR products of β actin, MH class I, MH class II, COX-2, TNFα and IL-1 β were cloned into a TA-cloning vector (pCR 4-TOPO Invitrogen) and sequenced to confirm sequence of amplified products. Vectors were then linearized by NotI (1 h at 37°C) and used as standards for Real-Time studies (Chapter 3.0, published in Fast et al., *in press*).

Real-time quantitative PCR was performed using an iCycler iQ™ Real-Time detection system and SYBR green kits (Invitrogen). The SYBR green mastermix kit was used as per manufacturer's instructions with the following exceptions. Supermix was added (25 µl) to template cDNA (2.5 µl), water (17.5 µl) and specific primers (125 nM forward and reverse final concentration) giving a total volume of 50 µl prior to dividing into separate wells for duplication of readings. Primer concentrations were optimized at 125 nM after testing a range of concentrations from 90-900 nM. To ensure no genomic DNA contamination added to the quantified cDNA, non-RT controls for each RNA isolation were run under PCR and observed by 2.5% agarose gel electrophoresis.

Table 5.1: Sequences of oligonucleotide primers used in Real-Time PCR.

Genes	Primers	Sequences (5`-3`)
β-actin	β actin - forward	CAACTGGGACGACATGGAGA
	β actin - reverse	AGTGAGCAGGACTGGGTGCT
Cyclooxygenase-2	COX-2 – forward	CAGTGCTCCCAGATGCCAAG
	COX-2 – reverse	GCGAAGAAGGCGAACATGAG
MH class I	MH I – forward	TGCTCGTCGTTGCTGTTGTT
	MH I - reverse	TCAGAGTCAGTGTCGGAAGTGC
MH class II	MH II - forward	AAGGCTTGAAGACACGTTGC
	MH II - reverse	CAGTCCAGCAGTAACGTCCA
IL-1β	IL-1β - forward	GGTCCTTGTCCTTGAACTCG
	IL-1β - reverse	ATGCGTCACATTGCCAAC
TNF α	TNF α - forward	GGCGAGCATACCACTCCTCT
	TNF α - reverse	TCGGACTCAGCATCACCGTA

The PCR profile was as follows: two initial 2 min denaturation steps at 50°C and then at 95°C, followed by 40-45 cycles of denaturation (15 s at 95°C), annealing (30 s at 56°C) and extension (30 s at 72°C), and finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products such as primer dimers, indiscriminately detected by SYBR green (ie. SYBR green binds to all double stranded DNA), were evaluated by amplifying 10 fold dilutions of the clones (10 to 10^{-6} ng) and duplicate samples as well as by performing a blank without cDNA with each run. The relationship between the threshold cycle (Ct) and the log [RNA] was linear (-3.5<slope<-3.2) for all reactions.

Single product amplification was further verified by melt curve analysis. Melting curves were obtained following 40-45 cycles of amplification on the Lightcycler by integrating the signal every 0.1 s during a linear temperature transition from 95°C to 70°C. Fluorescence data was converted by icycler software in which background fluorescence and the effect of temperature on fluorescence were removed.

5.4 Statistical analysis

Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All values shown for Trial 1 data are means of individual fish ± SEM (expressing genetic variation), whereas values for Trial 2 data are means of pooled fish under duplicate trials ± SEM (expressing instrument variation). The statistical significance of gene expression differences was assessed on

expression changes relative to β-actin using one-way analysis of variance ($p<0.05$). A two-way analysis of variance (2-way) was used to determine if there were any significant differences between infected and control fish physiological parameters over time. Multiple comparisons were carried out using Tukey's and paired t-tests. All non-normal data were transformed using a ($\sqrt{x}$) function.

5.5 Results

5.5.1 Trial 1

Experimental infection of Atlantic salmon with *L. salmonis* resulted in mean lice numbers of 9.6 and 9.8 lice per fish at 12 and 21 dpi, respectively (Fig. 5.1). In uninfected fish mean serum cortisol levels ranged from <0.04 -129.1 ng/ml. In infected fish mean serum cortisol levels ranged from 2.3 – 116.5 ng/ml (Fig. 5.2a). Although serum cortisol levels were generally higher in control fish at 0 and 12 dpi these differences were not significant (Fig. 5.2a). Serum glucose levels, however, were significantly higher in uninfected fish when compared to infected fish throughout this trial (Fig. 5.2c).

There were no significant differences in constitutive MH class I gene expression in whole kidney tissue between infected and uninfected groups prior to infection (0 dpi) or at 12 dpi (Fig. 5.3a). At 12 dpi MH class I gene expression was significantly lower in infected and uninfected fish when compared to both groups prior to infection and the uninfected group at 21 dpi. However, MH class I gene expression was significantly (10-fold) lower in infected fish when compared to uninfected fish at 21 dpi. At both 0 and 21 dpi, 67% of the uninfected fish had

Figure 5.1: Mean (±SEM) intensity of *Lepeophtheirus salmonis* on Atlantic salmon, *Salmo salar*, at various days post infection (dpi) over two separate trials (n=6).

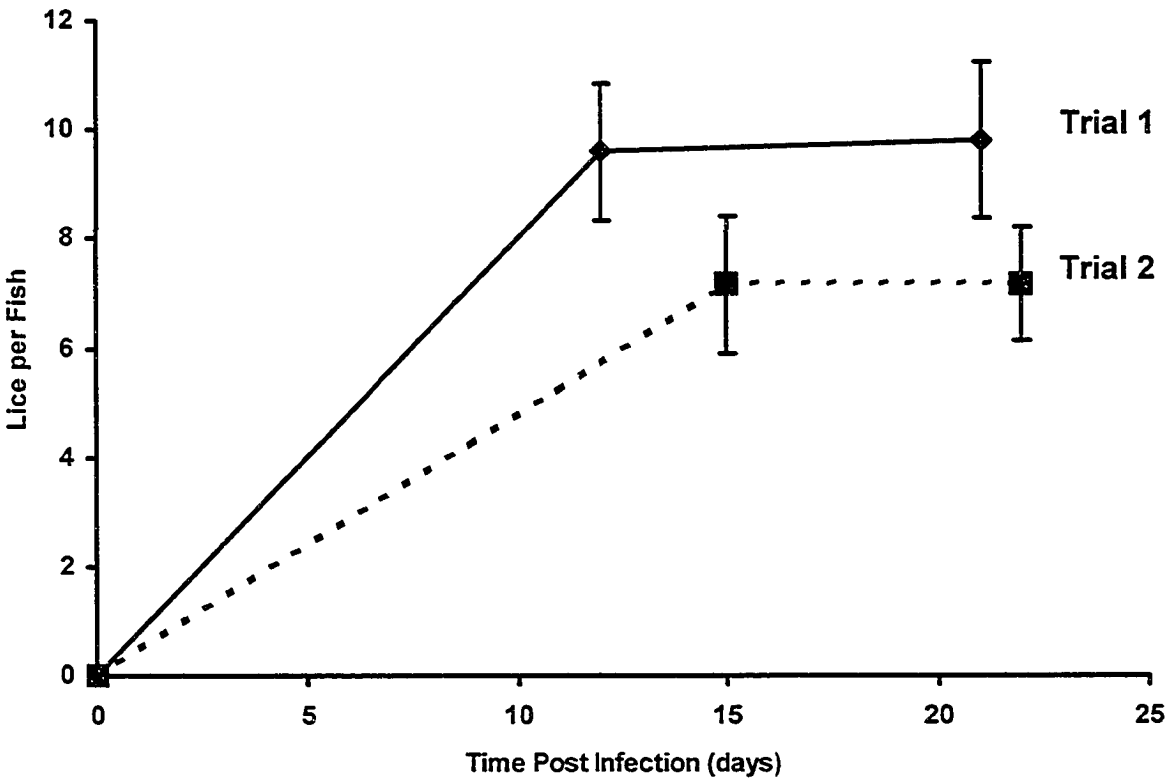
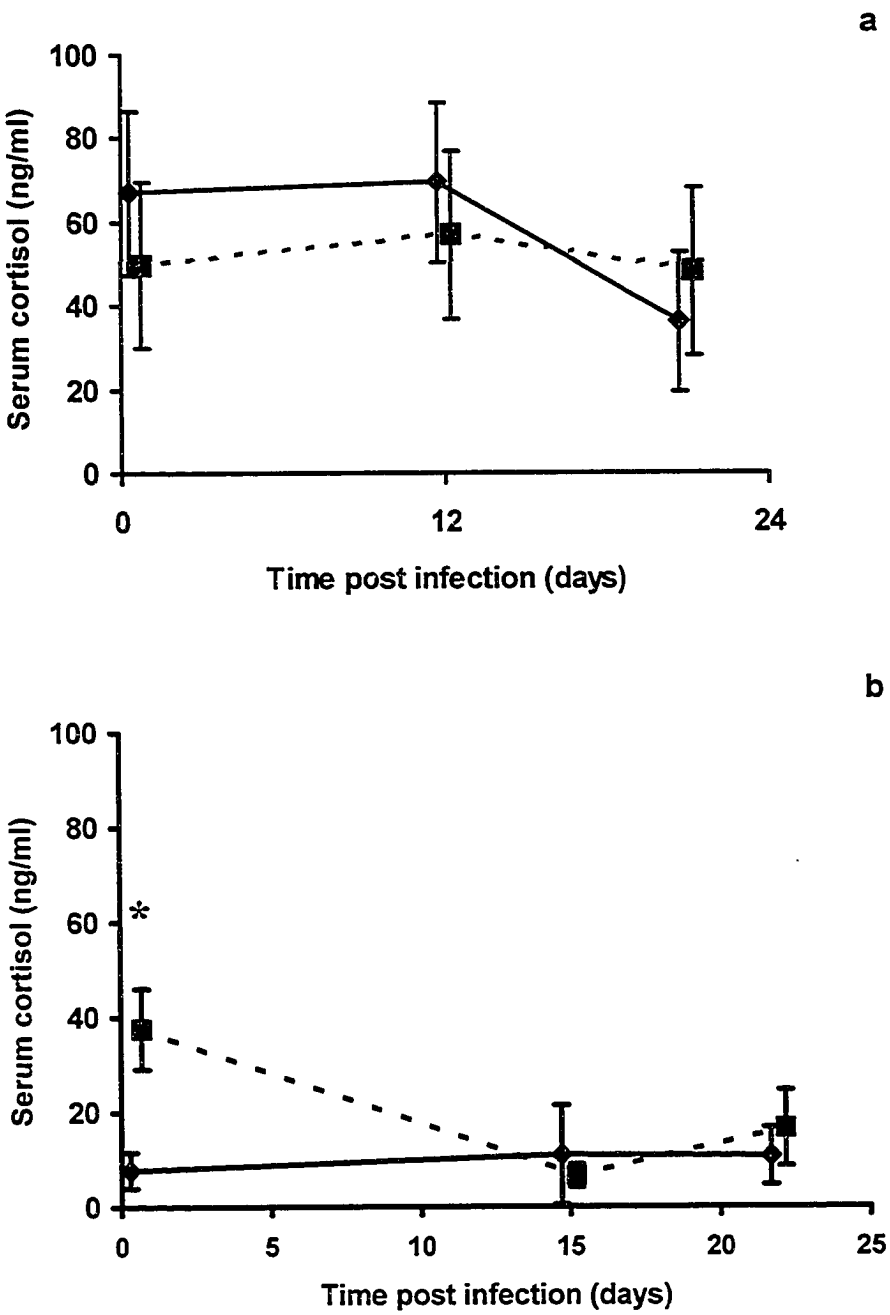


Figure 5.2: Comparison of mean (±SEM) serum cortisol (a, b) and glucose (c) levels between *Lepeophtheirus salmonis*-infected (- ■ -) and non-infected (-♦-) Atlantic salmon over time. Trial 1 (a, c), Trial 2 (b). * Significant differences between infected and control on that day ($p < 0.05$, n=6).



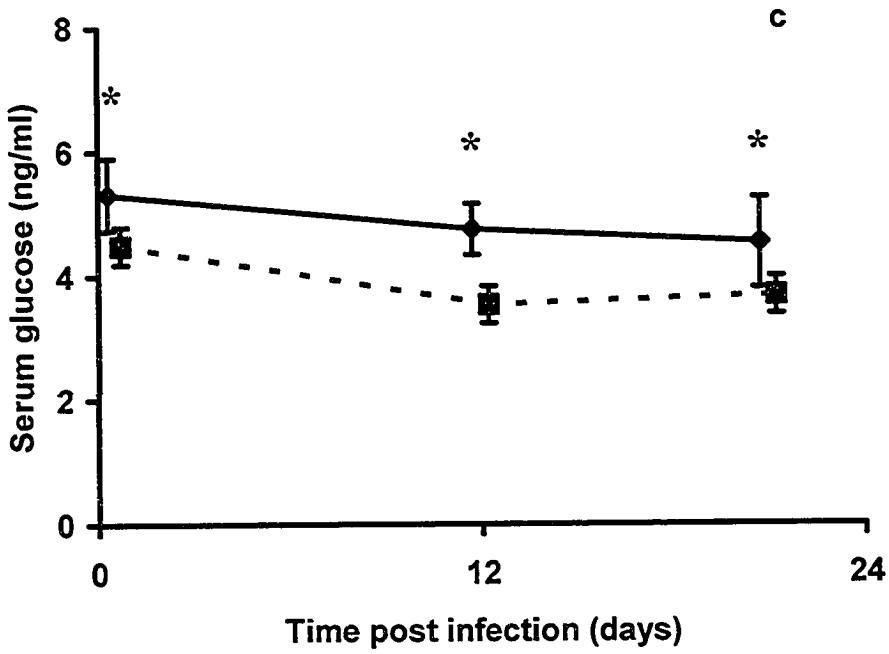
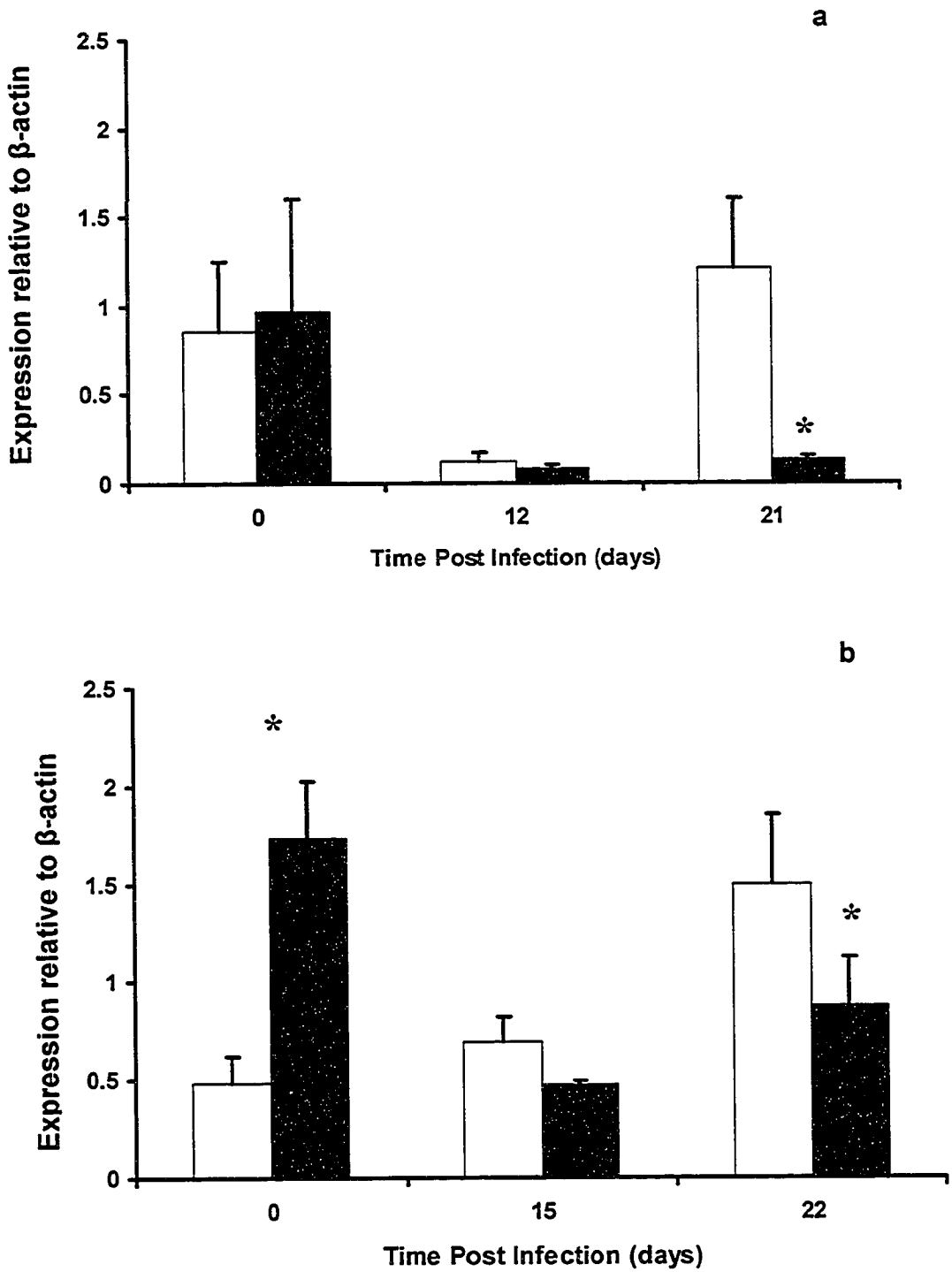

c
8
6
4
2
0
*
*
*
Serum glucose (ng/ml)
0
12
24
Time post infection (days)

Figure 5.3: Mean (±SEM) expression of major histocompatibility class I gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and non-infected (□) Atlantic salmon over time. Trial 1 (a), Trial 2 (b). * Significant differences from control on that day ($p < 0.05$, n=6).



expression levels around 1.0 relative to β-actin, whereas at 12 dpi all fish expressed levels of MH class I were <0.12 relative to β-actin. Similar to 12 dpi, all infected fish at 21 dpi had an MH class I expression of <0.19 relative to β-actin.

Prior to infection constitutive MH class I gene expression of isolated head kidney macrophages was similar between groups (Fig. 5.4). The levels of constitutive MH class I gene expression in uninfected fish remained relatively constant over time, whereas there was a significant increase in MH class I gene expression in infected fish at 12 dpi. By 21 dpi MH class I gene expression in infected fish had returned to a similar level as that seen in uninfected fish.

Stimulation of macrophages, collected from both groups prior to infection and at 12 dpi, with LPS resulted in significant increases in MH class I gene expression (Fig. 5.4). At 21 dpi LPS stimulation resulted in increased MH class I gene expression only in macrophages obtained from uninfected hosts.

Constitutive expression of the MH class II gene was very low in whole kidney tissue of uninfected fish when compared to MH class I expression. Mean expression levels relative to β-actin, in uninfected fish were 0.06, 0.05 and 0.2 at 0, 12 and 21 dpi, respectively. Expression of MH class II was observed to increase throughout the experiment in infected fish (Fig. 5.5a). At 12 and 21 dpi, the mean levels of gene expression were significantly (>100-fold) increased in infected fish when compared to uninfected fish (Fig. 5.5a). However, the large increases in MH class II gene expression were observed in only 3 out of 6 fish on both sampling days.

Figure 5.4: Mean (±SEM) expression of major histocompatibility class I gene, relative to β-actin, in LPS-stimulated and unstimulated head kidney macrophages isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon over time. (□) denotes uninfected-unstimulated macrophages, (▨) denotes uninfected-LPS-stimulated macrophages, (■) denotes infected-unstimulated macrophages, and (▤) denotes infected-LPS-stimulated macrophages.
* Significant differences from unstimulated control on that day ($p < 0.05$).
† Significant differences from uninfected fish under same incubation conditions ($p < 0.05$, n=5).

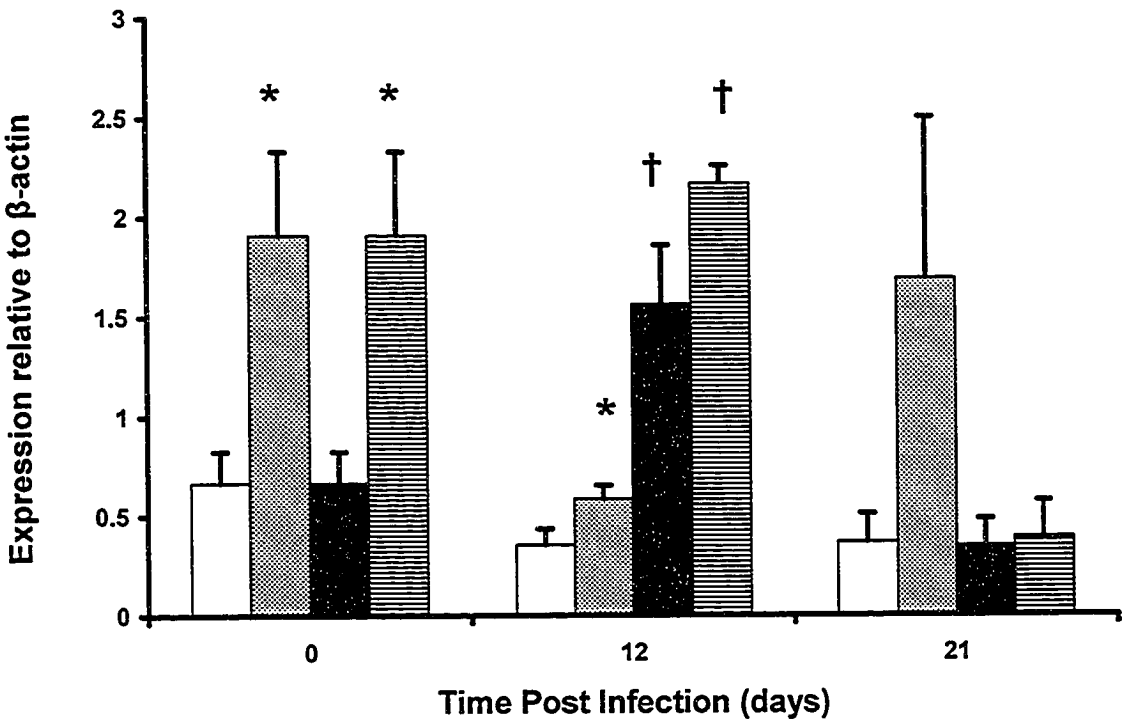
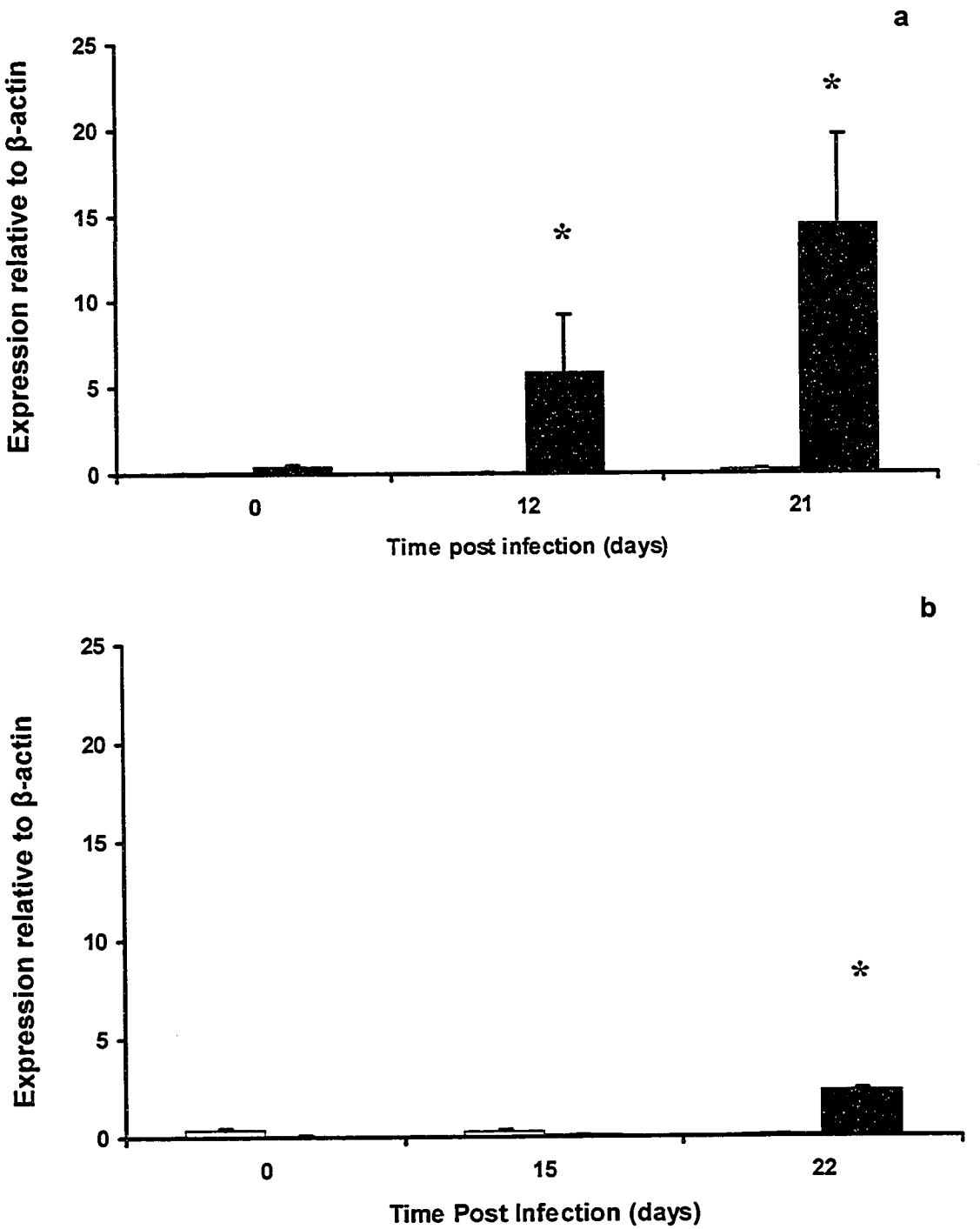


Figure 5.5: Mean (±SEM) expression of major histocompatibility class II gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and non-infected (□) Atlantic salmon over time. Trial 1 (a), Trial 2 (b). * Significant differences from control on that day ($p < 0.05$, n=6).



Isolated macrophages from uninfected fish showed relatively low constitutive expression of the MH class II gene similar to that seen in whole head kidney tissues from uninfected fish (Figs. 5.5a, 5.6). Levels of expression were variable over time and were not significantly affected by either LPS-stimulation or *L. salmonis* infection (Fig. 5.6).

Interleukin-1β was constitutively expressed in whole kidney tissues from uninfected fish, but again at much lower levels than MH class I (Figs. 5.3a, 5.7a). Interleukin-1β expression decreased at 12 dpi in both uninfected and infected fish, similar to that reported for MH class I gene expression (Figs. 5.3a, 5.7a). By 21 dpi, IL-1β expression in uninfected fish returned to level similar to that seen at 0 dpi, however, infected fish showed a significantly (3-fold) higher level of IL-1β expression when compared to uninfected fish (Fig. 5.7a). Similar to MH class II gene expression, the increase in IL-1β expression at 21 dpi was attributable to increases in only 3 out of 6 infected fish. However, this did not occur in the same fish as the MH class II increase was observed in.

Levels of constitutive interleukin-1β expression in head kidney macrophages from uninfected fish were lower than those observed in whole head kidney tissue (Figs. 5.7a, 5.8). Macrophages from uninfected fish showed variable responses to LPS stimulation, such that, LPS-stimulation resulted in a small increase in IL-1β expression at day 0, a significant (2.5-fold) increase at 12 dpi and no change at 21 dpi (Fig. 5.8). At 12 dpi, expression of IL-1β in unstimulated macrophages from infected fish was significantly (6-fold) higher when compared to unstimulated macrophages from uninfected fish. At 12 dpi

Figure 5.6: Mean (±SEM) expression of major histocompatibility class II gene, relative to β-actin, in LPS-stimulated and unstimulated head kidney macrophages isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon over time. (□) denotes uninfected-unstimulated macrophages, (▨) denotes uninfected-LPS-stimulated macrophages, (■) denotes infected-unstimulated macrophages, and (▤) denotes infected-LPS-stimulated macrophages ($p < 0.05$, n=5).

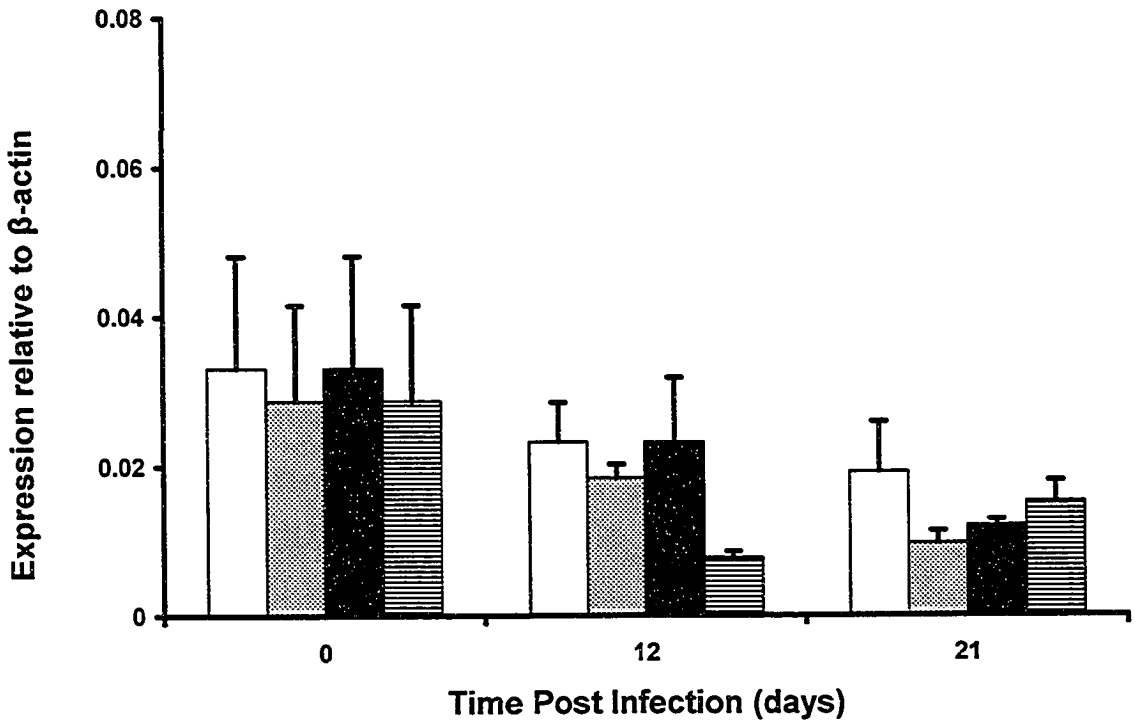


Figure 5.7: Mean (±SEM) expression of Interleukin-1β gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and non-infected (□) Atlantic salmon over time. Trial 1 (a), Trial 2 (b). * Significant differences from control on that day ($p < 0.05$, n=6).

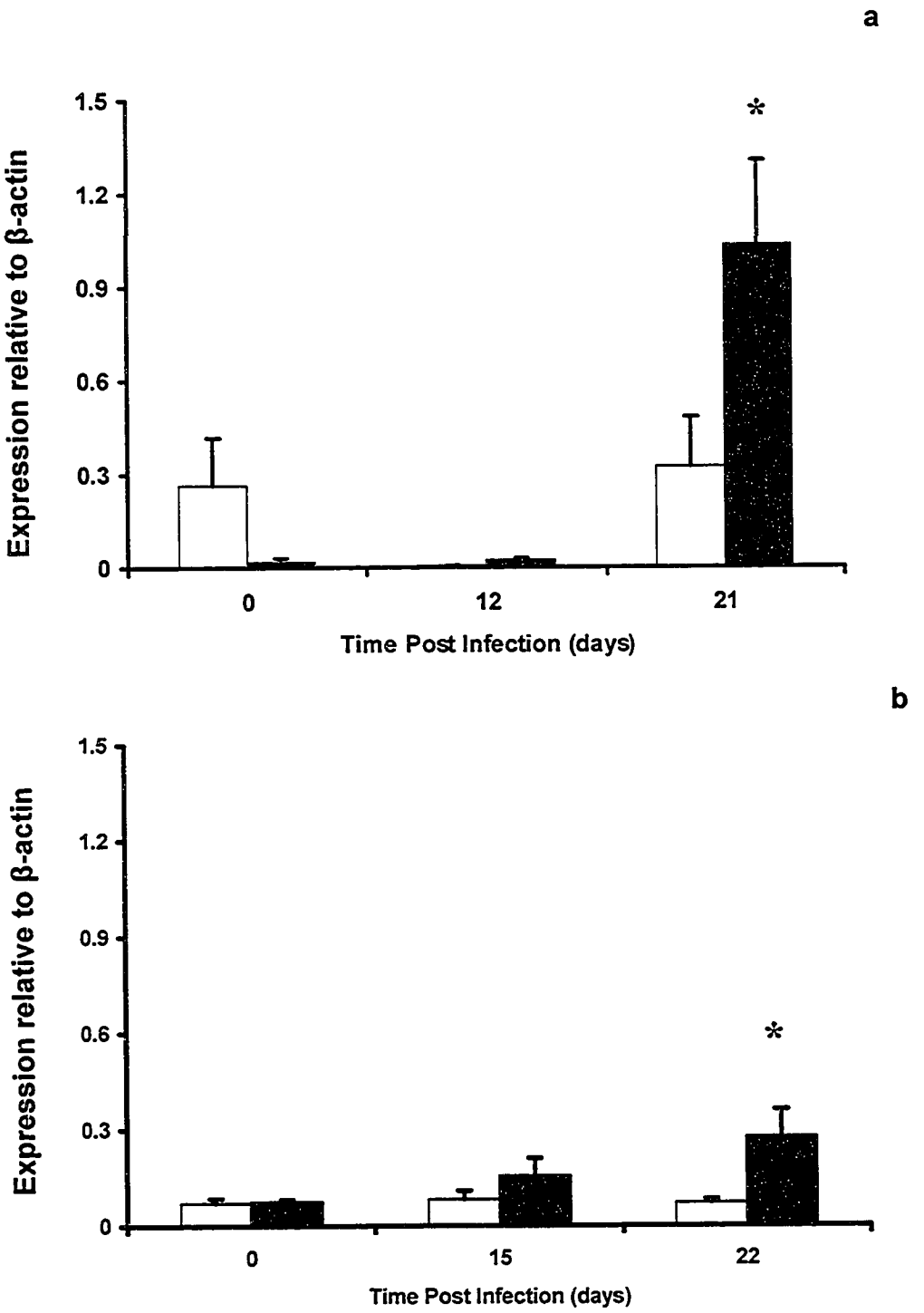
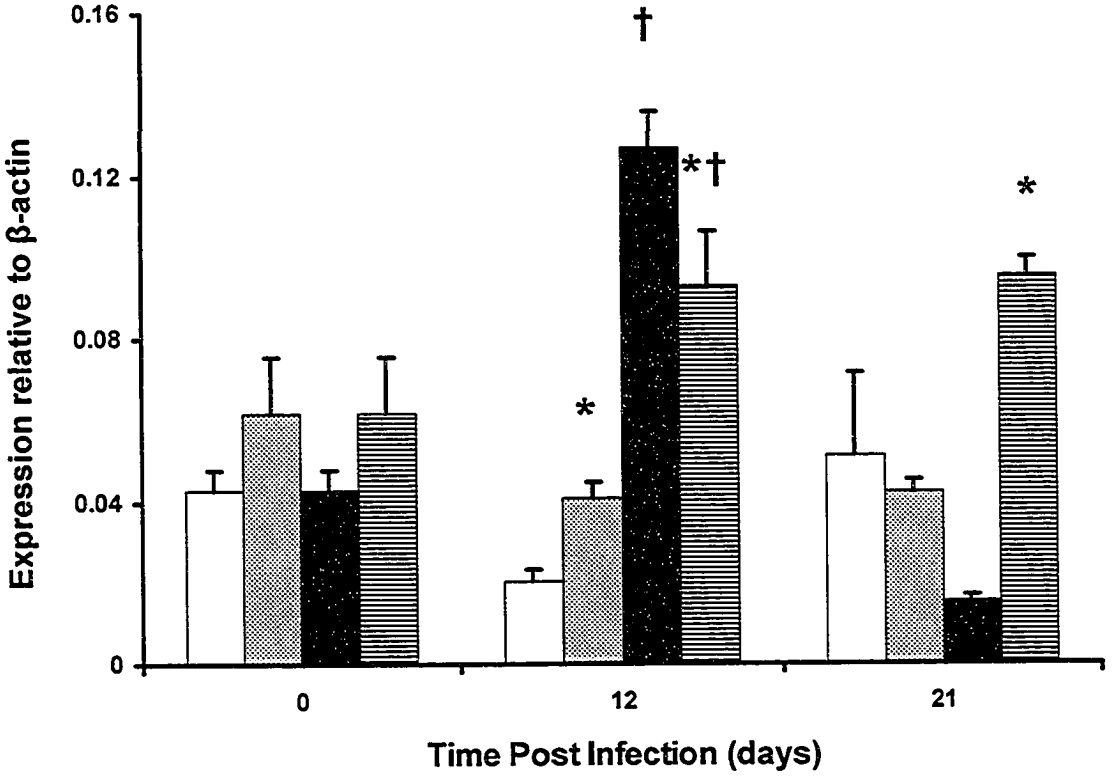


Figure 5.8: Mean (±SEM) expression of Interleukin-1β gene, relative to β-actin, in LPS-stimulated and unstimulated head kidney macrophages isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon over time. (□) denotes uninfected-unstimulated macrophages, (▨) denotes uninfected-LPS-stimulated macrophages, (■) denotes infected-unstimulated macrophages, and (▤) denotes infected-LPS-stimulated macrophages.* Significant differences from unstimulated control on that day ($p < 0.05$). † Significant differences from uninfected fish under same incubation conditions ($p < 0.05$, n=5).



LPS stimulation of macrophages from infected fish resulted in significantly lower levels of expression when compared to unstimulated macrophages. However, this level was still significantly (2-fold) higher when compared to unstimulated and LPS stimulated macrophages from uninfected fish (Fig. 5.8). At 21 dpi, IL-1β expression was relatively low in unstimulated macrophages from infected fish, whereas, LPS-stimulated macrophages showed similar levels of expression to those seen at 12 dpi (Fig. 5.8).

When compared to the other genes studied there was very low and highly variable levels of COX 2 expression in whole kidney tissues from uninfected and infected fish throughout this trial (Fig. 5.9). There were no significant differences in the levels of COX-2 gene expression between uninfected and infected fish at any time.

Constitutive expression of COX-2 was relatively higher in isolated head kidney macrophages when compared to whole kidney samples (Figs. 5.9, 5.10). Stimulation of macrophages with LPS resulted in significantly increased expression of COX-2 in both groups at day 0 (3-fold) and in the uninfected group at 21 dpi (2-fold) (Fig. 5.10). At 12 dpi COX-2 expression was significantly lower in LPS stimulated macrophages from infected fish when compared to all other groups. At 21 dpi, COX-2 expression was significantly (up to 4-fold) higher in unstimulated macrophages from infected fish when compared to unstimulated and LPS stimulated macrophages from uninfected fish (Fig. 5.10). At 12 and 21 dpi LPS stimulation of macrophages from infected fish did not further increase COX-2 expression (Fig. 5.10).

Figure 5.9: Mean (±SEM) expression of cyclooxygenase-2 gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and non-infected (□) Atlantic salmon over time (Trial 1) ($p < 0.05$, n=6).

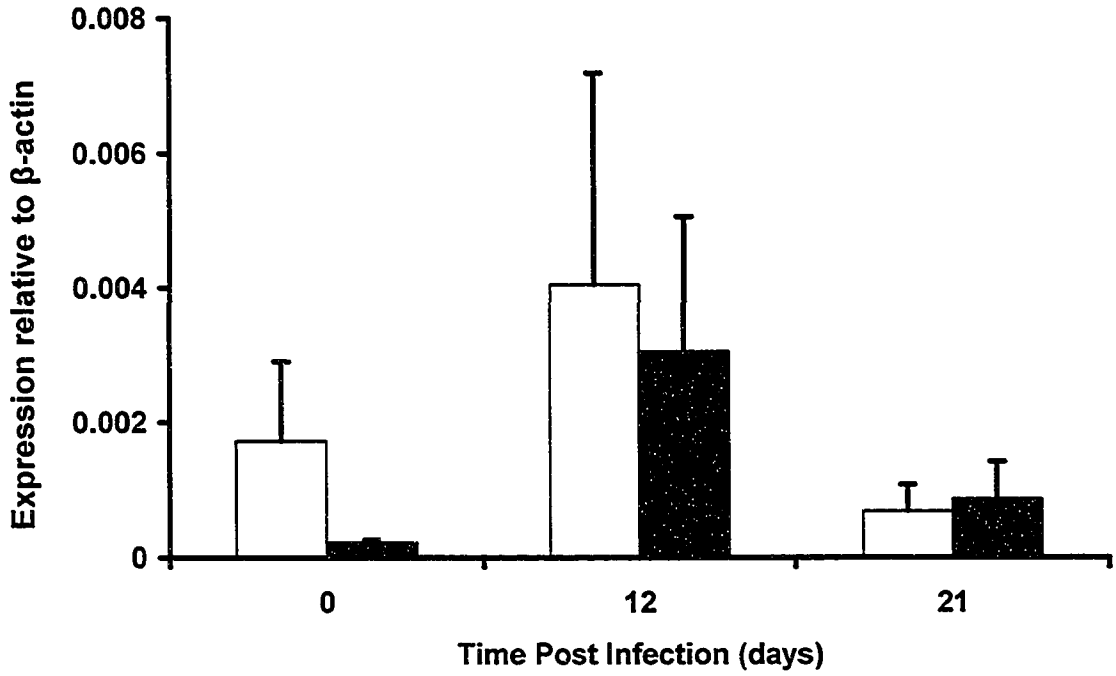
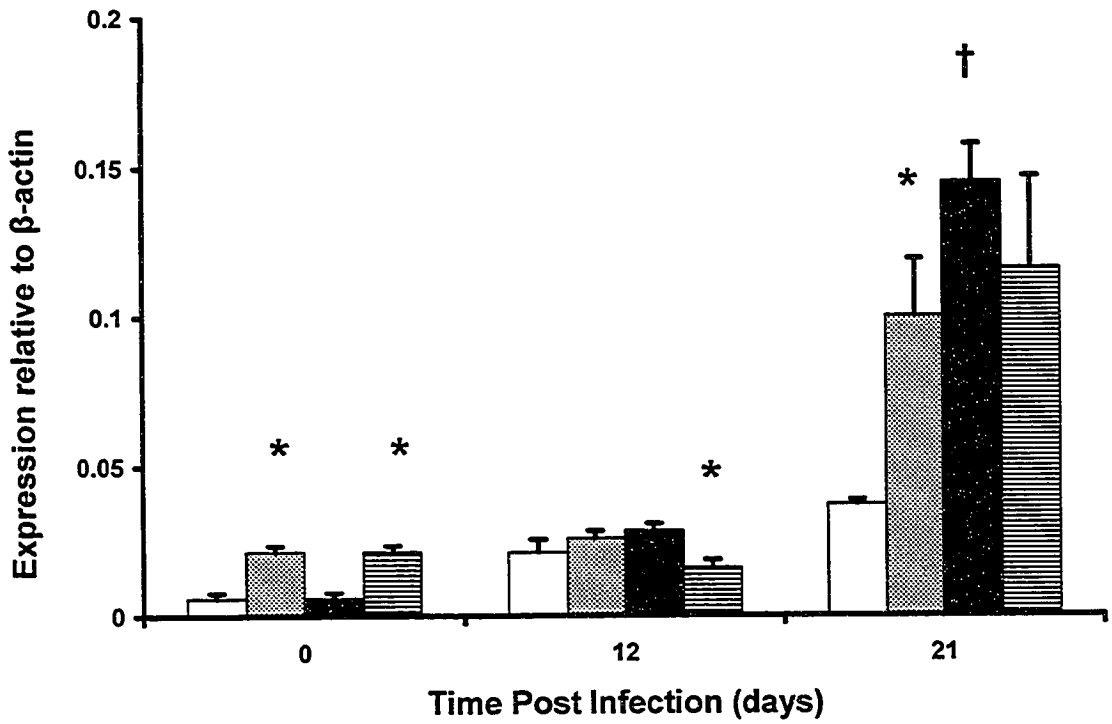


Figure 5.10: Mean (±SEM) expression of cyclooxygenase-2 gene, relative to β-actin, in LPS-stimulated and unstimulated head kidney macrophages isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon over time. (□) denotes uninfected-unstimulated macrophages, (▨) denotes uninfected-LPS-stimulated macrophages, (■) denotes infected-unstimulated macrophages, and (▤) denotes infected-LPS-stimulated macrophages.* Significant differences from unstimulated control on that day ($p < 0.05$). † Significant differences from uninfected fish under same incubation conditions ($p < 0.05$, n=5).



We were unable to detect TNFα–like gene expression in either whole kidney tissue or isolated macrophages from both groups (data not shown).

5.5.2 *Trial 2*

In Trial 2, *L. salmonis* infection levels were slightly lower than in the first trial (Fig. 5.1). Serum cortisol levels in control and infected fish were lower although not significantly different than the first trial (Fig. 5.2a, b). Serum cortisol levels ranged from <0.04 - 58.8 ng/ml in uninfected fish and <0.04 - 64.1 ng/ml in infected fish, over the course of this trial. Prior to infection (0 dpi) the group that was later infected with *L. salmonis* had significantly higher serum cortisol levels than the control group, however, by 15 dpi both groups had similarly low serum cortisol levels (Fig. 5.2b).

Similar to serum cortisol levels, MH class I gene expression, prior to infection, was significantly (3-fold) higher in the group that was later infected with *L. salmonis* when compared to the group that remained uninfected (control) (Fig 5.3b). At 15 dpi, there was no significant difference in whole head kidney MH class I gene expression between uninfected and infected fish. At 22 dpi, MH class I gene expression was significantly lower in head kidneys from infected fish when compared to those from uninfected fish (Fig. 5.3b).

In Trial 2, expression of MH class II gene was higher in fish from the uninfected group when compared to the uninfected group in Trial 1 (Fig. 5.5a, b). Major histocompatability class II gene expression in uninfected fish was 0.38, 0.28 and 0.06, relative to β-actin, on 0, 15 and 22 dpi, respectively. Major

histocompatability class II gene expression increased significantly (30-fold) at 22 dpi in whole kidney tissues from infected fish when compared to tissues obtained from uninfected fish (Fig. 5.5b).

Interleukin-1β showed relatively low constitutive expression in whole head kidney tissues from uninfected fish at all time points. Similar to Trial 1 expression of IL-1β, at 22 dpi, was significantly (3-fold) higher in whole kidney tissue from infected fish when compared to tissues from uninfected fish (Fig. 5.7b).

5.6 Discussion

Over the past decade numerous studies have been conducted on the disease state induced by heavy infections of *L. salmonis* on salmonids (Johnson and Fast, 2004). These works have provided insight into the pathogenesis of the disease, they have done little to improve our understanding of *L. salmonis* host-parasite interactions in the absence of disease (Johnson and Fast, 2004). Based on field observations and experimental data for other parasites it is assumed that even low levels of *L. salmonis* infection have significant effects on their hosts. *Lepeophtheirus salmonis* may interfere with host immune responses through the development of chronic stress or through the production of immunomodulatory factors that are released at the site of attachment and feeding. Two immunomodulatory compounds, trypsin and prostaglandin E_2 (PGE2) occur in the secretions of *L. salmonis* (Fast et al., 2002; 2003; 2004; Firth et al., 2000; Ross et al., 2000). Prostaglandin E_2 is a potent vasodilatory compound with anti-inflammatory activities (Demeure et al., 1997). The presence of compounds

such as PGE_2 may explain the lack of inflammatory responses observed in Atlantic salmon infected with *L. salmonis* (Johnson and Fast, 2004; Fast et al., *in press*).

In this study, the effects of infection with low levels of *L. salmonis*, between 9-12 lice (0.04-0.12 lice/g) per fish on constitutive and inducible immune-related gene expression in Atlantic salmon, were examined. As repression of NFκB-dependent gene expression (IL-1β, COX-2, etc.), for instance, is a major immunosuppressive activity of glucocorticoids (Brostjan et al., 1996; De Bosscher et al., 2000) low levels of infection were chosen to reduce the possibility of a parasite-induced cortisol stress response affecting gene expression. Although there were no significant differences in serum cortisol levels between infected and uninfected fish in either trial, it is difficult to prove that none of the fish were chronically stressed to some degree. Pickering and Pottinger (1989), for instance, have suggested that normal plasma cortisol levels in salmonids are in the range of 0-5 ηg/ml, similarly, Fast et al. (2002) found resting levels of plasma cortisol in the range of 3.5-7.2 ηg/ml. In trial 1, serum cortisol levels were very high in all Atlantic salmon to begin with, and only came down at 21 dpi. In trial 2, prior to infection, the treatment group also had high cortisol values. Thus, while some fish may have undergone chronic stress leading up to the experiment, it appears to have occurred in both groups and not affected the relative differences between uninfected and infected fish.

Numerous immune-related genes have been identified in different teleost species, allowing studies that have examined host gene expression under viral,

bacterial and parasitic infection (Ewart et al., 2005; Hansen and LaPatra, 2002; Saeij et al., 2003a, b; Sigh et al., 2004a, b; Tafalla et al., 2005). In this study the effects of *L. salmonis* infection on immune-related gene expression in whole kidney tissue and isolated kidney macrophages of Atlantic salmon, were determined. As Atlantic salmon like many other fish species show a great deal of variability between individuals with respect to immune and stress responses we conducted 2-trials, analysing both individual (Trial 1) and pooled samples (Trial 2). We report marked differences in expression between whole kidney tissues and isolated macrophages for several of the genes studied. As monocytes/macrophages make up only a small percentage of head kidney tissue, observed differences are most likely due to influences from granulocytes and lymphocytes that play an important role over the course of infection.

The major histocompatability class I and II genes were examined to determine the ability of host fish to elicit T-cytotoxic ($CD8^+$) and T-helper cell ($CD4^+$) responses, important in adaptive immunity. Generally, T-helper cells ($CD4^+$) interact with MH class II molecules on the surface of antigen presenting cells (APC), whereas T-cytotoxic cells ($CD8^+$) interact with MH class I molecules on the surface of APC. In both trials MH class I gene expression was significantly lower in whole kidney tissue from infected fish in the last samples collected (21 and 22 dpi). Down-regulation of the MH class I gene expression in whole kidney samples of Atlantic salmon may be a result of active immunomodulation from *L. salmonis*. Prostaglandin E_2 down-regulates MH class I and II gene expression in antigen presenting cells of mammals (Harizi and

Gualde, 2002; Snyder et al., 1982). As MH class I and II are important in stimulating adaptive immune responses, their regulation by PGE_2 during parasitic challenge has been studied. Inhibition of MH class I and II gene expression by PGE_2 has been observed in *Leishmania donovani* infections of the murine macrophage cell line P388D1, as well as, *Entamoeba histolytica* infections of BALB/c mice bone marrow-derived macrophages (Kwan et al., 1992; Wang and Chadee, 1995).

Decreased MH class I gene expression may be linked to the corresponding increase in MH class II gene expression during the experiment. The importance of MH class II molecules in ectoparasitic infection was observed in other arthropod parasite-host interactions. The sheep blowfly, *Lucialia cuprina*, and the European castor bean tick, *Ixodes ricinus*, stimulate influx of $CD4^+$ but not $CD8^+$ cells to sites of infection, suggesting they play a role in the host response to parasite invasion (Mbow et al., 1994; Nash et al., 1996). If an influx of CD4+ cells is occurring at the site of *L. salmonis* infection in Atlantic salmon, it could then be possible for the increased MH class II expression in head kidneys to result from the return of these cells to the lymphatic tissue in the head kidney. A corresponding decrease in expression of MH class I gene could then result from a higher percentage of the head kidney being made up of cells expressing relatively high amounts of MH class II and relatively low amounts of MH class I genes.

MH class II gene expression was significantly up-regulated in the whole kidney tissue of infected fish as compared to uninfected fish, later on in both trials

(21 and 22 dpi). In trial one, this could be further broken down to show that half (3/6) of the infected fish responded to infection by up-regulating MH class II expression. It is necessary to note that while changes in gene expression are important, more work needs to be done to show whether corresponding protein levels changed. For instance, rainbow trout MH class II-associated invariant chain INVX has shown post-translational control, with increases in protein levels following LPS stimulation without gene expression changes (Braunstein et al., 2004). Since, MH class II molecules are important in expressing antigens from extracellular pathogens, it is not surprising that a host would increase expression during parasitic invasion. In rainbow trout infected with the ectoparasitic ciliate *Ichthyophthirius multifiliis*, MH class II expression was significantly elevated in the kidney at 4, 6 and 26 dpi (Sigh et al. 2004a). These authors suggested that the upregulation of MH class II might be due to the presence of the pro-inflammatory cytokine, TNFα. Increased expression of TNF-α1, has been demonstrated over a 26 day period in rainbow trout head kidneys as the result of infection with *I. multifiliis* (Sigh et al., 2004b). In the current study there was no evidence for TNFα-like expression in either of the trials or tissues examined. In Chapter 3.0 (Fast et al., *in press*) it was reported that there was no constitutive or LPS induced expression of this gene in a salmon head kidney cell line (SHK-1) and it was only after 24 hr of LPS stimulation in the presence of PGE_2 that expression was evident. Whether the TNFα-like gene, studied here, functions similar to mammalian TNFα remains to be determined.

In both trials IL-1β was significantly up-regulated at 21 and 22 dpi in infected fish. Interestingly, other parasitic infections in teleosts and mammals increase IL-1β expression, but often at much earlier time points. Carp (*Cyrpinus carpio*) infected with extracellular blood flagellates, *Trypanoplasma borelli*, exhibit increased IL-1β expression in kidney tissues at 1 and 2 dpi (Saeij et al., 2003a). Head kidney tissue from rainbow trout infected with *I. multifiliis* had significantly increased IL-1β 1 expression at 1 and 6 dpi, and IL-1β 2 at 26 dpi (Sigh et al., 2004a, b). Similarly, Nash et al. (1996) observed increased IL-1β gene expression at the site of infection at 6, 24 and 48 h and in the afferent lymph of sheep infected with *L. cuprina,* early on or prior to any substantial tissue damage or neutophil influx. The late induction (21-22 dpi) of IL-1β expression in Atlantic salmon head kidneys corresponds to the same time as an observation of mild inflammation in Atlantic salmon tissues, in another *L. salmonis* infection (Johnson and Albright, 1992a). No head kidney samples were taken from infected Atlantic salmon early on (<12 dpi) in this study, but a delayed IL-1β response may help to explain the lack of inflammation and subsequently the susceptibility of these hosts to *L. salmonis* infection.

Head kidney macrophages were isolated from infected and non-infected fish, and subsequently stimulated with LPS in order to investigate the effects of parasite infection on macrophage function. Cyclooxygenase-2 gene and MH class I expression is rapidly induced by LPS, cytokines and other inflammatory stimuli in mammals (rats) and fish cell lines (Brubacher et al., 2000; Chapter 3.0, published in Fast et al. *in press*; Futaki et al., 1997). In this study macrophages

from infected fish did not appear to increase MH class I and COX-2 gene expression following stimulation with LPS at 12 and 21 dpi, respectively. Macrophages from infected hosts, already expressing higher levels of MH class I and COX-2 (Figs. 4, 10) than uninfected host cells, may not be able to express these genes above a threshold already reached prior to stimulation. In another case, MH class I (21 dpi) and COX-2 (12 dpi) gene expression in infected fish macrophages was similar to uninfected macrophages, yet still their expression was not increased (COX-2 decreased) following LPS stimulation (Fig. 4). As suggested previously, the monocyte subpopulations of the head kidneys of infected fish may change by these time points and an inability to increase MH class I or COX-2 gene expression may reflect a change in the composition of leukocytes adhering to the plates. For instance, subpopulations of monocytes expressing low-levels of $CD14^+$ are observed in human blood. As $CD14^+$ is important in LPS binding and signalling, these cells show a reduced expression of pro-inflammatory genes such as IL-10 upon LPS stimulation (Frankenberger et al., 1996). Dannevig et al. (1990) reported that sinusoidal endothelial cells were present in significant numbers in suspensions of head kidney enriched macrophages for rainbow trout.

Down-regulation of MH class I expression in the head kidney as seen over the course of infection, coupled with a lack of LPS-stimulated expression in head kidney macrophages, may help explain the observed decreases in macrophage phagocytic capacity and respiratory burst seen in previous infections at similar parasite densities (Fast et al., 2002).

In this study we noted individual variability in macrophage IL-1β expression following LPS-stimulation. Due to variability between individuals macrophages isolated from un-infected fish did not exhibit consistent LPS-induced increases in IL-1β expression as seen in other studies (Brubacher et al., 2000; Chapter 3.0, published in Fast et al., *in press*; Zou et al., 2000). This may be explained by the use of cell lines in the first two studies, which would greatly reduce variability. However, if some fish in both groups were chronically stressed, prior to the trial as suggested above, the immunosuppressive effects of cortisol may contribute to the observed inconsistent IL-1β responses to LPS.

The lack of LPS-stimulated effects on MHC II expression in head kidney macrophages further supports previous findings. Fast et al. (*in press*) observed LPS-stimulation having no effect on SHK-1 cell expression of MH class II gene. Similarly, stimulation of the rainbow trout cell line RTS 11 with 10 µg/ml LPS for 4h had no apparent effects on MH class II gene expression. Knight et al. (1998) found little or no effect of LPS stimulation on MH class II molecule expression using primary isolates of rainbow trout head kidney macrophages.

It is reasonable to expect that *L. salmonis* modulates host immune responses at the site of infection and feeding. In this study we have demonstrated that infection with low numbers of *L. salmonis* can result in changes in immune-gene expression in tissues away from the site of attachment and feeding. When considering the implications for the host-parasite relationship we must put these expression changes into context. It would be most disadvantageous for *L. salmonis* to immunomodulate their entire host, due to the

fact that it is a long-lived parasite with a prolonged attachment to its host. Therefore any major changes resulting in decreased survivorship of the host would not benefit *L. salmonis*, especially since these parasites are expected to mainly infect their hosts in the short period upon entering the seawater estuary and prior to their lengthy migrations out at sea. The increases observed in MH class II expression over MH class I gene expression in the head kidney are most likely a result of cell populations switching within the lymph in response to parasitic infection. While there may be an increase in IL-1β expression at the feeding site (skin) early on in the infection, perhaps similar to mongenean *Gyrodactylus derjavini* infections of rainbow trout (Lindenstrom et al., 2003), it may be resolved by the immunomodulatory capabilities of the parasitic secretions. As the infection continues, however, neutrophils begin to migrate to the site of feeding and attachment, as seen in Johnson and Albright (1992a). The increased expression of IL-1β in the head kidney at 21 and 22 dpi may then result from the increase in tissue damage due to the more detrimental mobile stages of *L. salmonis* and influx of neutrophils.

A larger study involving a greater number of fish may be useful in elucidating any correlations between cortisol and immune gene expression. The temporal changes in cytokine expression as well as the modulation of macrophage immune gene expression, shown here, give further insight into the mechanisms by which *L. salmonis* maintain long-term infections in Atlantic salmon hosts.

Chapter 6.0: The effects of successive *Lepeophtheirus salmonis* infections on the immunological status of Atlantic salmon (*Salmo salar*).

6.1 Abstract

This study was conducted to determine the effects of successive infections of the parasitic copepod *L. salmonis* on the immunological status of Atlantic salmon. An initial low-level initial infection was carried out 14 d prior to a second infection in which twice as many parasites were introduced. Plasma cortisol and prostaglandin E_2 (PGE_2) levels were monitored concurrent to the expression of 6 immune-related genes over 5 sample times (9, 21, 26, 33 and 40 days post initial infection, dpii). The mean lice counts on the infected fish increased significantly from the first infection (16.3 ± 1.89 at 9 dpii) to the second (142.8 ± 12.8 at 26 dpii). Plasma cortisol levels increased significantly at 26, 33 and 40 dpii in infected fish compared to controls. Plasma PGE_2 levels were significantly higher in infected fish at 9, 33 and 40 dpii, when compared to controls. At 9 dpii, expression of interleukin-1β (IL-1 β), tumour necrosis factor- α (TNFα)-like cytokine, major histocompatibility class II (MH II), transforming growth factor-β (TGFβ)–like cytokine and cyclooxygenase-2 genes were increased in infected fish compared to controls. The expression of most of these genes returned to control levels at 21 dpii when the highest expression of the MH class I gene was observed in infected fish (significantly higher than controls). Major histocompatibility class I gene expression remained higher in infected fish at 26 and 33 dpii compared to controls and this was observed for the TNFα-like gene. By 33 dpii, MH class II and TGF β-like genes had higher expression in infected fish compared to controls. Interleukin-1β and TNFα-like gene were the only genes that showed significantly higher expression in infected fish compared to controls at 40 dpi, while MH class I gene expression was significantly depressed in infected fish at this time. The expression of nearly all immune-related genes studied here increased following initial infection with *L. salmonis*, however, immunological stimulation did not reduce parasite numbers protect against a successive infection.

6.2 Introduction

Molecular characterization of pathogenic organisms and their fish hosts has become a common approach for studying host-parasite relationships. Recently, several papers have been published on parasitic ciliates, flagellates and monogeneans and their interactions with their host species (Lindenstrom et al., 2004; Saeij et al., 2003a; Sigh et al., 2004ab). While these types of studies exist for terrestrial parasitic arthropods (Schoeler and Wikel, 2001), the literature is lacking with respect to economically important arthropod parasites of fish, such as the sea louse, *Lepeophtheirus salmonis*.

The ectoparasitic copepod *L. salmonis* becomes infective towards salmonid hosts (genera *Salmo*, *Salvelinus* and *Oncorhynchus*) upon reaching its free-swimming copepodid stage. Following infection, the parasite undergoes a series of moults passing through four attached chalimus stages (I-IV) and two mobile pre-adult stages (I-II) prior to becoming an adult. *Lepeophtheirus salmonis* infections on susceptible hosts (i.e. Atlantic salmon, *Salmo salar*) are notable in that no significant inflammatory response is elicited despite its feeding on host mucus, tissues and blood (Johnson and Albright, 1992a). However, a well-developed inflammatory response is associated with resistance to infection in coho salmon (*Oncorhynchus kisutch*) (Johnson and Albright, 1992a). Suppression of this response, by the administration of cortisol, results in the loss of resistance to infection (Johnson and Albright, 1992b). It has recently been proposed that immunomodulation of the host in the absence of stress (i.e, high levels of cortisol) is responsible for the lack of response of Atlantic salmon to *L.*

salmonis (Chapter 2.0, published in Fast et al., 2004; Johnson and Fast, 2004). The identification of trypsin and PGE_2 in the secretions of *L. salmonis* has given validity to this hypothesis (Fast et al., 2003; Firth et al., 2000; Chapter 2.0, published in Fast et al., 2004). Prostaglandin E_2 is a vasodilatory compound that inhibits several salmonid immune-related genes including interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2), and major histocompatibility (MH) class I and II (Chapter 3.0, published in Fast et al., *in press*). Prostaglandin E_2 may be elevated in Atlantic salmon infected with *L. salmonis* (Pike and Wadsworth, 1999).

The first molecular characterization of *L. salmonis* infection on Atlantic salmon was conducted in Chapter 5.0. While that work was necessary to determine immune-related gene responses to low-level infections, it did not address the even more natural occurrence of re-infection. Due to its importance to salmonid aquaculture, numerous studies on the biology, ecology and the host-parasite relationship have been published on *L. salmonis* (reviewed in: Heuch et al., *in press*; Johnson and Fast, 2004; Tully and Nolan, 2002), yet effort has not been directed towards the response of host fish to more than a single infection pulse. As salmonids are threatened by *L. salmonis* infection upon entering seawater, both wild and cultured populations are under repeated infection pressure. Therefore, an understanding of how salmon respond to repeated infection will be useful in understanding natural effects of this parasite on wild salmonid populations. In this chapter I examine the response of Atlantic salmon to re-infection with *L. salmonis* by investigating plasma physiology (cortisol and

PGE_2) and lymphoid immune-gene expression. The development of acquired resistance towards *L. salmonis* and the immunological capacity of infected post-smolts will be discussed.

6.3 Materials and Methods

6.3.1 Fish

Post-smolt Atlantic salmon (Saint John River strain) were maintained in 1000 L tanks containing 300 L of seawater (SW). Fish were initially obtained from a freshwater hatchery following smoltification and were naïve with respect to infection with *L. salmonis*. Tanks were supplied with flow-through SW at 11-13^{0}C and maintained under a 12-h light:12-h dark photoperiod. Fish were separated into two populations (1 uninfected, 1 infected) each with 50 fish. The average weights of the fish ranged from 281.9 g to 371.3 g over the course of the study.

6.3.2 Copepodid production

Ovigerous *L. salmonis* were collected from recently harvested fish at salmon processing plants in New Brunswick, Canada. They were transported back to the laboratory on ice (ca. 10-15^{0}C), where their eggstrings were removed and placed in culture buckets supplied with flow through SW (12^{0}C) and gentle aeration. Eggs were allowed to hatch and develop to the infectious copepodid stage at which time they were harvested for use in infection trials (Chapter 5.0).

6.3.3 Infection trials

Fish were infected twice with *L. salmonis*. For the first infection approximately 5000 infective copepodids (ca. 120/fish) were used. During the

infection, the water volume of the tank was reduced to 150 L and the outflow screened with a 100 μm mesh to prevent the loss of copepodids. For the first 6 h of infection water flow to the tank was shut off. Following that, water was turned on intermittently for 6 h before a constant reduced flow was supplied for 12 h. Temperature was maintained within the designated range stated above and dissolved oxygen was maintained between 75-102% saturation throughout the infection period. The uninfected population was submitted to the same conditions as the infected population without addition of copepodids.

A second infection involving 10,000 infective copepodids (ca. 300/fish) was carried out 14 d after the initial infection (14 dpii). The conditions under which the re-infection was conducted were identical to those used during the first infection and the uninfected population was submitted to the same conditions as the infected population without addition of copepodids.

6.3.4 Sampling

On each sampling day 8 uninfected and 8 infected fish were sampled. Fish were not fed for 24 h prior to sampling. Fish were euthanized by rapidly anesthetizing with tricaine methanesulfonate followed by bleeding and head kidney collection. Blood samples were centrifuged for 5 min at 4000 x *g*, the resulting plasma decanted and split into two aliquots (one for cortisol and the other for PGE_2 analysis), and stored at -80^{0}C until used. Head kidneys from each time point were stored in RNAlater (Ambion) at -80^{0}C prior to RNA

extraction. All fish were individually examined and *L. salmonis* counted and staged on body surfaces.

6.3.5 *Analysis of plasma samples*

Plasma cortisol levels were measured using an ELISA kit (NEOGEN) following the manufacturer's instructions (Chapter 5.0). Briefly, plasma samples were thawed on ice and then diluted 1:50 with the provided extraction buffer. Conjugate (120 µl/per plate) was mixed with enzyme immunoassay (EIA) buffer (6.0 ml) and then added 1:1 (50 µl:50 µl) with standards (ranging from 0.4–10 ng/ml) and diluted samples in the provided 96-well plate. All standards and samples were run in duplicate. The plate was shaken gently, covered and incubated at room temperature (20^0C) for 1 h. Enzyme conjugate was then removed and each well washed 3X with 250 µl of diluted wash buffer (20 ml wash buffer and 180 ml ddH_2O). After removal of wash buffer, 150 µl of K-Blue substrate was added to each well and the plate shaken gently. The plate was incubated at room temperature (20^0C) for 30 min and shaken gently again prior to reading on a Thermomax microplate reader (Molecular Devices) at 650 ηm. An endpoint reading was determined using SoftMax Pro software.

A monocolonal competitive EIA kit (Cayman Chemical) was used to determine plasma PGE_2 levels of uninfected and infected fish. The sample volumes required for this analysis necessitated the pooling of plasma samples for each group on each day. The manufacturer's instructions were followed with minor modifications. Plasma samples were thawed on ice and pooled to produce 9 ml of plasma per group (uninfected and infected) per sampling day. Samples

were acidified to pH 4.0 with 2M HCl (ca. 25 µl/ml plasma) and cloudy samples were centrifuged at 1000 x *g* to remove particulate matter. The samples were then applied to octadecylsilane (ODS) solid phase extraction columns (C-18 SPE, Waters), activated with 10 ml of 15% ethanol followed by 10 ml of ddH_2O. The ODS columns were washed with 10 ml 15% ethanol, 10 ml ddH_2O and followed by 10 ml hexane (Fisher Scientific). The prostaglandin-containing fraction was eluted using 10 ml of methyl formate (Sigma) and evaporated to dryness under nitrogen (Chapter 2.0, published in Fast et al., 2004). The residue was reconstituted with 450 µl of EIA buffer and vortexed. Prostaglandin acetylcholinesterase tracer and monoclonal antibody were added 1:1:1 (50 µl:50 µl:50 µl) with standards (ranging from 7.8-1000 pg/ml) and extracted samples in the provided 96-well plate. All standards were run in duplicate and samples were run in triplicate at two different dilutions. The plate was shaken gently, covered and incubated at 4°C for 18 h. After an 18 h incubation, wells were emptied and washed 5X with the provided wash buffer. Ellman's reagent was added to each well, including wells containing only PGE_2 tracer, which was used to measure total activity. The plate was covered with plastic film and developed in the dark on an orbital shaker for 1.5 h. The plate was read on a Thermomax microplate reader (as above) at 405 nm. An endpoint reading was determined using SoftMax Pro software. To measure the efficiency of the extraction protocol known amounts (0.5 ng/ml) of PGE_2 were added to five different ODS columns and measured together with the samples.

6.3.6 *Isolation of RNA and cDNA synthesis*

Total RNA was isolated from 50 mg of head kidney tissue of individual fish using a Nucleospin RNA II kit (Clontech) and measured by spectrophotometry. Total RNA (1.0 µg) from each sample was dissolved in molecular biological grade water and reverse transcription was carried out using the Retroscript (Ambion) kit with random hexamers as described in the supplier's instructions. The resulting cDNA was stored at –20°C until use in Real-Time PCR assays.

6.3.7 *Real-Time PCR*

Sequences for Real-time PCR primers were designed using Primer 3 software (Rosen and Skaletsky, 2000) and Dr. Michael Zuker's mfold server (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi). Primers were based on available Atlantic salmon genomic (β-actin: AF012125, MHC I: I51348 and MHC II: X70166) and EST sequences (COX-2 and IL-1β) (Table 3.1) (Chapter 3.0, published in Fast *et al.*, *in press*). The TNFα-like and $TGF\beta_{1/5}$ -like gene primers were based on the highly conserved regions of those genes in rainbow trout and plaice (*Pleuronectes platessa*) (Chapter 3.0; published in Fast et al., *in press*; Laing et al., 2001). The gene product developed for the Atlantic salmon TNFα-like gene showed 124/125 (99%) identity to rainbow trout (TNFα 1), while the $TGF\beta_{1/5}$–like gene product exhibited 107/108 (99%) identity to rainbow trout $TGF\beta_{1/5}$. To confirm single amplification products all primers were then tested on cDNA obtained from Atlantic salmon head kidney cells (Chapters 3.0 and 4.0). PCR products of β-actin, MH class I, MH class II, COX-2, TNFα-like, $TGF\beta_{1/5}$–like and IL-1 β were cloned into a TA-cloning vector (pCR 4-TOPO; Invitrogen)

and sequenced to confirm the identity of the amplified products. Vectors were then linearized by NotI (1 h at 37°C) and used as standards for Real-Time studies (Chapter 3.0, published in Fast *et al.*, in press). Forward and reverse primers are listed in Table 3.1.

Real-Time quantitative PCR was performed using an iCycler iQ™ Real-Time detection system and SYBR green kits (Invitrogen). The SYBR green mastermix kit was used as recommended by the manufacturer with the following exceptions. Supermix (25 µl) was added to template cDNA (2.5 µl), water (17.5 µl) and specific primers (125 nM forward and reverse final concentration) giving a total volume of 50 µl prior to dividing into separate wells for duplication of readings. Primer concentrations were optimized at 125 nM after testing a range of concentrations from 90-900 nM. To ensure no genomic DNA contamination added to the quantified cDNA, non-RT controls for each RNA isolation were run under PCR and the lack of products confirmed by 2.5% agarose gel electrophoresis.

The PCR profile was as follows: an initial 2 min denaturation step at 50°C and then at 95°C, followed by 40-45 cycles of denaturation (15 s at 95°C), annealing (30 s at 58°C) and extension (30 s at 72°C), and finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products such as primer dimers, indiscriminately detected by SYBR green (ie. SYBR green binds to all double stranded DNA), were evaluated by amplifying 10 fold dilutions of the clones (10 to 10^{-6} ng) and duplicate samples as well as by performing a blank without cDNA with each run. The relationship

between the threshold cycle (Ct) and the log [RNA] was linear (-3.6<slope<-3.3) for all reactions. Copy numbers were estimated based on the molecular weight of clones and OD 260.

Single product amplification was further verified by melt curve analysis. Melting curves were obtained following 40 cycles of amplification on the Lightcycler by integrating the signal every 0.1 s during a linear temperature transition from 95°C to 70°C. Fluorescence data was converted by iCycler software in which background fluorescence and the effect of temperature on fluorescence were removed.

6.4 Statistical Analysis

Statistical analyses were performed using SigmaStat for Windows Version 3.0 (SPSS). All values shown are means of individuals for each sampling day ± SEM. The statistical significance of gene expression differences was assessed on expression changes relative to β-actin (ERB) using one-way analysis of variance ($p<0.05$). Analysis of variance (2-way) was also used to determine significant differences between infected and control fish physiological parameters over time. Multiple range tests were carried out using Tukey's HSD tests. Pearson product moment correlations were used to determine whether there were correlations between lice counts, cortisol level and relative gene expression.

6.5 Results

At 9 days post initial infection (dpii) there was an average of 16.3 chalimus I or II stage lice/fish (Figs. 6.1, 6.2). A four-fold higher number of successful infections was observed after re-infection when compared to the initial infection, despite only twice as many infectious copepodids being introduced (Fig. 6.1). At 21 dpii (7 days post re-infection [dpri]) the majority of *L. salmonis*, that could be attributed to the re-infection, were chalimus I-II larvae, whereas, lice from the initial infection had reached the pre-adult stage (Fig. 6.2). While lice numbers from the initial infection remained constant between 21-33 dpii, the highest number of lice from the second infection was observed at 12 dpri (Fig. 6.1). *Lepeophtheirus salmonis* from the initial infection passed from pre-adult stages (21 dpii), to pre-adult and adult male stages (26 dpii), and most likely all became adults by 33 dpii. Following the larger sub-population of lice (80%) from the second infection, *L. salmonis* development did not appear to be hampered or delayed by the initial infection. *Lepeoptheirus salmonis* life stages progressed from chalimus I-II (7 dpri), to chalimus III-IV (12 dpri), to pre-adults (19 dpri), and finally adult males and pre-adults (26 dpri) on very much the same schedule as in the initial infection (Fig. 6.2).

Plasma cortisol levels averaged 34.7 ng/ml prior to infection in both uninfected and infected populations (Fig. 6.3). The infection process resulted

Figure 6.1: Mean (±SEM) intensity (-♦-) of *Lepeophtheirus salmonis* on Atlantic salmon, *Salmo salar*, at various days post initial infection, with relative contributions of first (- ■ -) and second (-▲-) infections. Arrow denotes time of second infection (n=8 for each group).

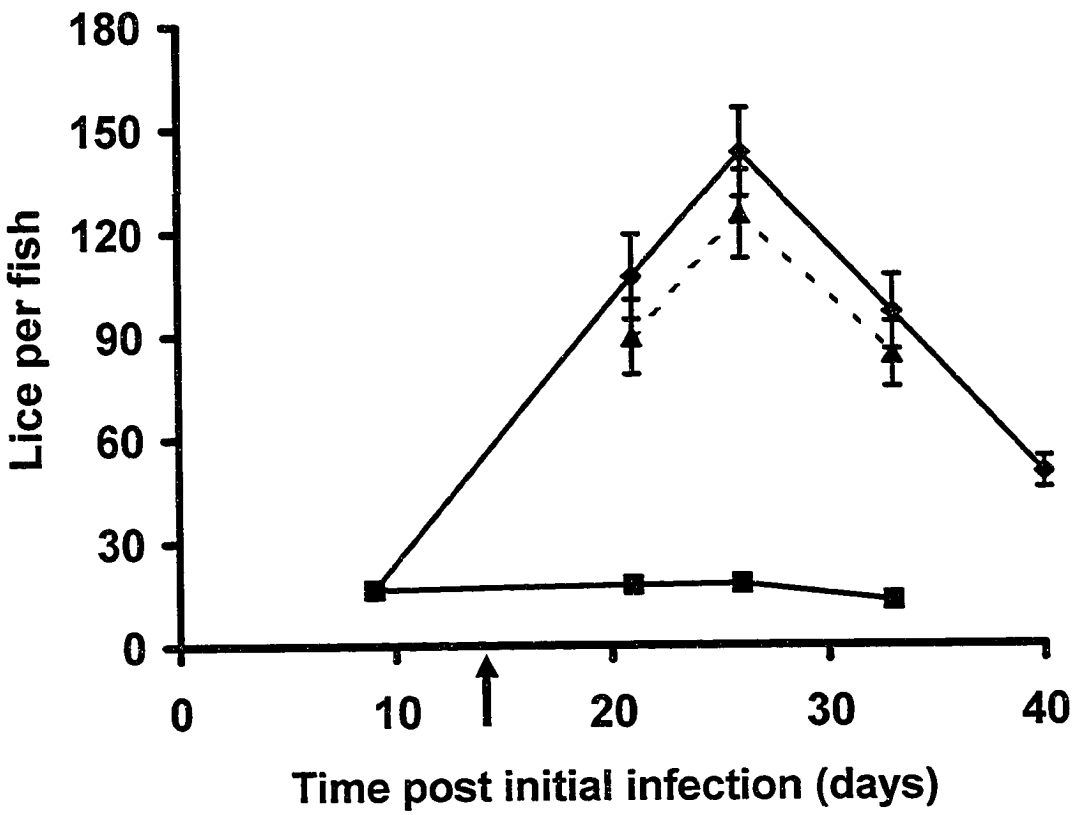


Figure 6.2: Percentage of each life stage of *Lepeoptheirus salmonis* present on Atlantic salmon at various days post initial infection. (■) chalimus I-II stage larvae, (□) pre-adult stage, (■) chalimus III-IV stage larvae, (▨) adult male, (▤) adult female, (▣) ovigerous female. Arrow denotes time of second infection (n=30 for each developmental stage).

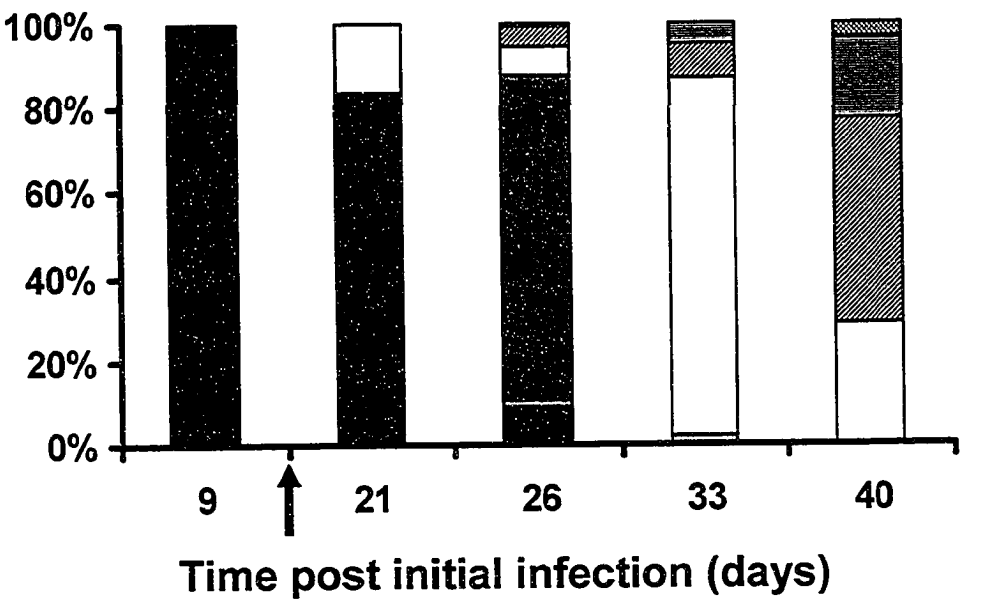
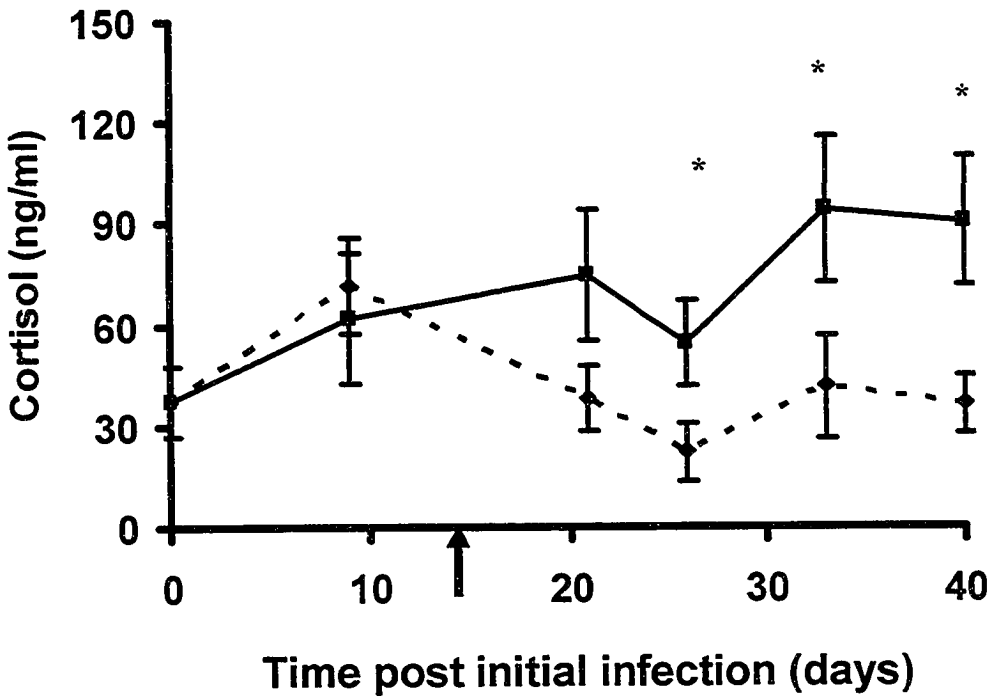


Figure 6.3: Comparison of mean (±SEM) plasma cortisol levels between *Lepeophtheirus salmonis*-infected (- ■ -) and uninfected (-♦-) Atlantic salmon over time. * Significant differences between infected and uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.



in elevated cortisol in uninfected (71.4 ng/ml) and infected (61.7 ng/ml) fish; however this was not significant (Fig. 6.3). Both groups were subjected to a second infection process at 14 dpii and by 21 dpii, cortisol levels of uninfected fish returned to an average of 38.2 ng/ml, while cortisol levels in infected fish remained elevated. Although plasma cortisol levels in infected fish dropped by 26 dpii, they rose and remained significantly higher than levels seen in uninfected fish throughout the rest of the study (33 and 40 dpii) (Fig. 6.3).

An EIA kit was used to determine PGE_2 concentration in the plasma of Atlantic salmon over the course of the study. Prior to infection, two pools of Atlantic salmon plasma contained an average of 1.34×10^{-11} M PGE_2 (Fig. 6.4). At 9 dpii the PGE_2 concentration determined for pooled plasma from infected fish was approximately double what it was for pooled plasma from uninfected fish, which remained at a similar level to that observed prior to infection (Fig. 6.4). At 33 and 40 dpii, PGE_2 levels were higher in infected fish when compared to uninfected fish; however PGE_2 levels from uninfected fish had decreased relative to levels seen earlier in the experiment (Fig. 6.4).

Expression of IL-1β was detectable in uninfected and infected fish on all days sampled (Fig. 6.5). At 9 and 40 dpii there was significantly higher IL-1β expression in infected fish when compared to uninfected fish. However, at 21 and 26 dpii, expression in infected fish was significantly lower when compared to uninfected fish (Fig. 6.5). There was no significant difference in IL-1β expression between the groups at 33 dpii.

Figure 6.4: Mean (±SEM) prostaglandin E_2 concentrations in the plasma of infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. Arrow denotes time of second infection.

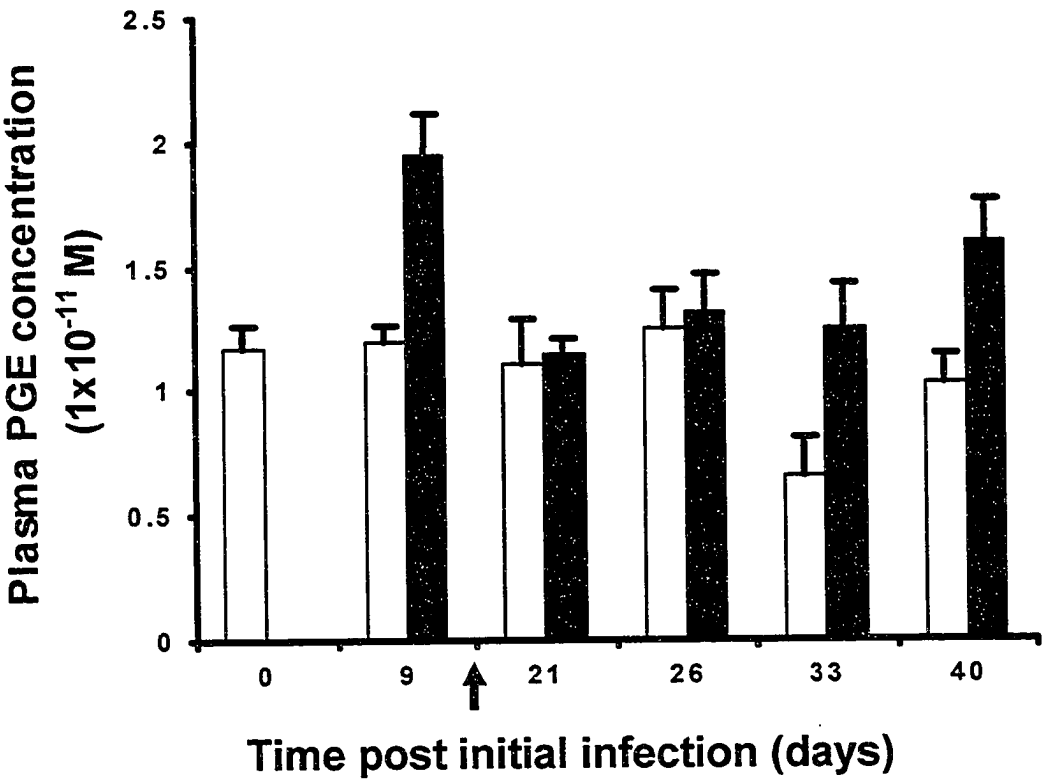
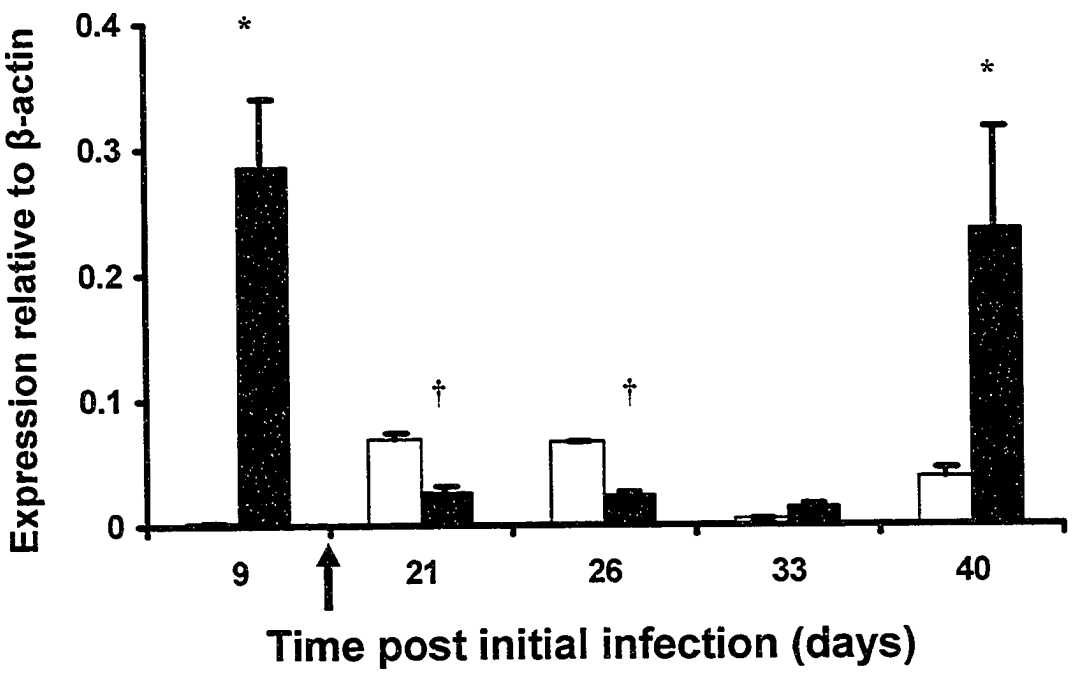


Figure 6.5: Mean (±SEM) expression of Interleukin-1β gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.



Expression of the TNFα-like gene was significantly higher in infected fish when compared to uninfected fish at 9 dpii, 26 dpii, 33 dpii and 40 dpii (Fig. 6.6). After the second infection process (7 dpri), there was no TNFα-like gene expression in either uninfected or infected fish, however it increased at subsequent time points (Fig. 6.6).

Similar to the TNFα-like gene, COX-2 expression was not observed in uninfected fish at 9 dpii or in either uninfected or infected fish following re-infection at 21 dpii (Fig. 6.7). In contrast to IL-1β and TNFα-like gene, COX-2 expression was much lower and exhibited greater variability. Infected fish head kidneys expressed higher levels of the COX-2 gene at 9, 26 and 40 dpii, however, the expression was only significantly higher at 9 dpii, when compared to uninfected fish (Fig. 6.7).

Transforming growth factor β–like gene was expressed at relatively higher levels than the other cytokine genes and was detected in both uninfected and infected fish on all sampling days (Fig. 6.8). Similar to COX-2, TGFβ-like gene was elevated in infected fish at 9, 26 and 33 dpii when compared to uninfected fish, but was only significantly higher at 9 dpii (Fig. 6.8). Although TGFβ-like gene expression increased from 21 to 33 dpii in infected fish, the high degree of variability did not result in further significant increases. While, a strongly negative correlation (0.868) between TGFβ-like gene expression and plasma cortisol was observed in uninfected fish, there was no correlation between TGFβ-like gene expression and cortisol, lice counts or other gene expression in infected fish.

Figure 6.6: Mean (±SEM) expression of tumour necrosis factor α-like gene , relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.

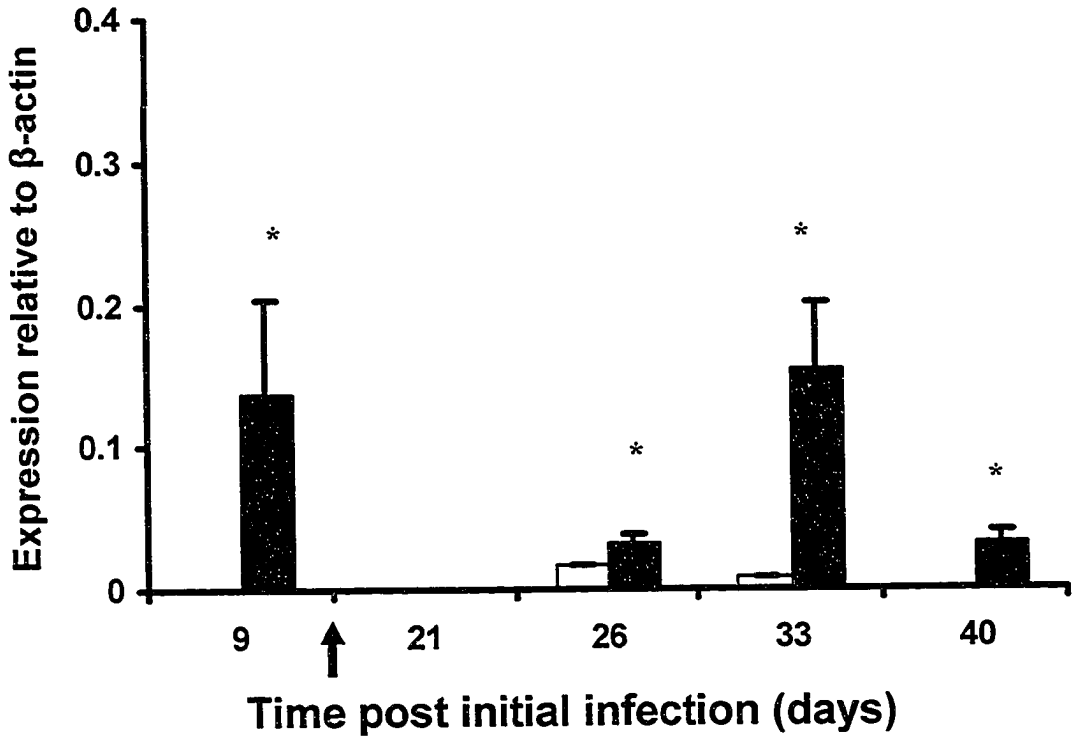


Figure 6.7: Mean (±SEM) expression of cyclopoxygenase-2 gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.

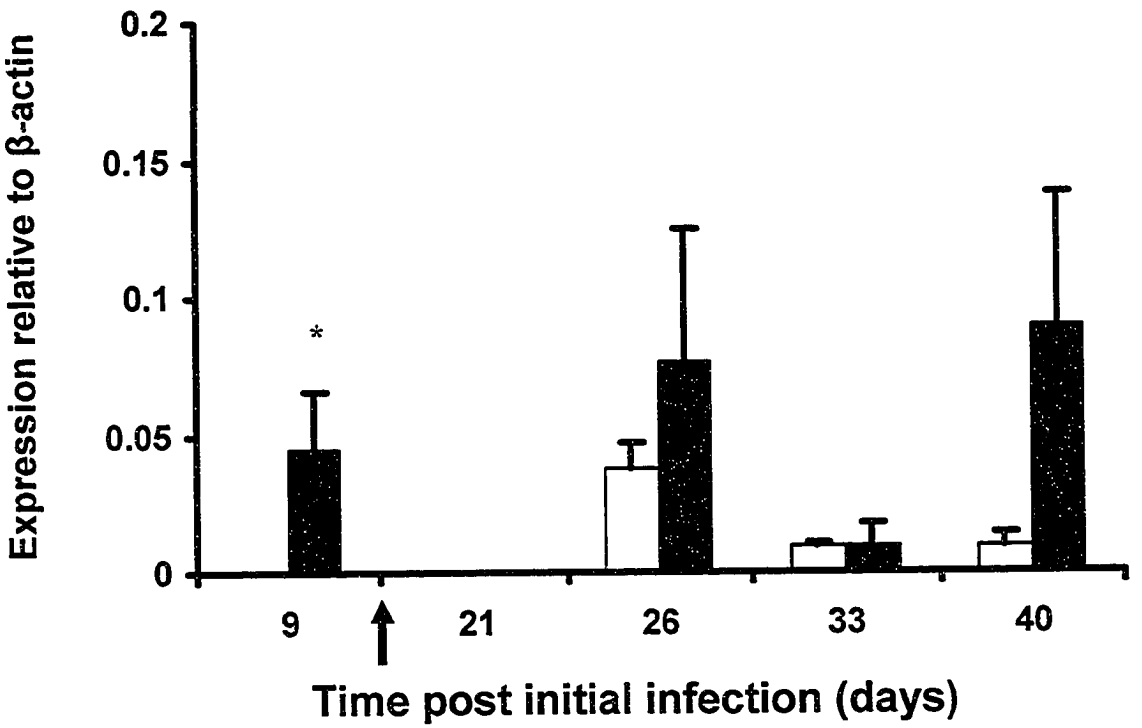
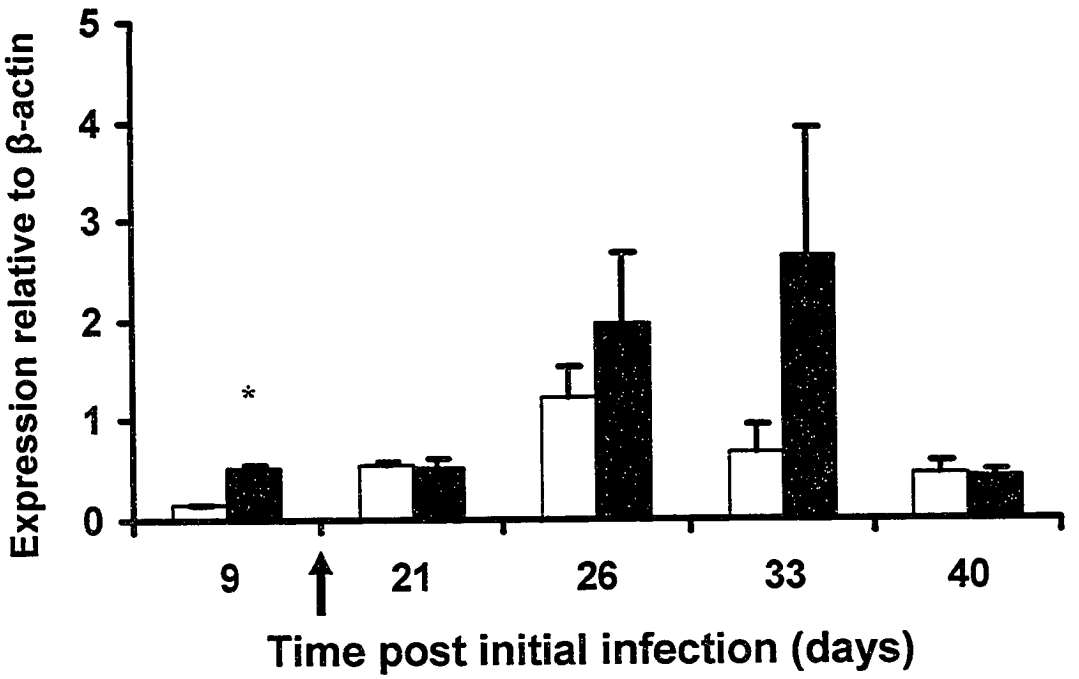


Figure 6.8: Mean (±SEM) expression of transforming growth factor β –like gene, relative to β -actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.



Expression of the MH class I and II genes exhibited variability over time. Initial expression in uninfected fish of the MH class I gene was 1.5 ERB and decreased throughout the rest of the experiment. Similarly, infected fish head kidney expression of MH class I peaked at 21 dpii and decreased over the rest of the experiment (Fig. 6.9). Major histocompatibility class I expression was significantly lower in infected fish at 9 and 40 dpii and significantly higher at 21 and 26 dpii, when compared to uninfected fish (Fig. 6.9). Expression of the MH class I gene showed a strongly positive correlation with plasma cortisol values in uninfected fish over time (0.901), however, there was no correlation between MH class I gene expression and cortisol, lice counts or other gene expression in infected fish.

Expression of the MH class II gene was absent from uninfected fish at 9 dpii and both uninfected and infected fish following re-infection (21 dpii), similar to COX-2 and TNFα-like gene (Fig. 6.10). Expression of the MH class II gene was significantly higher in infected fish when compared to uninfected fish at 9, 26, 33 and 40 dpii (Fig. 6.10). The highest expression of MH class II gene was observed at 26 and 40 dpii (Fig. 6.10). The expression of MH class II gene showed a strongly negative correlation with plasma cortisol values in infected fish over time (0.608) (Figs. 6.3, 6.10). This was due to strong negative correlations on 26, 33 and 40 dpii.

Figure 6.9: Mean (±SEM) expression of major histocompatability class I gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$). Arrow denotes time of second infection. † Significantly lower expression than uninfected fish on that day ($p < 0.05$, n=8 for each group).

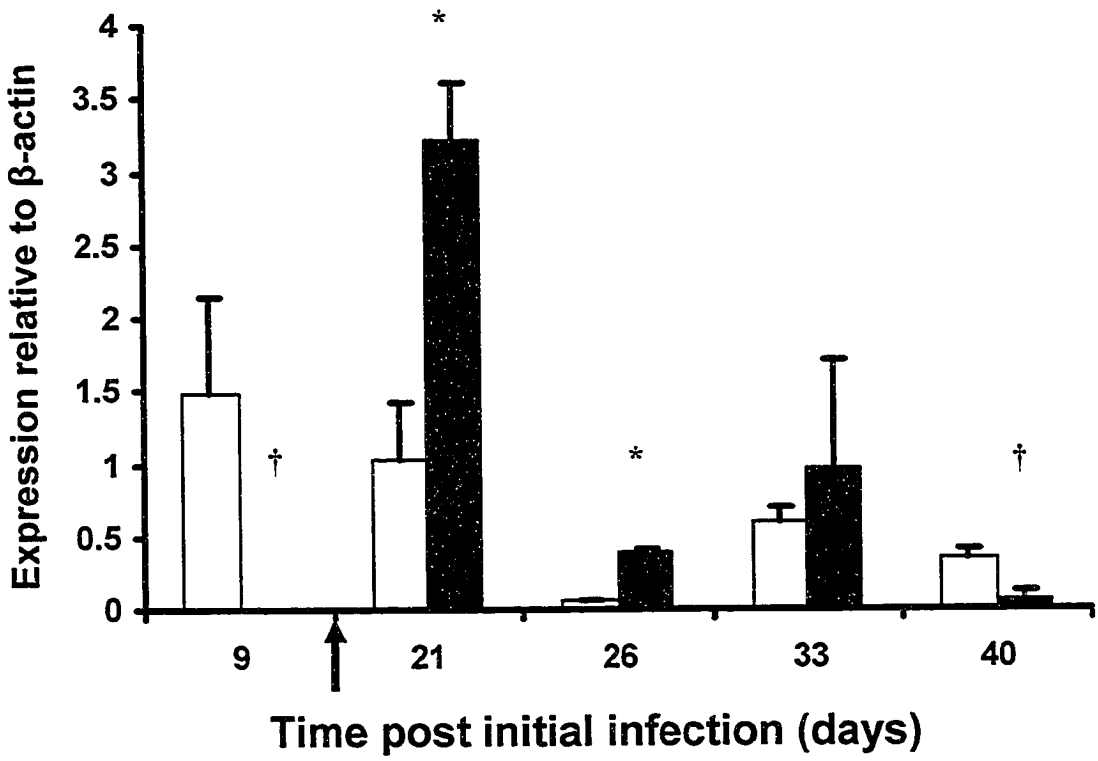
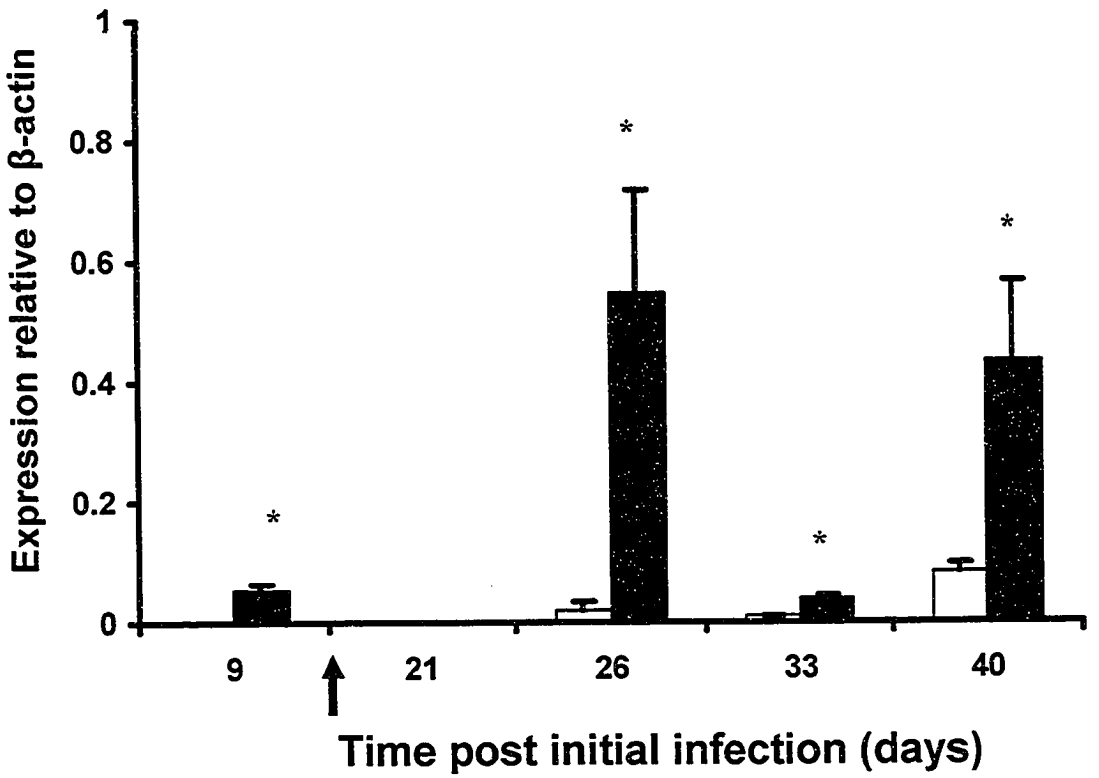


Figure 6.10: Mean (±SEM) expression of major histocompatability class II gene, relative to β-actin, in head kidneys of *Lepeophtheirus salmonis*-infected (■) and uninfected (□) Atlantic salmon at various days post initial infection. * Significant differences from uninfected fish on that day ($p < 0.05$, n=8 for each group). Arrow denotes time of second infection.



6.6 Discussion

The importance of Atlantic salmon in aquaculture and its susceptibility to infection with *L. salmonis*, has lead researchers to investigate genetic resistance in Atlantic salmon towards this parasite (Kolstad et al., *in press*; Jones et al., 2002), as well as the possibility of vaccine development (Raynard et al., 2002). While the development of a vaccine against *L. salmonis* and genetic selection of resistant Atlantic salmon strains are still several years away, progressing towards a better understanding of the host-parasite relationship should accelerate the process to reach these goals. In this chapter I studied the effect of repeated *L. salmonis* exposure on the stress response and immune-related gene expression in Atlantic salmon. These data complement Chapter 5.0 in showing differences between single low-level infections and re-infection at higher levels to provide a clearer picture of the host's response to this parasite.

In this study, numbers of *L. salmonis* arising from the first infection remained relatively constant over development. Following the re-infection lice numbers declined from an average of 142 lice per fish at 12 dpri to an average of 50 lice per fish at 26 dpri (65% decline). This decline in *L. salmonis* numbers from the second infection could be due to: routine mortality and loss during moulting of the parasite, the loss of mobile stages during sampling, older stages from the first infection interfering with survival and attachment of the younger stages as well as priming of the host immune response from the first infection.

Delayed maturity and/or rapid reductions (<10 d) in *L. salmonis* numbers as seen in relatively more resistant host species, such as chinook (*Oncorhynchus tshwatscha*) and coho salmon (*O. kisutch*), was not observed (Fast et al., 2002; Johnson, 1993; Johnson and Albright, 1992a). Development rates of *L. salmonis* were nearly identical between the first and second infections, both reaching ca. 30-50% pre-adults and 50% adult males by 26 dpi. Similarly, the appearance of adult males (50%) and adult females (20%) by 26 dpri, is in agreement with previous observations of the development rate of *L. salmonis* on naïve Atlantic salmon (Dawson et al., 1997; Johnson, 1993). Reductions in lice number (65%) observed from 26 dpii (12 dpri) to 40 dpii (26 dpri) are similar to previous findings over the same time (3-5 weeks post infection) period (Dawson et al., 1997). Therefore it is most likely that the reductions observed after re-infection were attributable to mortalities or loss at moulting and the loss of mobile lice during sampling.

There may have been a founder effect, where older (initial infection) established *L. salmonis* either dislodged or out competed *L. salmonis* from the second infection. *Lepeophtheirus salmonis* from the first infection did not exhibit large changes in density over the course of the infection, whereas *L. salmonis* from the re-infection did. Priming of the immune response following initial infection may have reduced *L. salmonis* from the re-infection. This has been suggested to occur in another species of parasitic copepod. In *Lernaea cyprinacea* it was shown that previously infected gouramis, *Helostoma temmincki*, were resistant to future infection with the copepod, unless the infection dose was very high, in which case hosts were susceptible (Woo and Shariff, 1990). As the re-infection

was carried out 2 weeks after the initial infection and reductions were observed within the following 3 weeks it is unlikely that this was due to a host antibody response. Salmon often do not show significant antibody responses to immunization until after 6 weeks at the water temperature used in this study (Grayson et al., 1995; Gudmundsdottir et al., 1997).

The increase in *L. salmonis* numbers observed from 21 dpii to 26 dpii (25%) may be due to losses of copepodid stages from the re-infection at 21 dpii (7 dpri), thereby underestimating numbers at that time point. In *L. salmonis* infections in sea trout (*Salmo trutta*), >10% increases in *L. salmonis* numbers have been observed between 7 and 14 dpi (Bjorn and Finstad, 1997).

Fish in this study had resting cortisol levels of 34.7 µg/ml prior to the infection which in comparison to other work may be considered high (Fast et al., 2002; Pickering and Pottinger, 1989). Whereas short-term acute stressors may prime an immune response and result in decreased parasite loads, chronic or daily handling stressors increase host susceptibility (Haond et al., 2003; Nolan et al., 2000; Saeij et al., 2003b). However, resting cortisol levels in this study were not different from those seen in other groups of fish obtained in different years from the same source (Chapter 5.0) and may be characterisitic of this population of Saint John River strain Atlantic salmon. In this study plasma cortisol levels generally increased as *L. salmonis* developed towards the mobile pre-adult and adult stages, as seen in other studies (Bowers et al., 2000; Ross et al., 2000). This decrease observed in both uninfected and infected fish at 26 dpii, may have been due to sampling of significantly larger fish at this time point, which may have been less stressed by the number of copepodids present when compared to smaller hosts.

Average plasma PGE_2 levels of 1.34×10^{-11} M, observed prior to infection, are similar to those found in healthy humans (Samuelsson, 1973). The observed elevation of PGE_2 in infected fish confirms the report that PGE_2 was elevated in *L. salmonis*-infected individuals, which was based on unpublished data (Pike and Wadsworth, 1999). Prostaglandin E_2 levels in infected Atlantic salmon are in the same range (5.33×10^{-11} M) as those observed in rainbow trout head kidney leucocytes following calcium ionophore stimulation (Rowley et al., 1995). As *L. salmonis* secretions contain PGE_2 it is possible that some of the PGE_2 measured was parasite-derived (Chapter 2.0, published in Fast et al., 2004). The EIA kit used does show some cross reactivity with PGE_1 and PGE_3, in mammalian samples. Therefore a portion of the observed changes may not be fully attributable to PGE_2.

In chapters 2.0 and 3.0, the role of PGE_2 in toxic shock was discussed. While PGE_2 increased in infected fish at 3 time points, levels were not in the order of 2-3-fold higher as is often the case in shock models of mammals (Lefer, 1983). Toxic shock-like mortalities in Atlantic salmon infected with *L. salmonis* often occur following the parasitic moult to the pre-adult stage (Ross et al., 2000). The PGE_2 levels at 33 and 40 dpii, where large numbers of pre-adult and adults were observed, although higher in infected fish, were no different from PGE_2 levels at the beginning of the experiment. While it does not appear that PGE_2 levels in infected fish increased towards a "toxic" level, there was no morbidity or mortality in these fish either, and therefore may not have been expected to be observed at this infection level. Although the numbers of *L. salmonis* at the end of the experiment were high (96.5 lice/fish) these are still below the level (ca. 200 lice per fish) at which

large scale mortality with the moult to the mobile stages has been reported (Ross et al., 2000).

Increased head kidney expression of IL-1β in infected fish was observed at 9 and 40 dpii. This result is dissimilar to findings of Chapter 5.0, where IL-1β expression significantly increased at 21 and 22 dpi in infected fish. While Chapter 5.0 did not include time points prior to 12 dpi, the 21 dpi time point was shared by both studies. The lack of response in the current study at 21 dpii, is probably due either to re-infection or the higher level of infection.

The expression of TNFα-like gene increased in head kidneys of infected fish at 9, 26, 33, and 40 dpii. However, as in the case for IL-1β, this increase was not observed at 21 dpii (7 dpri). In the head kidneys of rainbow trout infected with *Ichthyophthirius multifiliis*, TNFα-1 was observed to increase at 4 and 26 dpi but not at 6 dpi (Sigh et al., 2004b). The low-levels of *L. salmonis* infection observed in Chapter 5.0, however, did not elicit a response in head kidney expression of TNFα-like gene, suggesting that re-infection and/or the higher level of infection resulted in a different response.

Increased expression of IL-1β and TNFα-like gene in infected fish corresponded with elevated PGE_2 levels at 9 and 40 dpii. While these inflammatory mediators were observed to increase at 9 dpii, they dropped in the subsequent 2 weeks. This suggests that while initial infection may elicit a systemic inflammatory response it is quickly resolved, probably due to early parasite stages not resulting in much damage to host tissues. However, as the infection progresses, especially at higher lice burdens, mobile stages elicit a second inflammatory response, which is sustained for a longer period of time, perhaps due to the immune system's inability

to remove the parasitic stimuli. This second response to *L. salmonis* infection may be a precursor to shock or morbidity as seen in rainbow trout infected with *Ichthyopthrius multifiliis* (Chapter 3.0, published in Fast et al., *in press*; Sigh et al., 2004b).

Up-regulation of COX-2 in head kidneys of infected fish was only observed at 9 dpii. Similar to Chapter 5.0, COX-2 expression was not observed to change in infected fish at 21 dpi. This suggests that any effect *L. salmonis* infection has on COX-2 expression in Atlantic salmon head kidneys probably occurs early on (≤9 dpi). The increase in COX-2 expression in head kidneys of infected fish was observed on the same day as the highest plasma PGE_2 concentration. As PGE_2 is a product of COX-2 activity, up-regulation of COX-2 and a subsequent increase in PGE_2 is not surprising (Chapter 3.0, published in Fast et al., *in press*; Tjandrawinata and Fulford, 1997). However, the increased expression of COX-2 found in head kidneys of infected fish was not observed at 33 or 40 dpii and therefore suggests that either the source of PGE_2 was not due to input from the head kidney or the activity of COX-2 in this case was not related to expression levels.

While TGFβ-like gene, similar to COX-2, showed significantly higher gene expression in infected fish only at 9 dpii, it appeared to increase further up to 33 dpii. Transforming growth factor β has numerous effects on the immune system including inhibition of macrophage activation, the production of pro-inflammatory agents, and inhibition of T-cell differentiation (Bogdan and Nathan, 1993; Gorelik and Flavell, 2002). Increasing expression of TGFβ-like gene over time may be in response to increasing expression of inflammatory mediators IL-1β and TNFα-like gene and/or PGE_2, as a means of homeostatic control (Gorelik and Flavell, 2002). Tick

(*Rhipicephalus sanguineus*) infestations and tick saliva also stimulate TGFβ secretion by cells of C3H/HeJ mice (Ferreira and Silva, 2001).

Expression of the MH class II gene increased at 9, 26, 33 and 40 dpii in infected fish compared to uninfected fish, but not at 21 dpii as seen previously (Chapter 5.0). Expression of MH class II in the head kidneys of rainbow trout infected with *I. multifiliis* showed significant increases compared to uninfected fish at all three time points observed after 2 dpi (4, 6 and 26 dpi) (Sigh et al., 2004a). The relative level of MH class II expression in infected fish was lower than that seen during low-level single infections with *L. salmonis* (Chapter 5.0). It may be that due to the second infection, elevation of MH class II expression was delayed from 21 dpii to 26 dpii, which was also observed during the second infection at 26 dpri. This is in contrast to what was seen for carp infected with *T. borelli* and rainbow trout infected with *G. derjavini*, where MH class II gene expression in head kidney and skin tissues was depressed shortly after infection compared to uninfected fish (Lindenstrom et al., 2004; Saeij et al., 2003a). As MH class II molecules are important in presenting extracellular pathogenic peptides to T helper cells, it is not unexpected to find this gene upregulated during parasitic infection. Similar to previous work, MH class I gene expression was depressed in infected fish compared to uninfected fish, but only after the re-infection (26 dpri). Previously, MH class I gene expression was decreased at 21 and 22 dpi with *L.salmonis* (Chapter 5.0). Again, the Atlantic salmon immune system appears to be responding differently under multiple and heavy infection pressure. Here MH class I gene expression was elevated at 21 and 26 dpii or 7 and 12 dpri.

Expression of the MH class I gene had a highly positive correlation with cortisol in control fish in this study. Over this same time period, a negative correlation was found between TGFβ-like gene and cortisol in the same fish. Positive correlations have previously been found between cortisol and lysozyme in Atlantic salmon and rainbow trout (Fast et al., 2002). Small increases in cortisol, as seen in uninfected fish, can have an immunostimulatory effect (Nolan et al., 2000). As TGFβ is a potent inhibitor of cytotoxic T lymphocyte activation, which involves MH class I molecule presentation, a negative correlation between these two genes is expected (Gorelik and Flavell, 2002). Conversely, in infected fish, a negative correlation was found between MH class II gene expression and cortisol. As high levels of circulating cortisol, as seen in infected fish, result in apoptosis of lymphocytes and other cells, we may be observing a subsequent apoptotic loss of cells, that highly express MH class II genes (van der Salm, 2000). As these correlations were strong only after the re-infection, it suggests there is a cortisol threshold beyond which negative impacts can be observed on MH class II expression and possibly MH class II expressing cells. Cortisol administration inhibits responses in carp to *T. borreli* in terms of expression of TNFα and IL-1β as well as inducible nitric oxide sythetase and serum amyloid A (Saeij et al., 2003b). Similarly, we observed IL-1β and MH class II expression in infected fish to be lower than that seen in fish with low-level infections without elevated cortisol (Chapter 5.0).

Mammalian host responses to long-term endoparasitism often mirror those seen in successive ectoparasitic infections (Lawrence and Devaney 2001; O'Neill

et al., 2001; Schoeler et al., 1999; 2001). Briefly, a class switching occurs in which Th2 cytokines are up-regulated in favour of Th1 cytokines. As *L. salmonis* is both an ectoparasite and maintains a long-term association with its host, we expect and indeed found that it would elicit a similar response from its hosts. Atlantic salmon exhibits similar initial responses to *L. salmonis* infection as is seen in host-endoparasitic relationships of other fish. Primary infection does not appear to provide any added protection to Atlantic salmon, from *L. salmonis* re-infection, within the first 6 weeks. As was mentioned above, however, the 2-week period prior to re-infection would not have been long enough to immunize the fish and provide a significant antibody response towards re-infection.

The initial increases in expression of genes for inflammatory mediators, such as COX-2, IL-1β, TNFα-like cytokine, TGFβ-like cytokine and even PGE_2 appear to subside as juvenile attached parasites progress through the chalimus stages and possibly in conjuction with re-infection. Interestingly, expression of inflammatory genes (COX-2, IL-1β, TNFα 1) in the skin of rainbow trout infected with *G. derjavini* show these same initial increases (8 dpi), which are not present following secondary infection at the same time point (Lindenstrom et al., 2003; 2004). As *L. salmonis* infections develop into mobile pre-adults and more mature stages, cortisol levels as well as the expression of pro-inflammatory mediators (IL-1β and TNFα) studied here increase. Increases in pro-inflammatory cytokine expression may be a result of the host's inability to resolve infection and lead to an unregulated systemic inflammatory response and TNF-α-mediated toxic shock as seen in Chagas disease (Holscher et al., 2000). The increases in anti-

inflammatory molecules such as PGE_2 may be an attempt to counter these pro-inflammatory cytokines by the host to retain homeostasis, or from the parasitic secretions to assist in feeding. In the future it is hoped that a significant number of antibodies will become available for Atlantic salmon cytokines, and the effects of pro-inflammatory gene elevation in this model will become clearer.

Chapter 7.0 Conclusion

The economical importance of parasitic copepods has initiated numerous studies into the physiological, pathological and biochemical effects of infection, but immunological studies of parasitic copepod host-parasite relationships have rarely been undertaken. The first studies looking at the host's immunological status during *L. salmonis* infection showed that over time, the lymphocyte percentage of total white blood cells decreased, macrophage phagoctosis and respiratory burst decreased and susceptibility to secondary infection increased in infected hosts (Bjorn and Finstad, 1997; Mustafa et al., 2000a; b). These were all attributed to high cortisol levels in infected fish. In a later study, macrophage function decreased in infected individuals without correspondingly high cortisol levels (Fast et al., 2002). Based on these results and the available information for other arthropod parasites the question of whether *L. salmonis* actively modulated the host's immune response, as a means of feeding and maintenance on the host, was posed.

The discovery of *L. salmonis*-derived trypsin-like proteases in the mucus of Atlantic salmon, provided the first evidence of a potential immunomodulatory compound secreted/excreted by this parasite (Fast et al., 2002; Firth et al., 2000; Ross et al., 2000). It was suggested that the trypsin-like protease Hypodermin A, which cleaves C3, thereby inhibiting the complement pathways, as well as other inflammatory and immune reactions, is used by *Hypoderma* spp larvae for survival in their hosts (Sandeman, 1996). Further evidence towards trypsin's possible role in the *L. salmonis*-

salmonid host relationship was revealed when trypsin-like proteases were found to be secreted/excreted more consistently in the presence of mucus from susceptible host species' as compared to mucus from resistant or non-host species (Fast et al., 2003).

Trypsin was previously the only known *L. salmonis* secretory/excretory product (SEP). Finding PGE_2 in the SEPs and SPs of *L. salmonis* indicated that immunomodulatory compounds were produced by the parasite. The amount of PGE_2 produced per parasite was also in agreement with amounts found in the saliva of other arthropod ectoparasites. While it has been postulated that PGE_2 may be used by other parasites for vasodilatory or anti-inflammatory activities, its effects on salmonid cells were unknown. Through the use of a salmonid cell line (Chapter 3.0), it was shown that, at physiological concentrations, PGE_2 inhibited lipopolysaccharide (LPS)-induced expression of immune-related genes: interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and the major histocompatability (MH) class I and II. Therefore, PGE_2 could be used by *L. salmonis* not only to increase blood to the feeding site but to prevent leucocyte recruitment and presentation of parasitic antigens to T lymphocytes. This agreed with work showing no inflammation at the site of *L. salmonis* feeding, but in the periphery, in Atlantic salmon epithelia (Johnson and Albright 1992a; Jonsdottir et al., 1992).

As secretions of many ectoparasitic arthropods contain other immunomodulatory compounds, it was expected that this would also be the case for *L. salmonis*. In Chapter 4.0, it was observed that fractions containing

trypsin and PGE_2 had inhibitory capabilities towards IL-1β, as well as those fractions that did not contain trypsin and PGE_2. A few of the peptides found within these fractions exhibited increased gene expression in *L. salmonis* after infection of salmonid hosts, suggesting that they are involved in evasion of the host's immune response. Excretory/secretory products of *L. cuprina* cleave MHC II surface proteins of macrophages (Sandeman, 1996). Cleavage of MH class II molecules from macrophages and a subsequent failure to present parasite antigens to T helper cells in salmonid hosts may explain why increased expression of this gene during infection (Chapters 5.0 and 6.0), does not assist in mounting a response that is detrimental to *L. salmonis*.

At infection levels similar to those found naturally on wild salmonid populations, changes in immune-gene expression in tissues away from the site of attachment and feeding were observed (Chapter 5.0). Increases were observed in MH class II expression and decreases in MH class I gene expression, most likely due to a change in lymphatic tissue cell populations in response to parasitic infection. While an increase in IL-1β expression at the feeding site (skin) may occur early in infection, it may also be resolved by the immunomodulatory capabilities of the parasitic secretions. As *L. salmonis* infection continues, increased expression of IL-1β and other pro-inflammatory genes in the head kidney would probably result in tissue damage due to the more detrimental mobile stages of *L. salmonis*. During the course of re-infection no added protection appears to be provided by the acquired immune system, at least within

the first 6 weeks, and declines in parasite density do not differ from single infections of previously naïve hosts (Chapter 6.0).

At high infection levels cortisol and pro-inflammatory cytokine expression elevation may be a result of the host's inability to resolve infection due to *L. salmonis* immunomodulation of the feeding site (Chapter 6.0). The constant stimulation by large numbers of parasites all over the body may then lead to an unregulated systemic inflammatory response and TNF-α-mediated toxic shock. This may explain sudden fish-kills observed in heavily infected fish shortly after the parasitic moult to the pre-adult stage.

In summary my thesis research has demonstrated that the nature of the *L. salmonis* host-parasite relationship with Atlantic salmon shares many similarities with that of terrestrial arthropod parasites and their hosts. This is not particularly surprising since teleost immunology has begun to be recognized as quite similar to that in higher vertebrates. This is especially true with respect to innate immunity due to the length of time required for antibody and adaptive responses to occur. Evidence of convergent evolution in parasitic relationships with their hosts, is already present in the phylum Arthropoda, as seen between the blood-feeding flies (Class: Insecta) and mites and ticks (Class: Chelicerata), and now copepods. This suggests that immunomodulation at the feeding site is a successful strategy for these parasites in coping with host inflammation, in order to feed and/or remain on the host. Furthermore, commonly used pro- and anti-inflammatory compounds, such as proteases and prostaglandins, appear to be the most successful and widespread, in terms of use, across the different

parasitic groups. A caveat is that proteases and prostaglandins may just be the most easily discovered, or the only ones investigated, of all the parasitic immunomodulatory compounds.

Several factors make studies of parasitic copepod host-parasite relationships difficult. There is a large amount of variability between hosts with respect to physiological and immunological responses, making it difficult to demonstrate significant trends in these responses. Studies on higher vertebrates such as inbred mice do not have this problem. Another problem is that due to the lack of a reliable method to maintain and produce *L. salmonis* in the laboratory it is necessary to collect ovigerous females from the field to produce copepodids for infection trials. As collection from a species at risk of endangerment (Atlantic salmon) has its own problems, *L. salmonis* are obtained from Atlantic salmon routinely treated for this parasite (i.e. fish farms), resulting in very low abundances. Availability of proper cell lines is another hurdle, which must be overcome in the study of this host-parasite relationship as well as other aspects of fish immunology. In Chapters 3.0 and 4.0, I used the SHK-1 cell line to characterize whether *L. salmonis* secretions could affect salmon immune-gene regulation, but further characterization of this and other cell lines is needed for future work on fish immunology.

7.1 Future research directions

As fish culture is constantly expanding to include new and diverse species, so must we expect the possibility of encountering new and diverse species of parasitic copepods that thrive under culture conditions. As it stands now,

parasitic life cycles and host-parasite interactions are understood at a very basic level for a few well-studied species, but a large gap exists in our knowledge of most others. There are serious limitations of the data that does exist and evidences the need for controlled laboratory experiments to confirm observations made from natural infections. Often we are faced with making conclusions from data biased towards heavy infection levels of these parasites, which limits our ability to understand the underlying host-parasite relationship. We must then tease apart the data and see which effects can be attributed to the parasite and to which we must attribute effects from the host stress response. Further development in the area of fish immunology is needed. Advances in host-parasite interactions in mammalian systems are expedited by the availability of antibodies towards an array of mouse cytokines as well as knock–out mice lineages. Current construction of salmon and other teleost genomes will no doubt lead to these possibilities in the near future. Combine that with available microarray and Real-Time PCR techniques and unlocking the intricacies of parasitic copepod interactions with their hosts will definitely become more attainable.

As suggested by Schoeler and Wikel (2001), EST databases must be expanded to include more ectoparasitic arthropod information. So far very few have been done and genes unique to these organisms are lacking in available EST databases. *Amblyomma americanum*, *Anopheles gambiae*, *B. microplus* , and *Lutzomyia longiplapis*, are the only parasitic arthropods extensively added to dbESTY in GenBank (Schoeler and Wikel 2001). Parasitic copepod data are not

present in any of the existing public databases with the exception of several trypsin genes. With this information, proteomic studies on parasitic copepod secretions could be conducted followed by expression of these bioactive molecules and cell-based testing. Investigations into parasitic copepod secretions are limited by the large number of animals needed to collect relatively minute volumes of secretions.

Using the above technologies, genes centrally involved in maintaining the host-parasite relationship can be more efficiently isolated and possibly targeted for vaccine development. Finally, the relationship parasitic copepods have with other aquatic pathogens and their ability to act as vectors needs study. Currently, we lack evidence to support copepods as efficient vectors of disease, yet the similarities they share with other arthropod parasites suggest they could. The economic impact of parasitic copepods is no doubt dwarfed by that of their terrestrial parasite cousins, however, these impacts are not small and growing aquaculture industries will benefit from further study in these areas.

Appendix I

Salmon gene sequences

Cox-2 (13X Coverage)

ATCTTACTCACTACAAAGGGGTGTGGAATGTCATCAACAAGATCACTTTTGTGAGAA
ATGCTATCATGAGCTATGTCTTGACATCCCGCTCACATTTGGTGGACAGCCCACCG
ACTTACAATTCTGATTATGGCTACAAGAGCTGGGAAGCTTACTCCAACCTGTCTTAC
TATACACGGACCCTTCCCCCATTGCCAAAGGGCTGTCCTACACCTATGGGAACTGC
AGGAAGAGCCGTGCTTCCAGATGCCAAGCTTGTGGTGGAGAAGGTTATGTTGAGG
AAGCGGTTCATTCCGGATCCTCAGGGTTCCAATCTTATGTTCGCCTTCTTCGCCCA
GCACTTCACCCACCAGTTCTTCAAATCAGACTTCATGAAAGGACCAAGC

IL-1β (8X Coverage)

TACTGTGATGTACTGCTGAACCCTGCTTCCCTCCTGCTCGTAGGGGGCGCCGACTC
CAACTCCAACACTATATGTTCTTCCACAGCACTCTCCAGCAAGAAGTTGAGCAGGT
CCTTGTCCTTGAACTCGGTTCCCATGGTAACCCCCTCGCCACCCTTTAACCTCTCC
ATGGCGATGATGAGGTTGGCAATGTGACGCATGGTGATGGGGTGATGGGATACCT
CCAGATCCAGACCCTGAGGCAGCTTGGAGCTCCATGCCTTACTTTCAGAGGCGTTC
TTTATTAAACTGTAGTTTGACTCAAATTCCATGTCTGTATAGTTAGATCTTGAGTTGT
TTTGTAGTTTGTAGGTAGGTAACAGTGTCTTGTATTCAGTCCTTAGTTCTTGTGAATC
CA

TGF$\beta_{1/5}$ (10X Coverage) (100% identity to rainbow trout)*

ATCGGAGAGTTGCTGTGTCGAAAACTTTACATTGACTTCCGTAAGGACCTGGG
CTGGAAGTGGATCCATGAACCCACTGGCTACTTTGCTAACTACTGCATCGGCCC

TNFα (6X Coverage) (100% identity to rainbow trout)*

GGCGAGCATACCACTCCTCTGAGTCACATATTTGGCGCTATTCGGACTCCATCGGG
GTTAATGCTAATCTTCTTAGCGGGGTAAGGTCAGTTTGTCAACAAAACTACGGTGAT
GCTGAGTCCGA

* Variability in sequencing (≈5%)

Appendix-II

MS results

Figure A.1: Time of flight - Mass spectrometry scan of the tryptic peptide, IAVSDITYHEK (a) and TOF-MS 2 of IAVSDITYHEK at 7.25 min (b).

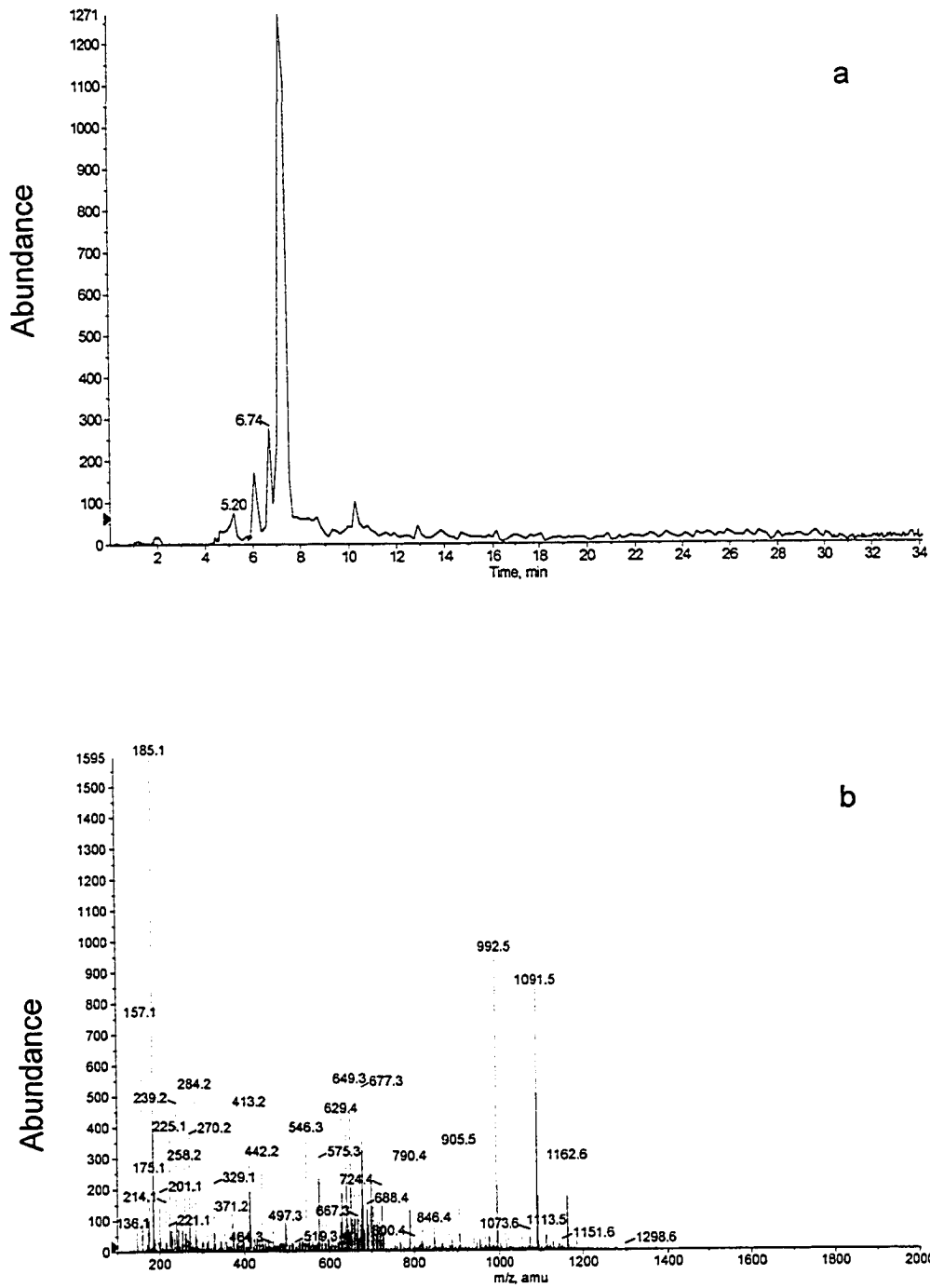
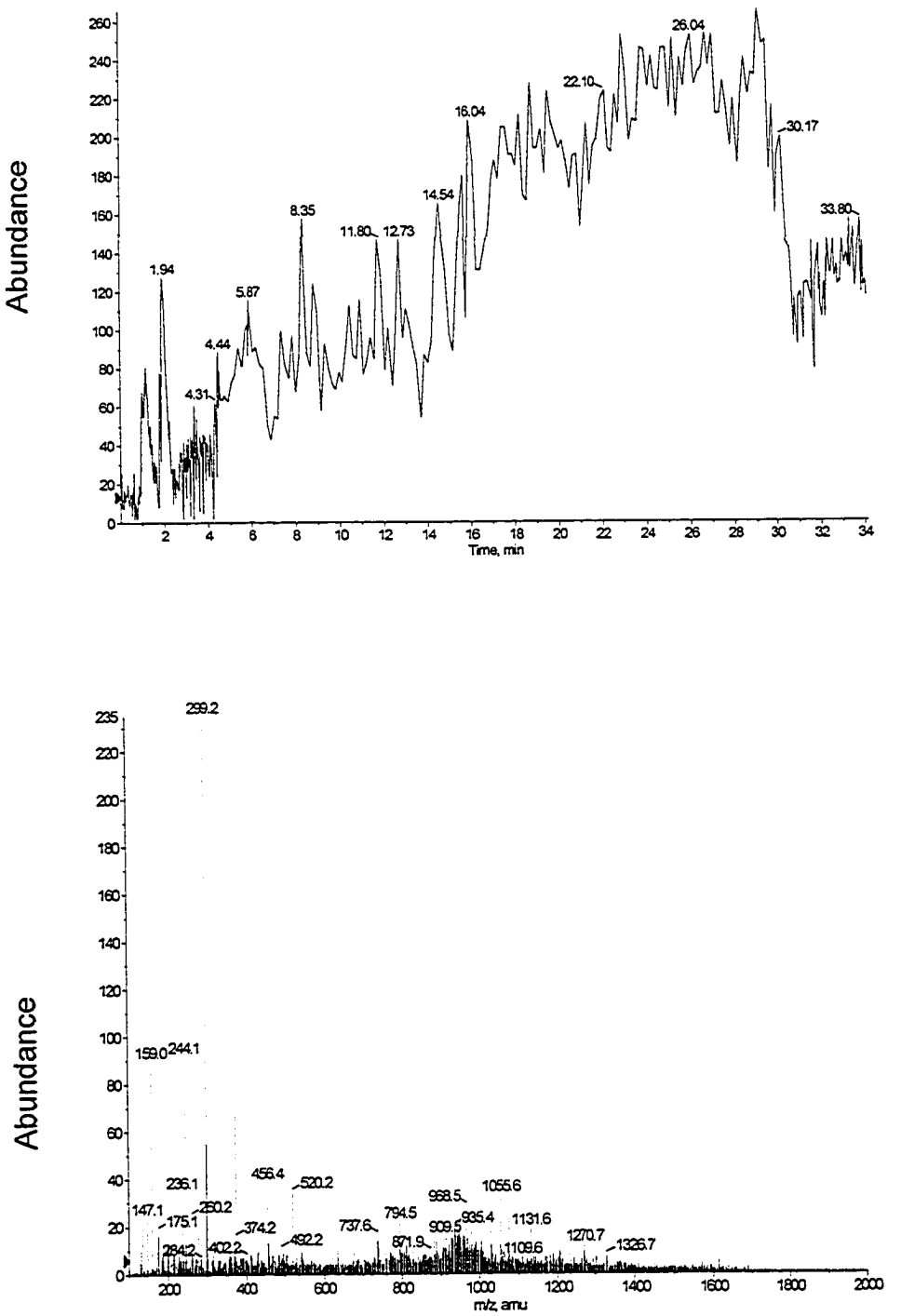


Figure A.2: Time of flight - Mass spectrometry scan of the tryptic peptide, DQEFIGDVVVSGWGTISSSGPPSPVLK (a) and TOF-MS 2 of DQEFIGDVVVSGWGTISSSGPPSPVLK at 20.61 min (b).



Appendix III

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