Host – parasite interactions between *Lernaeocera branchialis* (Copepoda: Pennellidae) and its host *Gadus morhua* (Teleosti: Gadidae).

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By

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To my darling Rémi, and my always supportive family without whom I never would have got this far. Thank you x

Declaration

I declare that this thesis has been compiled by myself, and is the result of my own investigation. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Sarah Elizabeth Barker

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Abbreviations

α2M: alpha-2-macroglobulin

AB-PAS: alcian blue pH 2.5 – periodic acid Schiff's

AG: antennal gland

APES: 3-aminopropyltriethoxysilane

Apol: apolipoprotein A-I

ASC: antibody secreting cells

BLASTN: Basic Local Alignment Search Tool nucleotides

cDNA: complementary deoxyribonucleic acid

CDR: complement determining region

CFT-G: complement fixation test dilution buffer with 0.1% (w/v) gelatine

CG: circum-oral gland

conA: concanavalin A

DAB: 3', 3 diaminobenzidine tetrahydrochloride

DEPC: diethylpyrocarbonate

DHR123: dihydrorhodamine-123

$DiOC_6(3)$: 3, 3-dihexyloxacarbocyanine

DNA: deoxyribonucleic acid

d.p.e.: days post exposure

d.p.i.: days post-infection

DPOG: dorsal post-oral glands

DSctrl: dopamine/seawater control

DTT: DL-dithiothreitol

ELISA: enzyme-linked immunosorbant assay

FCS: foetal calf serum (heat inactivated)

FI: fluorescence intensity

FSC: forward scatter channel

FGC: frontal gland complex

G: Gauge

GLM: general linear model

xg: multiple of gravity

H&E: haematoxylin and eosin

H_2O_2: hydrogen peroxide

HSW: high salt wash-buffer

IFN: interferon

Ig: immunoglobulin

IHC: immunohistochemistry

IL: interleukin

ISH: *in situ* hybridisation

IU: international units

L-15: Leibovitz -15 media

LPS: lipopolysaccharide

LSW: low salt wash-buffer

M: molar

mAb: monoclonal antibody

MAC: molecular attack complex

MAF: macrophage activating factor

MAX: maxadilan

MC/EGCs: mast cells/eosinophilic cells

MW: molecular weight

MWCO: molecular weight column

NADPH: nicotinamide adenine dinucleotide phosphate-oxidase

NBF: neutral buffered formalin

NO: nitric oxide

OD: optical density

pAb: polyclonal antibody

PAMP: pathogen associated molecular pattern

PB: phosphate buffer, pH 7.0

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline with Tween®20

PCR: polymerase chain reaction

PGE_2: prostaglandin E_2

PI: propidium iodide

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

Polyc:IC: polyinosinic: polycltidylic acid

PRR: pattern recognition receptors

RER: rough endoplasmic reticulum

RNA: ribonucleic acid

ROS: reactive oxygen species

RT: room temperature (22°C)

RT-PCR: reverse transcription-polymerase chain reaction

SE: standard error

SEM: scanning electron microscopy

SEPs: secretory / excretory products

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SGE: salivary gland extract

SH: serum haemolysis

sIgM: secretory IgM

SRBC: sheep red blood cells

SSC: side scatter channel

SSC buffer: standard sodium citrate buffer

TBS: tris buffered saline

TEM: transmission electron microscopy

TGF: transforming growth factor

tIgM: transmembrane IgM

TMB: tetramethylbenidine dihdrochloride

TNFα: tumour necrosis factor alpha

v/v: volume/volume

w/v: weight/volume

ZAS: zymosan activated serum

Abstract

Lernaeocera branchialis (Linnaeus, 1767) is a parasitic copepod possessing a complex dual-host lifecycle. The "definitive" gadoid hosts, including *Gadus morhua* (Atlantic cod), *Melanogrammus aeglefinus* (haddock) and *Merlangius merlangus* (whiting), are infected by the fertilised female, which penetrates the host's ventral aorta or *bulbus arteriosus* whilst undertaking extensive metamorphosis and a haematophagous lifestyle. The pathogenic effects of this activity upon the host have been well documented and mortality may occur, especially when multiple parasites are present. These negative impacts on cod, particularly juveniles, by *L. branchialis* have the potential to adversely affect cod aquaculture in the future, and already vulnerable wild cod stocks. This PhD project therefore, investigated the immune response of wild haddock and cultured-cod post-infection by *L. branchialis,* and the possible mechanisms by which the parasite modulates / evades the host's immune response.

The systemic immune response of both wild haddock and cultured-cod post-infection by *L. branchialis* depended on the maturation stage of the parasite, and in the former host species, upon the infection intensity. Wild haddock harbouring fully metamorphosed females showed an increase in circulating thrombocytes and a decrease in serum protein levels however; if multiple mature *L. branchialis* were present the haddock possessed reduced circulating monocytes, and increased circulating thrombocytes and serum anti-trypsin activity. Infection by *L. branchialis* was also associated with a suppressive effect on haddock serum spontaneous haemolytic activity. These responses were thought to be due to the host trying to counteract the increased damage caused by the massive increase in size and the feeding of the mature parasite, which is more pronounced when multiple parasites are present, resulting in the increase in some parameters and the 'consumption' of others. However, the effect of parasite-derived secretions and other pathogens due to observations on wild fish could not be discounted. The laboratory-infection of cultured-cod from two different sources was also performed in order to study the immune response over time. The two groups of cod showed differences in their immune response to *L. branchialis*. The first group showed suppressed respiratory burst activity of phagocytes, as the parasite reached the early penella sub-stage, whilst no suppression in phagocyte respiratory burst activity was found in the second group. The parasite was found to migrate along the afferent branchial artery of the cod where a thrombus formed and was present throughout its migration into the ventral aorta. At 14 d post-infection, leukocytes expressing Interleukin 8 mRNA were observed within the free-flowing blood at the periphery of the organising thrombus within the lumen of the ventral aorta. This was speculated to aid the recruitment and activation of leukocytes to the site, and the maturation and neovascularisation of granulation tissue. The infection of the second group subsided with the death of the parasite, and none of the parasites metamorphosed past the early penella sub-

stage. The live parasites infecting the first group of cod did not possess IgM or complement component C3 binding on their cuticle, however, both IgM and C3 binding occurred on the dead parasites in the second infection trial. This may highlight the importance of these opsonins and the cytotoxic effect of phagocytes in the elimination of *L. branchialis* by some cod. However, the first infection was terminated as the parasite reached the early penella sub-stage due to a loss of stock cod prior to the study, so the long-term success of the infection can not be concluded. Therefore, the immune response to infection needs to be determined over the entire metamorphosis of *L. branchialis* to determine whether the infection was successful or not, and preferably in populations with varying susceptibility to *L. branchialis*. This will not be possible without further studies into the resistance of different stocks of cultured-cod.

Many arthropod parasites, such as ticks and salmon lice, have been previously documented to produce pharmacologically active secretions, aiding host invasion and parasite feeding, preventing the host immune response from working effectively against the parasite, all aimed at improving survival of the parasite. Therefore, the effects of the secretory/excretory products (SEPs) produced during the initial infective stage and by the mature, fully metamorphosed female on the immune response of cultured-cod *in vitro*, and the location of exocrine glands associated with the oral region of the parasite were investigated. The SEPs from the infective stage of the parasite were found not to affect the intracellular hydrogen peroxide (H_2O_2) production of phagocytes. The practical difficulties in collecting large quantities of the SEPs from the infective stage meant that their effects could not be tested on the other host immune parameters studied. The SEPs from fully metamorphosed female *L. branchialis*, however, had a number of suppressive effects on the host immune response *in vitro* including: 1) suppression of the intracellular production of cytotoxic H_2O_2 during the respiratory burst of phagocytic leukocytes post-PMA stimulation, 2) suppression of the production of macrophage activating factor by leukocytes with a priming effect on naïve phagocyte function, and 3) suppression of the chemo-attraction 'power' of zymosan activated cod serum, *i.e*. anaphylatoxin activity, on head kidney-derived leukocytes. These effects were dose-dependent, and highlight the capacity of *L. branchialis* to suppress its host's innate immune response at the local feeding area. Further work is required to establish the mechanisms by which the parasite-derived SEPs suppress these host immune parameters, and to identify which molecules produced by the parasite are responsible. The correlation between these *in vitro* results, and systemic immune parameters measured from laboratory-infected Atlantic cod and wild infected haddock are discussed.

Host immuno-modulation by other arthropod parasites is mediated by pharmacologically active secretions produced by exocrine glands. Therefore, the exocrine glands of the infective and fully metamorphosed female *L. branchialis* were also investigated in order to identify those that might be responsible for the secretion of host-modifying products. Adult female exocrine glands were mapped using diaminobenzidine (DAB), most commonly known to stain peroxidases and catalases. These compounds are known to be involved in the neutralisation of harmful free radicals which are released during the respiratory burst and tissue damage. Such products may therefore be important protective secretory components at the site of feeding / infection. Exocrine glands were located in the infective stage associated with the oral region, one pair termed the anterior gland complex (AGC), and the other pair extending either side of the oral cone termed the circum-oral glands (CG). These were further investigated using light microscopy and transmission electron microcopy. The AGC and CGs possessed multi-component secretions and they possessed secretory vesicles, abundant and highly active rough endoplasmic reticulum and Golgi apparatus suggesting that protein is an important component of the secretory products. These glands were also observed in the fully metamorphosed females where they had increased in size within the cephalothorax post-metamorphosis. It is hoped that the identification of these glandular structures, which are thought to secrete within the local vicinity of the oral cone, will aid future studies regarding the identification and secretion kinetics of parasite-derived molecules during the infection and feeding process. These studies together with the investigation of the immune response to infection in this thesis have shed more light on the interactions between this host and parasite, which will lay the foundation for further research which could eventually lead to the development of targeted control measures. This could include research into vaccine development against parasite-derived compounds involved in the modulation of the host's immune response and important for parasite survival, or the investigation of the use of immuno-stimulants to counteract the host immunosuppression experienced during infection by *L. branchialis.*

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Chapter 1 General introduction

1.1 Background

Lernaeocera branchialis (Linnaeus, 1767) is a parasitic copepod belonging to a family of entirely parasitic crustaceans, the Pennellidae, which infect marine teleosts, elasmobranchs and cetaceans. There are approximately 150 reported species within this family, all possessing complex life cycles. *L. branchialis* was the first pennellid species for which the dual host lifecycle characteristic for this group was revealed, by Metzger in 1868.

Maturation and copulation in *L. branchialis* occur on the gills of an "intermediate"[1] host, usually a Pleuronectiform flatfish. Fertilized adult females subsequently leave their intermediate hosts in order to infect their "definitive" hosts. These primarily consist of several gadoid species, such as *Gadus morhua* (Linnaeus, 1758; Atlantic cod), *Merlangius merlangus* (Linnaeus, 1758; whiting) and *Melanogrammus aeglefinus* (Linnaeus, 1758; haddock; Kabata, 1970; Kabata, 1979; Evans *et al*., 1983). Once attached to the 'definitive' host, the female undergoes extensive metamorphosis resulting in a mesoparasitic, haematophagous lifestyle.

The 'definitive' hosts, the gadoids, are of great economic importance. In particular, Atlantic cod has been one of the most economically important marine fish species in the North Atlantic (Kurlansky, 1998; Hemmingsen & MacKenzie, 2001). The Atlantic cod stocks in both Eastern and Western Atlantic fisheries are now in acute decline after hundreds of years of exploitation (Kurlansky, 1998 cited in Hemmingsen & MacKenzie, 2001). However, the demand for this food source has not diminished,

[1] An intermediate host is one which harbours the parasite for a short period e.g. the completion of a developmental stage of the parasite, and the definitive host is one where parasite maturation and copulation occur. However, due to the complex lifecycle of *L. branchialis* where maturation and copulation occur on the first host followed by egg production on a second host by the females only, this has led to them being termed the intermediate and definitive host, respectively within the literature. This current convention will be followed within this thesis.

leading to an increase in prices and the relatively recent advent of commercial Atlantic cod farming in Canada, Iceland, Norway and Scotland. *L. branchialis* is documented as one of the most serious metazoan pathogens of cod, and Burt & MacKinnon (1997 cited in Hemmingsen & MacKenzie, 2001) included it in their list of 'candidate' parasites that they perceived could become serious pathogens of farmed marine fish. This parasite may also pose a threat to over-exploited wild cod stocks, especially since Khan (1988) observed a mortality rate of 30% in laboratory-infected cod, and Sundnes (1970) reported the total natural mortality of infected cod from Norwegian coastal waters to be approximately 6% higher than that of *Lernaeocera*-free cod.

1.2 Life History of *Lernaeocera branchialis*

Sproston (1942) extensively described the developmental stages of *L. branchialis,* suggesting that they possess seven life stages prior to life on their definitive host, comprising a free-living nauplius, a free-living infective copepodid, four chalimus stages, and finally the adult male / adult female stage. However, Pedashenko (1898 in Schram, 1979), Wilson (1917), Whitfield *et al*. (1988) and Brooker (2007) have described an additional nauplius stage for *L. branchialis* prior to moulting into the copepodid stage (Figure 1.1). Therefore, the nauplius I hatches from the egg, moults into a nauplius II, and, once the moult into the copepodid stage has occurred it infects an intermediate host, becoming attached via chelated antennae to host gill filament tips. Once a firm attachment is made, the second maxilla grasps the gill filament in order to bring the oral appendages into close proximity with the host surface ready for browsing (Sproston, 1942).

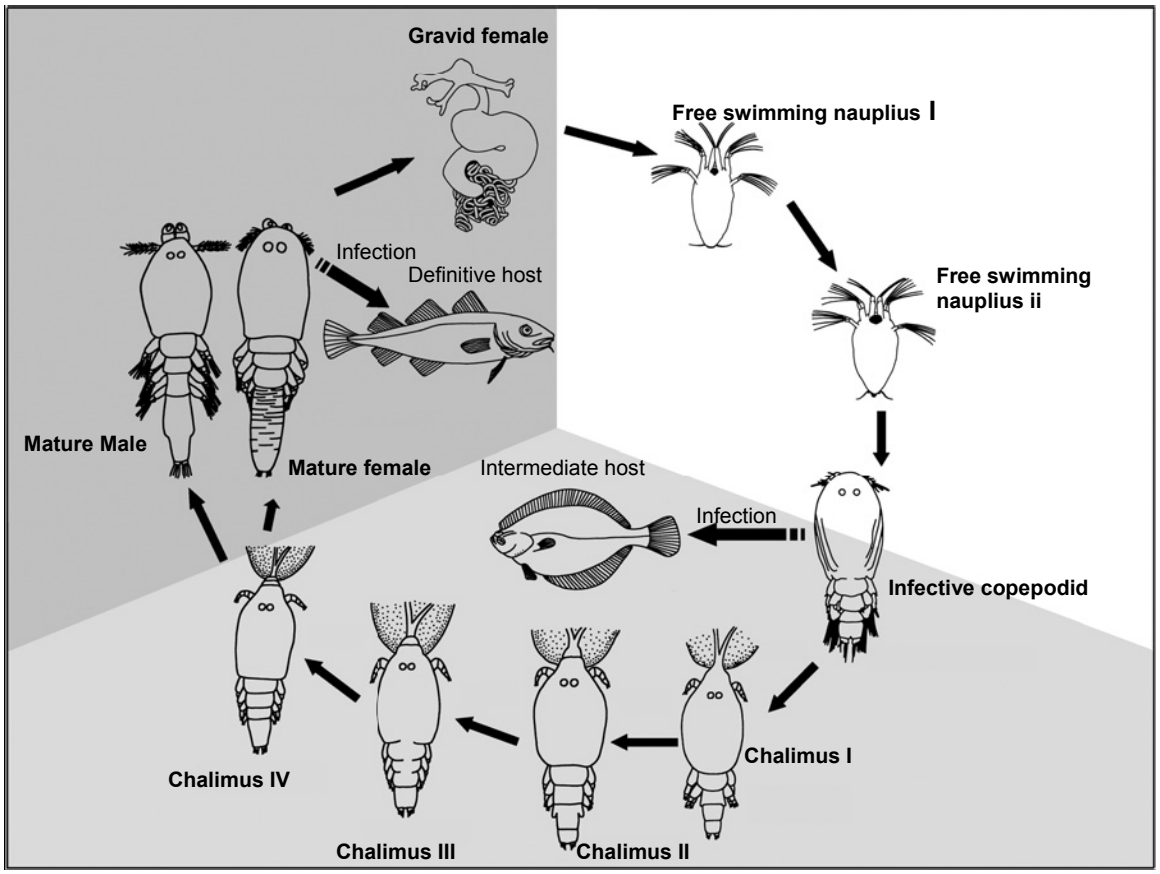

Figure 1.1 Life cycle diagram of *Lernaeocera branchialis* from Brooker (2007)

During the moult into the first chalimus stage a frontal filament is inserted into the host gill tissue, which is characteristic of the order Siphonostomatoida (Bron *et al*., 1991). The frontal filament is produced by the mid-frontal region, attaching the chalimus larva onto its host's gill filament and securing it against the water currents produced in the branchial cavity (Sproston, 1942). This is followed by successive chalimus moults where sexual dimorphism becomes apparent at the fourth chalimus stage (Whitfield *et al*., 1988), and finally into the adult stages ready for copulation to occur.

Post-copulation the female's abdomen begins to elongate, starting the metamorphosis process (Smith & Whitfield, 1988), and females detach from the intermediate host, releasing the frontal filament to become free-living for a short

period thought to only last a few days whilst she finds a definitive host (Schuurmans Stekhoven & Punt, 1937; Sproston & Hartley, 1941; Capart, 1948; Kabata, 1958). Successful females settle on the ventral portion of the gill arches (Kabata, 1979). The female's cephalothoracic region penetrates deep into the host tissue, initially through the lumen of the afferent branchial artery and eventually residing in the host's ventral aorta, often extending as far as the *bulbus arteriosus* or the heart ventricle (Sproston & Hartley, 1941; Kabata, 1979; Smith *et al*., 2007). Attainment of the final attachment area is associated with the development of three branched cephalic antlers (one dorsal, two lateral), acting as an elaborate holdfast (Kabata, 1970; Smith & Whitfield, 1988; Figures 1.2 and 1.3). Capart (1948) suggested 'arteriotropism' to be the mechanism behind the directness with which the adult female reaches her final location. The female is now permanently immobilised and undertakes a haematophagous lifestyle (Kabata, 1970).

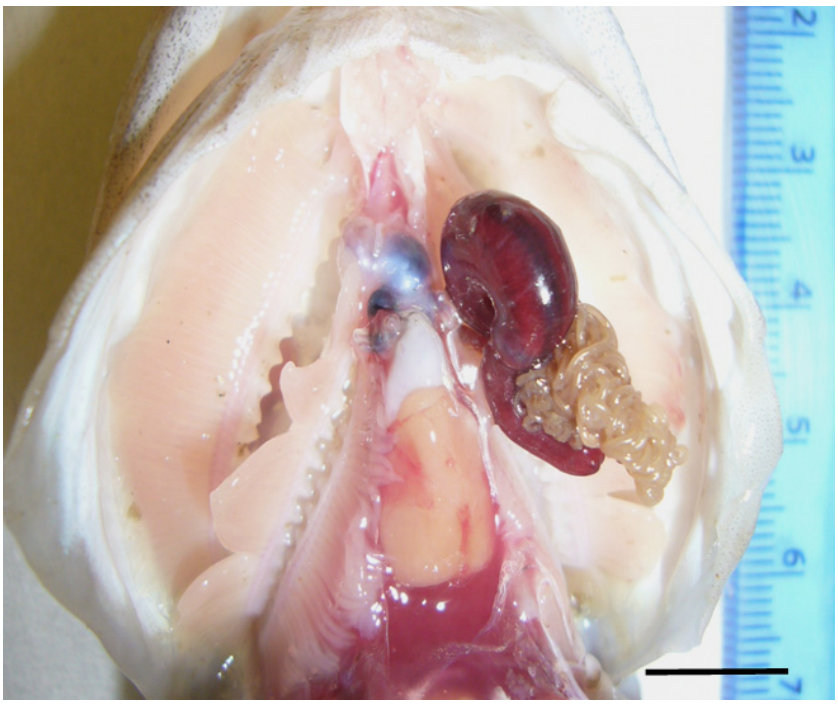

Figure 1.2 Wild haddock infected with one fully metamorphosed adult female *Lernaeocera branchialis* in the ventral aorta; Bar = 1cm

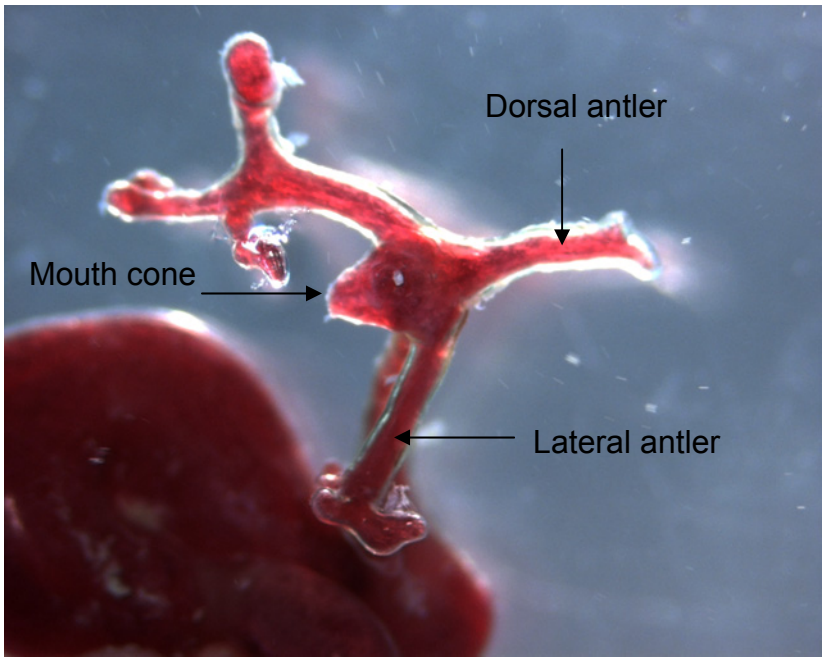

Figure 1.3 Cephalic holdfast of fully metamorphosed adult female *Lernaeocera branchialis* showing two lateral and one dorsal antler.

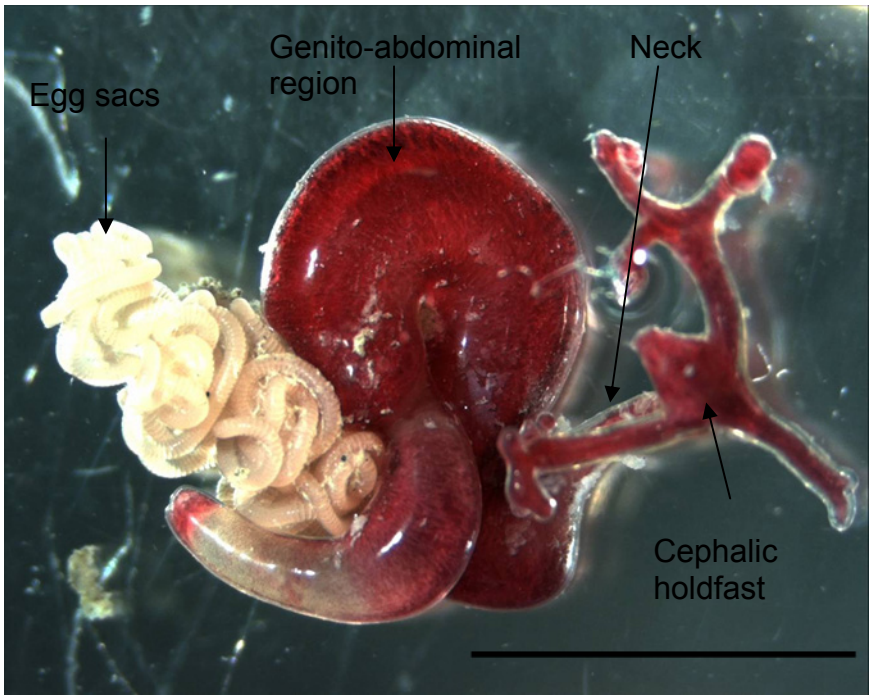

Figure 1.4 Fully metamorphosed gravid adult female *Lernaeocera branchialis*. Bar = 1cm.

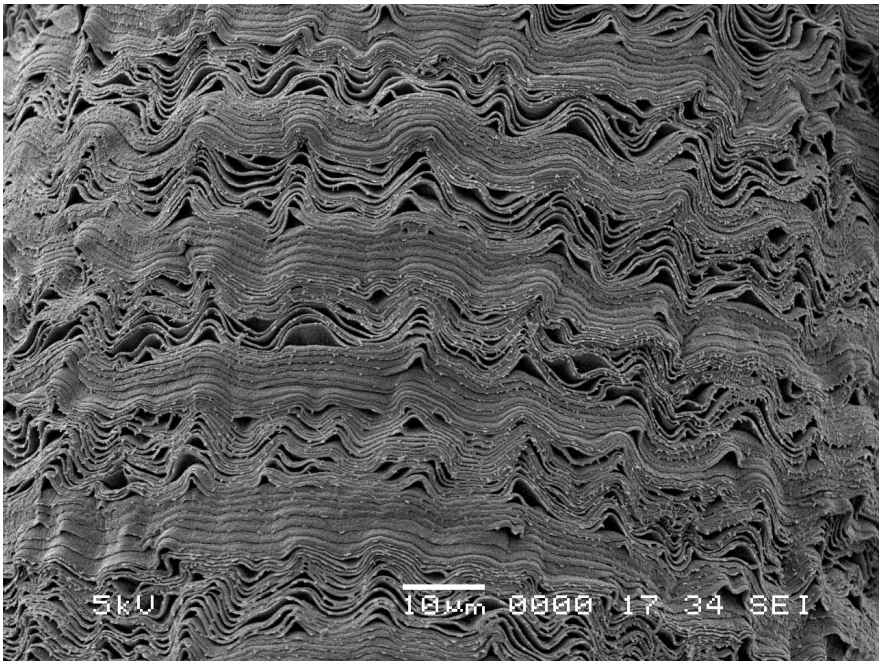

Figure 1.5 Scanning electron micrograph of the transverse sectional face of cuticle from a pre-metamorphosed adult female genito-abdominal region showing the cuticular folds which allow rapid cuticular expansion during metamorphosis.

Shortly after attachment the genito-abdominal region undergoes extensive elongation, dwarfing the cephalothorax (Smith & Whitfield, 1988). This is ensued by flexion of the genito-abdominal region, in order for the parasite to fit into the confined space of the host's branchial cavity, and by swelling of the genito-abdominal region (Figure 1.4). This extreme metamorphosis results in an approximately 20-fold increase in the abdomen length and girth (Smith & Whitfield, 1988). This extensive increase is performed without a moult, and is enabled partly by the straightening out of densely packed transverse abdominal folds (Figure 1.5) (Smith & Whitfield, 1988). Smith and Whitfield (1988) described 4 - 6µm deep transverse folds of the epicuticle and outer procuticle layers of the abdomen of pre-fertilized *L. branchialis* females at a density of 1 – 1.2 folds.µm^{-1} abdominal length. However, these authors stated that the straightening of these folds could only produce an approximately 6-fold length increase. In its final form the slim, cylindrical

neck originating from the cephalothorax embedded in the host via the holdfast, broadens into the unsegmented, sub-cylindrical, sigmoid-flexed genito-abdominal region, ventrally bearing four pairs of 'dwarfed' legs just behind the cephalothorax (Kabata, 1970; Kabata, 1988). The genito-abdominal region remains exterior to the host for dispersal of nauplii following egg-hatching. Whitfield *et al*. (1988) noted that females can produce on average 1445 eggs per pair of egg sacs, and can produce another pair of egg sacs just 48 hours after removal of her previous pair. Anstensrud (1990) suggested that females probably produce one pair of egg sacs every two weeks depending on water temperature. The recognised developmental sub-stages of the adult female *L. branchialis* are outlined in Table 1.1.

Adult males of *L. branchialis* survive 75 – 100 days post-maturation, in which time they copulate with several females and do not leave their intermediate host (Kabata, 1979; Anstensrud, 1990). However, Khan (1988) observed the female penella stage to take 9 – 10 months to attain full maturation on the definitive host (Atlantic cod) *i.e.* Y stage, in the laboratory. He noted that on reaching full maturation the female disperses her ripe eggs and begins to degenerate, turning grey, and that ultimately only a thin, translucent membranous covering connected to a 'stalk' remains, dropping off approximately 2 years post-infection leaving a 'stub'. Van Banning (1974) found one *L. branchialis*-infected Atlantic cod caught in Dutch coastal waters to possess two mussels (*Mytilus edulis* L.) attached to the body of *L. branchialis*. He suggested that the life span of the parasite's gadoid phase was 9-12 months by working out the age of the mussels, assuming that the unconventional nature of the habitat had not affected mussel development. Other estimates range through 8 weeks (Sproston, 1942), approximately 1 year (Schuurmans Stekhoven, 1936; Schuurmans Stekhoven & Punt, 1937), 1 – 1.5 years (Kabata, 1958) or even

up to 10 or more years (Capart, 1948). However, the life span of the gadoid phase of adult female *L. branchialis* in the wild can only be estimated in light of these investigations.

Table 1.1 *L. branchialis* sub-stages during metamorphosis on the definitive host specified by Sproston & Hartley (1941); Sproston (1942), Khan (1988); Smith & Whitfield (1988) and Van Damme & Hamerlynck (1992). * Stages Z and R are not 'true' metamorphic stages.

Sub-stage	Life stage Abbreviation	Description	Approximate timescale of onset of each sub-stage post-infection of definitive host based on Atlantic cod (Khan, 1988)
Penella	P	Elongated straight abdomen; rudiments of cephalic antlers.	~ 1 week post-infection.
Immature post-penella	U	Beginning of abdomen torsion and elongation of cephalic antlers.	~ 2 weeks post-infection.
Immature	V	Abdominal sigmoid torsion complete with four right-angled flexures; genital region partially swollen; beginning of antler branching.	~ 6-8 weeks post-infection
Mature	W	Completely metamorphosed; cephalic antlers fully branched; no egg sacs.	~ 8 weeks post-infection.
Mature gravid	X	Egg sacs present.	~ 12-14 weeks post-infection.
Mature post-gravid	Y	Egg sacs partly or wholly exhausted.	~ 9-10 months post-infection.
Dead parasite*	Z*	Parasite dead with transparent cuticle, only holdfast remaining embedded in host.	Not known conclusively.
Rejected parasite*	R*	Extensive proliferation of gill arch tissue in absence of parasite.	Not known conclusively.

1.3 Systematics, distribution and host utilisation

1.3.1 Systematics

The genus *Lernaeocera* (Blainville, 1822) belongs to the order Siphonostomatoida, and the family Pennellidae. The order Siphonostomatoida contains many successful marine and freshwater fish parasites, characterised by a unique frontal filament formed in the larval stages to enable them to securely attach to their host, and the virtually fused tube buccal apparatus with a pair of stylet-like mandibles surrounded by the labrum and labium (Kabata, 1979). Pennellid copepods are further characterised by their complex life cycle involving two hosts, as described in detail above for *L. branchialis*. The genus *Lernaeocera* possesses great polymorphism between adult female individuals of the same species (Kabata, 1988; Tirard *et al*., 1993). Kabata (1988) recognised this morphological variability and ascribed it to the adult female's size and it's attachment within the cramped space of the host's branchial cavity, leading to morphology partially based on efficient space utilisation.

This morphological plasticity has been a major factor in the past debates on the systematics of the genus *Lernaeocera* by many authors, mostly due to the fact that no thorough morphological study of the specimens discovered has been carried out, with most species descriptions concentrating on the highly modified morpho-anatomy of the adult female (Tirard & Raibaut, 1989; Tirard *et al*., 1993). Van Damme & Ollevier (1995) summarised some of the morphological characters used in past studies to differentiate between species, these comprising: antler shape (Scott & Scott, 1913; Schuurmans Stekhoven, 1936); neck width (Kabata, 1957); trunk shape (Kabata, 1957); abdomen flexure (Kabata, 1957) and cephalic appendage structure (Schuurmans Stekhoven, 1936). The majority of these features are now agreed not to be valid in terms of species description within the

genus *Lernaeocera* (Kabata, 1979; Van Damme & Ollevier, 1995). Sproston (1942) gave detailed morphological accounts of the larval and adult stages of *Lernaeocera branchialis* and Brooker (2007) recently reviewed and updated this data; however such studies are non-existent for other species, such as *Lernaeocera lusci* (Tirard *et al*., 1993). Kabata (1957) reported on the existence of five *Lernaeocera* species occurring off Western Europe, including *L. branchialis* (Linnaeus, 1767), *L. lusci* (Basset-Smith, 1896), *L. minuta* (Scott, 1900), *L. brevicollis* (Schuurmans Stekhoven, 1935), and *L. obtusa* (Kabata, 1957). However, since then Kabata (1979) has recognised only two *Lernaeocera sp.* in the North Atlantic to be valid on the basis of morphological evidence, *L. branchialis* (Linnaeus, 1758) and *L. lusci* (Bassett-Smith, 1896), which parasitise *M. merlangus* (whiting) and *Trisopterus luscus* (bib), respectively. *L. lusci* is differentiated from *L. branchialis* due to the fact that it possesses antennary processes (Slinn, 1970; Kabata, 1979; Van Damme & Ollevier, 1995). Bastide-Guillaume *et al*. (1987) questioned the validity of *L. lusci* as a species due to its morphological closeness to *L. branchialis*. Tirard *et al*. (1993) found reproductive isolation between *Lernaeocera* infecting whiting *i.e. L. branchialis* and those infecting bib, *i.e. L. lusci* showing the former to be monomorphic for the loci glucose phosphate isomerase (GPI) and mannose phosphate isomerase (MPI), and the latter to be polymorphic for these loci. Tirard *et al*. (1993) concluded that *Lernaeocera branchialis* and *L. lusci* are indeed two distinct species but that they co-inhabit large areas in the Atlantic Ocean, the Bay of Biscay, and the North Sea. These authors also challenged the proposal made by Delamare-Debouteville (1950 cited in Tirard *et al*., 1993) that *Lernaeocera* found in the Mediterranean were *L. branchialis*; they found *Lernaeocera* parasitizing bib and whiting in the Gulf of Lions to show more genetic homogeneity with *L. lusci* specimens from the Atlantic and North Sea than with *L. branchialis*.

The remaining proposed species of the North Atlantic have since been discounted due to the fact that they were described by identification criteria not thought to be taxonomically significant or correct. *L. minuta* (Scott, 1900) has been demoted to a junior synonym of *L. lusci* due to the fact that they are morphologically very similar, with the main characteristic originally used to distinguish them being their size. Van Damme & Ollevier (1995) reported that *Lernaeocera* size can be significantly influenced by host size, refuting its use as a valid taxonomic criterion in this species. Van Damme and Ollevier (1995) went on further to suggest that *L. lusci* comprises three forms; *L. lusci f. minuta, L. lusci f. lusci and L. lusci f. lyra* parasitizing *Pomatoschistus minutus* (sand goby), *Trisopterus luscus* (bib) and *Callionymus lyra* (dragonet), respectively. The American form of *L. branchialis* was first described by Wilson (1917), however Schuurmans Stekhoven (1936) contested that the species described was *L. branchialis.* The author stated that the American form differed from those of European forms sufficiently, in terms of their antennules, first maxillae and caudal rami, so as to warrant a new species, *Lernaeocera wilsoni* (Schuurmans Stekhoven, 1936). Capart (1948) later opposed this view suggesting that not enough was known about the American form, in particular the larval forms which were not reported in this area until 1951 (Fleming & Templeman, 1951). Kabata (1961) went on to study and compare larval and adult *L. branchialis* specimens from both sides of the north Atlantic from *Cyclopterus lumpus* (lumpsucker) and *G. morhua*, as well as larval forms from flounder (*Pleuronectes flesus*; Linnaeus, 1758) off the north east coast of Scotland for comparison. He reported no morphological differences between the European and American form concluding that they are conspecific, and suggesting that previous reported differences were due to inadequate earlier descriptions (Kabata, 1961).

1.3.2 Distribution and host utilisation

L. branchialis is an Arctoboreal species stretching from the northwest to the northeast Atlantic Ocean, North Sea and surrounding areas, paralleling its definitive gadoid hosts, with a limited southern distribution (Sherman & Wise, 1961). Their favoured host varies depending on the geographical location, even when species diversity is similar between these locations. Kabata (1979) summarised these differences in host utilisation by geographical location; off the coast of the Faroe Isles and Iceland, Atlantic cod is the main host, in the northern North Sea haddock is a definitive host (Kabata, 1957), and in the southern North Sea whiting becomes the main definitive host for *L. branchialis*. However, Tirard *et al*. (1993) found *L. lusci* to infect both their usual host bib as well as whiting in the Mediterranean, the latter the usual definitive host of *L. branchialis* becoming the preferred host for *L. lusci* in this region. The utilisation of intermediate hosts also differs between geographical locations, with the utilisation of different pleuronectiform species with their northerly distribution in European waters (Kabata, 1979), and *C. lumpus* in the northwest Atlantic (Fleming & Templeman 1951; Templeman *et al* 1976). The typical intermediate host of *L. branchialis* larvae in the North Sea is *P. flesus*, off the Faroe Isles is *Microstomus kitt* (lemon sole), and in the White Sea *P. flesus*, *Liopsetta glacialis* (Arctic flounder) and *C. lumpus* (Kabata, 1960).

Tirard *et al*. (1993) concluded that host utilisation / settlement by *L. branchialis* can be influenced by host availability. The differences in host utilisation in different geographical areas seems to support this conclusion as well as other observations, such as those of Begg and Bruno (1999) who found a small percentage (0.039%) of the usual intermediate host of *L. branchialis* (Kabata, 1979; Begg & Bruno, 1999), *Limanda limanda* (common dab) off the east coast of Scotland, to act as the

definitive host too. This is usually a rare event but other authors such as Slinn (1970) have observed intermediate hosts of *Lernaeocera sp*. to also act as a definitive host. This could be a result of a limited supply of preferred definitive hosts, or adult females lacking the capacity to leave their intermediate host and swim to find a definitive one, although further research is needed in order to elucidate the reasons.

Many authors have described the infection rates of *L. branchialis* on their gadoid hosts from both sides of the Atlantic. Several of these studies describe infection rates of cod and whiting with adult females to be higher in inshore areas than in offshore areas, with rates declining with the distance from the coast (Templeman *et al*., 1976; Larsen *et al*. 1997). This pattern seems to be mostly due to the fact that the distributions of intermediate and definitive hosts spatially overlap inshore during certain times of the year (Templeman *et al.,* 1976; Van Den Broek, 1979; Van Damme & Hamerlynck, 1992; Larsen *et al.,* 1997; Van Damme *et al.,* 1997).

The distribution of *L. branchialis* is not only affected by host availability, and seasonal host spatial overlap but also other environmental factors such as water salinity. Panikkar & Sproston (1941) originally showed the osmotic regulation of adult female *L. branchialis* to depend principally on their host. However, Sundnes (1970), and Knudsen and Sundnes (1998) opposed this, reporting *L. branchialis* to be osmotically dependent mostly on its surrounding medium rather than the host's plasma, suggesting that parasite survival would be compromised below 16 – 20‰. This was later confirmed in field studies by Sundnes *et al*. (1997) who found cod not to be infected in waters below 18‰. Mellergaard and Lang (1999) suggested that the distribution of *L. branchialis* is greatly determined by the water salinity, reporting

the highest prevalence of *L. branchialis* on cod in the Baltic to occur in the southwest whilst no *L. branchialis*-infected cod were found in the northeast Baltic. These authors concluded that the spatial distribution of *L. branchialis* is restricted to those areas with salinities above 8‰. Many other authors reported similar findings with the prevalence of infected cod in the Baltic being highest in the regions with higher salinities (Arntz, 1972; Lang, 1989). K∅ie (1999) also reported that only flounder caught in the Southwestern Baltic were found to posses *L. branchialis*. The distribution of *L. branchialis* in the Baltic is hypothesised to be due to a salinity gradient from about 20‰ in the western Baltic to very low or absent in the north-eastern Baltic (Mellergaard & Lang, 1999), resulting from fluctuations in the influx of seawater through the Danish Belts.

The distribution of *L. branchialis* is influenced by many factors including viable host availability, spatial overlap of intermediate and definitive hosts, and environmental parameters such as water salinity. These factors act together to determine the distribution of *L. branchialis* in the Atlantic, and the seasonal peaks in abundance of different life stages of this dual host-parasite interaction in inshore areas.

1.4 Pathogenicity of *Lernaeocera branchialis*

The pathogenic effects of *Lernaeocera* spp. on their gadoid hosts have been debated by numerous authors with contradictory findings on the effects of infection in gadoids on host; 1) growth and condition factor, 2) maturation and fecundity, 3) haematology and 4) mortality.

1.4.1 The pathogenic effect on host weight and length

Several authors have suggested a negative association between infection and host length both in the laboratory and in the wild (Templeman *et al.,* 1976; Van de Broek,

1979; Khan & Lacey, 1986; Khan, 1988; Alsuth & Ebeling, 1989; Lang, 1989; Khan *et al.,* 1990; Jones & Taggart, 1998). Debrosses (1948) reported the head of infected whiting to be larger, and the body length shorter than that of uninfected whiting, concluding that infection has a negative effect on host growth. However, Mann (1952) carried out similar studies on older groups of whiting, and found there to be no significant differences between the head length: total length ratio of infected and uninfected whiting. He went on to suggest that older hosts are less affected than their juvenile counterparts, and that this parasite has a more substantial effect on host weight.

Several other authors have also reported on the negative effect of parasitism by *L. branchialis* on host weight (Khan & Lacey, 1986; Kabata, 1958; Mann, 1970; Van Den Broek, 1978; Hislop & Shanks, 1981; Kabata, 1970; Khan & Lee, 1989; Khan, 1988). Mann (1952) reported substantial weight losses in infected gadoids and also reported the degree of weight loss to be dependent on the intensity of the infection with whiting, haddock and cod infected with one parasite showing weight losses of 0 - 20%, 0 - 47%, and 0 – 28%, and those with two parasites 19 – 42%, 28 – 36%, and 0 – 35%, respectively. Van den Broek (1978) observed only fully metamorphosed female *L. branchialis* to cause significant weight reduction in whiting, whereas the immature females had no effect on host weight. Khan (1988) found the growth of cod, especially those <40cm in length, to be significantly reduced by single and multiple infections. He suggested that this was due to a decrease in food intake in the initial stages of infection. Khan and Lee (1989) noted a steeper rise in total body length and weight in uninfected control cod compared to infected cod up to 10 months post-infection. Subsequent to this stage cod with single and multiple infections gained weight, and following the end of parasite

reproduction (13 – 16 months post-infection) no significant differences in weight were observed between infected and uninfected cod. Khan and Lee (1989) also found infected immature cod to posses lower food consumption, food conversion efficiency, and condition factors compared to uninfected cod; however they found adult cod infected by young parasites to consume more food and to be heavier than uninfected cod even though their food conversion efficiency was lower. Following parasite maturation and reproductive activities, Khan and Lee (1989) observed the hosts' food consumption, weight gain, and K – factor to be greater than that of uninfected adult cod, but their food conversion efficiency remained below that of the uninfected controls of comparable length. Therefore, Khan and Lee (1989) concluded that only adult cod have the capacity to compensate for infection with young parasites by increasing food consumption, and that slight effects on the host such as lower food conversion are still present once the parasite has completed her reproductive cycle, although this is likely to disappear as the parasite disintegrates. On the other hand Kabata (1970) observed young haddock to gain weight when infected with immature parasites, concluding that juvenile hosts were able to compensate for the infection until parasite maturity. Khan *et al*. (1993) went on to conclude that adult cod are capable of compensating for infection with one female *L. branchialis*, by consuming more food, in the autumn rather than the winter. These conclusions were based on the fact that adult cod infected with one female consumed more food, and had comparable weight gain, even though their food conversion efficiency and condition factors were lower than those of uninfected adult cod. However, some authors such as Sproston and Hartley (1941) deny that *L. branchialis* has any effect on the weight of infected whiting and pollack, and Sherman and Wise (1961) reported infected cod to possess similar condition factors to uninfected cod.

Mann (1964) further investigated the effect of *L. branchialis* infection on the weight of a range of host tissues, reporting the greatest weight loss of the host to occur in the muscle tissue, which decreased from more than half of the body weight in uninfected fish to only approximately a third of the body weight. Muller (1983) also found Atlantic cod infected with *L. branchialis* to have lower fillet fat content and higher water content by approximately 8% and 1%, respectively, as well as reduced condition factors. This will obviously incur consequences for gadoid fisheries in areas where *L. branchialis* prevalence is high due to the fact that the desired economic end-product is the fish muscle / fillet. Khan and Lacey (1986) found the weights of hearts, livers, spleens and alimentary tracts of cod infected with one parasite to be similar to those of uninfected groups of all size groups. Kabata (1958) investigated the effect of the various stages of infection on haddock liver fat content, reporting that haddock infected with more than one mature parasite possessed liver fat contents half that of uninfected haddock livers. Remarkably the liver fat content of haddock infected with immature parasites increased slightly in comparison to uninfected haddock (Kabata, 1958). Van Den Broek (1978) observed liver lipid contents and phospholipid levels of whiting infected by mature females to be reduced by approximately 51.4% and 30.3%, respectively, compared to uninfected fish, and that immature females had no effect on liver lipids. They also reported the fatty acid composition of whiting infected by mature females to be similar to that of uninfected fish, and cholesterol levels to be greater in whiting infected by mature females than uninfected ones. He suggested that once the females reach the mature stages they exert physical damage on their host leading to a negative effect on the host's metabolism, as suggested by the reduction in liver lipid concentrations in this study. Khan *et al*., (1990) found infected wild caught cod held in cages to show reduced weight gain, lower liver somatic indices, lower liver lipid values and

blood haemoglobin values than uninfected controls, suggesting that this parasite has the potential to negatively affect cod ranching enterprises.

1.4.2 The pathogenic effects on host maturity and fecundity

Several authors have also suggested that infection with *L. branchialis* delays the onset of host maturity with the possibility of affecting their fecundity. Templeman *et al*. (1976) noted the prevalence of *L. branchialis* to be higher on immature Atlantic cod in the Newfoundland area than on mature cod of the same length, suggesting that the parasite delayed the start of sexual maturation. Khan (1988) observed multiple infections of cod to delay gonad maturation, with infected cod possessing significantly reduced gonad somatic indices compared to control cod. They suggested that any delay in gonad development had the capacity to prevent spawning or sperm production / release, eventually affecting recruitment in heavily exploited populations, due to the fact that the spawning period of cod is only 1 – 2 weeks in the north western Atlantic. Kabata (1958) reported infected male and female haddock to possess mean gonad weights 36% and 26% lower, respectively, than those of uninfected haddock. However, he suggested that this observation should be treated with caution as the data from uninfected and infected groups overlapped a great deal, and that he was unable to conclude that *L. branchialis* infection leads to delayed sexual development of the host due to the lack of knowledge on the typical maturation of haddock. Hislop and Shanks (1981) decided to further investigate the findings of Kabata (1958) because they found his observed mean gonad weights to be quite low suggesting that he had measured them from immature haddock or those outside of their spawning season. These authors reported that the fecundities, weight for weight, of infected haddock only accounted for 83% of that of uninfected haddock. They concluded that *L. branchialis* infection

of haddock significantly decreases their fecundity, but suggested that in areas where exploitation was relatively low, the effect of infection on egg production over the haddock's reproductive life and hence stock recruitment may be low, due to the evidence by many authors that after parasite degeneration the host usually resumes good health assuming that re-infection does not occur. However, Lysne & Skorping (2002) found the existence of small groups of inherently susceptible cod which were infected and re-infected if the parasite was lost, and groups of resistant hosts with a lower chance of infection. Therefore, if haddock have been infected and lose their parasite, the likelihood that they will become re-infected may be quite high. Hislop and Shanks (1981) also suggested that the combination of *L. branchialis* infection and heavy exploitation of a population of haddock may have the capacity to adversely affect total egg production and therefore stock recruitment. They recommended that the estimation of stock size should use fecundity data based on fecundity at length relationships founded on a regional basis, due to the fact that haddock in areas such as the Moray Firth where *L. branchialis* infection is higher in relation to offshore areas, may possess lower fecundities per unit length than in areas with relatively lower *L. branchialis* prevalence, such as the Baltic.

1.4.3 The pathogenic effects on host haematological parameters

A few studies have investigated the effect of adult female *L. branchialis* on the hosts' blood erythrocyte count and haemoglobin values (Mann, 1952; Kabata, 1958; Guillaume *et al.,* 1983; Khan, 1988; Van Damme *et al.,* 1994), due to the fact that this is a haematophagous parasite with a gut capable of holding 100µl of host blood (Sproston & Hartley, 1941). Mann (1952) reported blood erythrocyte counts and haemoglobin levels in infected whiting to be lower and approximately half that

observed in their uninfected counterparts, respectively. This data, however, is limited with respect to its interpretation, as the author did not note the stage and number of parasites present on the hosts. Several years later Kabata (1958) highlighted this point and went on to investigate the effect of *L. branchialis* on haddock in Scottish waters at different stages, including the effect on blood haemoglobin levels. He observed haddock infected with mature females to possess lower haemoglobin levels than uninfected haddock and this reduction was most significant with multiple infections with mature females. However, Kabata (1958) found haddock infected with immature females to possess higher haemoglobin levels than uninfected haddock, suggesting that haddock were trying to compensate for blood loss by stimulation of their haematopoietic tissues. This is in agreement with his other findings of host compensation for infection whilst the parasite remains immature (Kabata, 1958) which is no longer possible once parasite maturation occurs due to the increased nutrient requirements of the parasite and hence larger blood loss for the host. Khan and Lacey (1986) found that blood haemoglobin values were lower in juvenile cod infected with two or more parasites compared to uninfected juvenile cod, and noted them to possess paler gills. Van Damme *et al*. (1994) found whiting infected by *L. branchialis* to possess reduced haematocrit levels; these were approximately 21%, 38% and 9% lower than uninfected fish when one live, two live or dead parasites were found attached. Khan (1988) carried out Giemsa-staining of blood smears on Atlantic cod, observing that those with multiple infections show erythrocytic changes, pale gills and reduced haemoglobin and haematocrit values. These changes involved a higher proportion of polychromatic erythrocytes and lower abundance of erythroblasts in infected cod compared to uninfected cod (Khan, 1988). These studies highlight the fact that

hosts suffer secondary anaemia from infections with mature females, this being exacerbated when multiple females are present.

1.4.4 Host mortality post-infection by *L. branchialis*

Infection with *L. branchialis* in gadoids has also been reported by several authors to result in host mortality, both in the laboratory and in the wild (Kabata, 1970; Sundnes, 1970; Khan & Lacey, 1986; Khan, 1988; Khan & Lee, 1989; Khan *et al.,* 1990; Khan *et al.,* 1993; Jones & Taggart, 1998). Sundnes (1970) and Jones and Taggart (1998) carried out tagging experiments on wild Atlantic cod from Norwegian coastal waters and northeast Newfoundland, and found the total mortality of infected cod, to be about 6% and 8% respectively, higher than that of uninfected cod. However, the mortality induced by *L. branchialis* infection may be underestimated as Sproston & Hartley (1941) and Khan (1988) suggested that *L. branchialis* infection has detrimental effects on the swimming behaviour of its host. Therefore, this may increase the likelihood of capture in fishing gear for infected fish above that of their uninfected counterparts, leading to tagged infected fish being over-represented in the recapture experiment. Khan (1988) studied the infection time course of experimentally infected cod reporting a much higher mortality rate of 36% for infected cod in the laboratory; noting that the majority of cod deaths occurred in the first four months of infection, with the greatest proportion of deaths in the smaller size classes (29 - 35cm and 36 – 41cm). He also noted that these deaths were usually associated with multiple infections, although he did observe 23% of cod that died to only carry one female *L. branchialis*. Khan (1988) reported open haemorrhagic lesions 1-2mm in diameter after some parasites had been shed, emaciation, haemorrhaging at the holdfast penetration site, necrotic tissue surrounding the parasite, and low haemoglobin, all associated with mortality of

infected young cod. However, in infected adult cod he suggested that mortality was likely to have been a result of blockage or thrombus formation in the ventral aorta or branchial arteries. Khan (1988) also noted that when parasites were shed the resulting open lesions often became necrotic, accompanied by a caseous discharge, and in some cases, especially in adult cod the lesions healed. He stated that adult cod infected by one female usually survived infection but were highly susceptible to stress, often dying under such conditions. Khan *et al*. (1990) tried to investigate the effect of *L. branchialis* on cod ranching by infecting wild cod in the laboratory, and observing naturally infected wild cod in areas adjacent to sea cages. They observed 64% of wild cod to become infected in the laboratory with 33% of these fish dying over a four-year period. However, the majority of these deaths (74%) occurred within the first four months of infection, with parasite prevalence and host mortality significantly greater in immature cod than in adults. Mortality was seen to be associated particularly with multiple infections, even though juvenile cod died possessing single infections (Khan *et al*., 1990). These authors reported similar features of infection to be associated with host death to those of Khan (1988), including necrotic, caseous tissue around the penetration site, pale gills, and low blood haematocrit and haemoglobin levels. However, Khan and Lacey (1986) observed no effect of infection on subadult (42 – 47cm) and adult cod (>47cm), and mortality to only occur in juvenile cod infected with both *L. branchialis* and the protozoan *Trypanosoma murmanensis*.

1.4.5 Other reported pathogenic effects on the gadoid host

It is hypothesised by many authors that the reduction in food conversion efficiency due to infection, and the fact that *L. branchialis* consumes considerable amounts of its host's blood leads to a more limited energy supply for the fish to carry out

foraging, migration, growth and sexual development compared to uninfected fish, and may eventually cause mortality of the host. The attachment site of this parasite also prevents the complete closure of the operculum, and has often been reported to cause atrophy of the parts of the gill where it resides (Kabata, 1970; Van Den Broek, 1978; Khan & Lee, 1989). Kabata (1970) also reported that in serious cases where the pressure of the parasite occluded branchial blood vessels the gill filament became necrotic, eventually disintegrating. This in turn would affect host respiration as the host's area for gaseous exchange is reduced, playing a part in restricting the supply of oxygen to the host, possibly to an insufficient level. Mann (1952) found infected whiting to consume less oxygen than their uninfected counterparts concluding that infection may have a negative impact on the host's metabolism. Rokicki (1988) found *L. branchialis* infection of one *Pollachius pollachius* (pollack) to result in a deformed gill cover, perforation of the ventral artery with production of a haematoma, and possible deformation of the bones of the viscerocranium. He states however, that he was unable to determine whether the extensive deformation in this pollack was 'normal' for this species and therefore, that *L. branchialis* is more pathogenic to pollack than to cod, haddock and whiting. Sproston and Hartley (1941) have also hypothesised blood loss caused by the feeding activities of *L. branchialis* to be the reason behind infected whiting and pollack from the southern North Sea remaining inshore whilst the remaining uninfected population migrate offshore. They stated that blood loss would lead to fluid loss in infected fish, making it necessary for them to remain in waters of lower salinity, such as those found inshore, in order to balance water loss by re-absorption of water from the surrounding media. These hypotheses have not been confirmed, and numerous other reasons are possible for the delayed offshore migration of infected fish. These may include: 1) reduced muscle mass and energy resources caused by reduced

food conversion efficiency, respiratory efficiency and weight gain compared to uninfected fish, and 2) factors which possibly pre-disposed the fish to infection by *L. branchialis*.

In conclusion, *L. branchialis* has been hypothesised to have various pathogenic effects on its gadoid hosts, including reduced growth rate and weight gain, reduced respiratory and food conversion efficiency, anaemia, suspected metabolic disruption shown by reduced liver lipid weight and reduced muscle weight contribution, delayed onset of sexual maturation, reduced condition, and mortality. These detrimental effects are likely to result from the feeding activities of the parasite and / or the attachment of the parasite, and are exacerbated on juvenile hosts and with mature and multiple infections. However, the literature contains many contradictions as to the exact pathogenicity, with some authors such as Sherman and Wise (1961) finding *L. branchialis* not to possess any pathogenic effect on Atlantic cod from New England waters. Many possible reasons for the presence of discrepancies between the conclusions drawn from various studies are apparent, such as the effect of host age, maturity stage of the adult female *L. branchialis*, differences in susceptibility within a host species in various geographic areas and between host species to the effects of *L. branchialis,* and the fact that some studies are carried out in the wild whilst others are from experimental infections in the laboratory. The effects of other infections in combination with that of *L. branchialis* may also have a role to play in these discrepancies, especially in studies undertaken on wild fish as they are likely to have numerous other infections. For example, Evans *et al*. (1983) found a positive relationship between infection of bib with adult female *L. lusci* and the metacercarial cysts of the digenean *Cryptocotyle lingua* in Southampton waters. This was highlighted by Khan (1988), and Khan and Lacey (1986) who showed the

dual infection of Atlantic cod with *L. branchialis* and *T. murmanensis* to potentiate the effects on the host. Therefore, no final conclusions can be drawn from studies on wild fish until controlled studies on the pathogenic effects of *L. branchialis* infection of gadoids have been performed in the laboratory, the exact pathogenic effects of this parasite remain contradictory.

1.5 The host immune response to parasitic copepods

The vertebrate immune system has developed two arms, comprising the innate (non-specific) and the adaptive (specific) immune response. The innate and adaptive immune responses however, are not isolated and interactions occur between them. The innate immune response plays a proportionally greater role in protecting teleosts against pathogens (poikilotherms), compared to homeothermic vertebrates (Jones, 2001). This is thought to be due to the fact that the adaptive immune system in poikilotherms is initiated over a longer time period and has a more limited repertoire, compared to that of homeotherms (Jones, 2001). For example antibody production in salmonids takes at least 4-6 weeks (Ellis, 2001). The innate immune response also provides the first line of defence to invasion by pathogens due to its 'fast' response to invasion and its relative temperature independency.

1.5.1 The innate immune response

The innate immune response has evolved to respond to two categories of molecular patterns. The first being motifs, which are highly conserved among the major groups of pathogens, such as repetitive mannose structures, lipopolysaccharides and unmethylated CpG dinucleotides. These are more commonly known as pathogen-associated molecular patterns (PAMPs) (Saeij *et al*., 2003). The second

category is composed of molecules not normally present on the surface of host cells, such as host DNA, RNA and heat shock proteins, which are revealed by damaged host tissue in the course of infection, necrosis, inflammation and natural cell death (Matzinger, 1994). The innate molecular pattern recognition receptors of teleosts are composed of humoral proteins such as C3, lectins, and C reactive protein or cell membrane bound receptors on immune cells such as Toll-like receptors. Once these are activated the following usually ensue: pro-inflammatory cytokine release, opsonisation and phagocytosis of 'foreign' particles, stimulation of natural cytotoxic cells, and activation of acute phase response, complement and lytic pathways. The three main components of the teleost innate immune system are discussed below.

1.5.1.1 Physical barriers

The first line of teleost defence against parasites is a physical barrier formed by the scales, skin and gill mucous epithelia, and the epidermis (Ellis 2001; Ingram 1980; Shephard 1994). The gills make up a large surface area of delicate epithelium for gaseous exchange to occur. Therefore, this organ is protected by mucus, by a highly responsive epithelium which undergoes hyperplasia upon infection, and by phagocytic cells that line the branchial capillaries. Soluble circulating antigens, such as bacterial LPS and collagen, are also taken up by the endocardial endothelial cells (EEC) of the heart in Atlantic cod (Smedsrod *et al.*, 1995; Seternes *et al.*, 2001). The EECs of cod line the muscular trabecula of the heart with the capacity to uptake and degrade foreign and physiological waste macromolecules from the circulation by scavenger – receptor – mediated endocytosis (Seternes *et al.*, 2001). However, the cellular uptake of circulating particulate antigens, such as latex beads and whole bacteria, occurs mainly in the spleen and kidney with few antigens found

in the cells of the heart (Smedsrod *et al.*, 1995; Arnesen *et al.*, 2002). The humoral and cellular components of the teleost innate immune response are initiated once the parasite has made intimate contact with the host. Fish skin mucus not only acts by trapping and sloughing off foreign material but it contains many factors involved in the innate defence mechanisms of teleosts, such as complement, lectins, lysozyme, C-reactive protein, anti-bacterial peptides, IgM, and haemolysins (Alexander & Ingram 1992; Rombout *et al* 1993; Aranishi & Nakane 1997). Jones (2001) suggested that "physico-chemical characteristics of skin mucus with the presence of bioactive substances may affect establishment and proliferation of ectoparasitic copepodids". The antiparasitic effect of fish mucus has been well documented for ectoparasite-teleost host interactions. Buchmann & Bresciani (1998) observed a significant negative correlation between the intensity of *Gyrodactylus derjavini* (monogean) infection and mucous cell density on the skin surface of rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792). Many authors have also found evidence that monogenean and copepod ectoparasites adapt their micro-environment by reducing host skin mucous cell density in order to reduce mucus secretion onto the skin surface (Wells & Cone 1990; Lindenstrom & Buchmann 1998; Nolan *et al* 1999).

1.5.1.2 Humoral immune response

The humoral immune response is composed of soluble molecules of serum and other bodily fluids, such as mucus. These mediate functions such as complement associated lysis, and/or opsonisation by natural antibodies, and include proteases, anti-proteases, pentraxins such as C-reactive protein, transferrin, lysozyme, chitinase and anti-bacterial peptides (see Alexander & Ingram, 1992; Jones, 2001).

Complement

The complement system of teleosts is well developed and possesses important roles in both the adaptive and the innate immune response. This system is composed of more than 30 serum soluble proteins in higher vertebrates involved in opsonisation, immune complex solubilisation and removal, inflammation processes, direct killing via formation of membrane attack complexes and chemo-attraction (Sakai, 1984; Boshra *et al.*, 2004; Rotlant *et al*., 2004; Kato *et al*., 2004). Three pathways of complement activation have been characterised including the antibody-independent alternative, antibody-dependent classical and lectin pathway, all of which have been found in various fish species (Sakai, 1992; Holland & Lambris, 2002; Sunyer & Lambris, 1998). The third complement component (C3) plays a central role in all of the above pathways, is a key opsonin of the complement system, and is essential for triggering the formation of the membrane attack complex, and is therefore the best characterised complement component to date.

In general, fish complement systems are well developed with similar characteristics to those of mammals, albeit with a few exceptions, including the facts that fish complement possesses lower optimal reaction temperatures, stronger anti-microbial activity, and has a tendency to be more heat labile and labile to storage at -20°C (Sakai, 1992). Complement is thought to be one of the most effective immune effectors of fish due to the fact that: 1) some species possess multiple forms of functionally active C3 with varying abilities to bind to different surfaces, 2) its high activity in fish compared to higher vertebrates especially alternative pathway titres, and 3) its capacity to work at low temperatures (Sakai 1992; Sunyer & Lambris 1998; Nonaka & Smith 2000). Multiple isoforms of C3 have been identified in numerous teleost species to date, and have been found to differ significantly in their

binding affinities for various molecules, such as zymosan, *Escherichia coli*, sheep and rabbit erythrocytes (Sunyer *et al*., 1996; 1997). Rainbow trout possess four isoforms of C3 (C3-1, C3-2, C3-3 and C3-4) (Zarkadis *et al*., 2001), hypothesised to provide a mechanism for immune diversity allowing a more efficient innate immune recognition capacity (Sunyer & Lambris, 1998). Lange *et al*. (2004) however, found Atlantic cod to only possess one isotype of C3. Complement activation results in the opsonisation of antigens promoting their phagocytosis, acting through the covalent binding of activated C3 and/or C4 to foreign molecules (Boshra *et al*., 2006).

The complement system has been associated with host immunity and host specificity with respect to a variety of parasites to date, infecting both teleosts and mammals. The deposition of host complement has been observed in guinea pigs repeatedly infected with the tick *Dermacentor andersoni*, at the dermo-epidermal junction (Allen *et al*., 1979). Wehnert & Woo (1980) used *in vitro* studies to show that *Cryptobia salmositica* (a haemoflagellate parasite of salmonid fish) was lysed by factors in the plasma of resistant hosts but not in that of rainbow trout, a susceptible host. These factors were found to be heat labile, and thought to share the characteristics of alternative complement activity. Forward & Woo (1996) suggested that alternative complement activation was partially accountable for brook charr (*Salvelinus fontinalis*; Mitchill, 1814) family resistance to *C. salmositica*. *Raja radiata* serum has also been shown to cause mortality in the tapeworm *Acanthobothrium quadripartitum* (Cestoda) in a heat labile manner, whilst the serum of *R. nauvus* (the normal susceptible host) had no obvious effect on the parasite's survival (McVicar & Fletcher, 1970). These authors suggested that the serum possessed a natural antibody to the worm. Harris (1972) found the incubation of *Pomphorhynchus laevis* (Acanthocephala) in sera and mucus of infected chub to

result in a precipitating antibody against the parasite. He stated that the antibody was produced against excretory and secretory antigens derived from mature parasites, and that it might have a role in neutralising toxic product(s) excreted / secreted by the parasite. Buchmann (1998) demonstrated rainbow trout plasma comprising intact complement factors to result in death of *Gyrodactylus derjavini* when incubated *in vitro*. He also found rainbow trout C3 bound to cephalic gland openings, the parasite's body and the hamulus sheath but no host immunoglobulin after incubation with infected and non-infected rainbow trout plasma. Buchmann (1998) suggested the lethal effect to be a result of complement activated by the alternative pathway due to the loss of lethal effect in heat-treated plasma and no loss of effect when plasma was treated with EGTA, a standard classical pathway inhibitor. However, lectin-binding assays revealed mannose-rich areas at the cephalic gland openings, therefore the complement activity observed in this study may also be a result of activation via the lectin pathway (Boshra *et al*., 2006). Harris *et al*. (1998) demonstrated the immense sensitivity of *Gyrodactylus salaris* to salmon blood and mucus components, suggesting a lethal factor within the host serum and mucus causing damage to the integument probably resulting in osmotic stress induced mortality, hypothesised to be host complement activated by the alternative pathway. Rubio-Godoy *et al*. (2004) observed the oncomiracidia of the monogean *Discocotyle sagittata* to be rapidly killed when incubated with naïve and immune serum from rainbow trout and brown trout (*Salmo trutta*; Linnaeus, 1758), thought to be due to alternative complement activity. The authors described surface disruption of these larvae after exposure to fish plasma. They found no significant difference in the larval survival time when incubated in naïve or immune fish blood, and took this as supporting evidence for the larvicidal activity to be complement activated by the alternative pathway, as larvicidal activity was lost after heating at

45°C, and by the addition of EDTA and not EGTA. These studies highlight the susceptibility of a wide variety of parasites to complement, particularly through the alternative pathway in teleosts.

Protease inhibitors

Protease inhibitors play key roles in many host-parasite interactions by inactivating and clearing parasite-derived proteases involved in parasite metabolism and virulence. Protease inhibitors comprise two classes; active-site inhibitors and α2-macroglobulins (α2M). They differ in their mechanisms for inactivating proteases as the former binds to and inactivates the active site of the protease, whereas the latter traps the protease in a molecular cage delivering it for degradation in secondary lysosomes (Armstrong, 2006). Plasma protease inhibitors such as α2M, α1-protease inhibitor and α1-antitrypsin are thought to have evolved as a natural defence mechanism of fish against bacteria and parasites possessing proteolytic virulence factors (Ellis 1987: Salte *et al* 1993), and are involved in the acute phase response to infection (Bayne & Gerwick, 2001).

Many recent studies have found Atlantic cod total serum anti-protease activity to be generally quite high, reduced under elevated environmental temperatures and not altered by infection or immunisation (Magnadóttir *et al*., 1999a: Magnadóttir *et al*., 2001: Magnadóttir *et al*., 2002). The protease secretions, if any, of *L. branchialis* have not been investigated to date, but other parasites have been found to secrete proteases thought to be involved in host entry, immuno-modulation and digestion, as described later.

Natural antibodies

Immunoglobulins are part of the adaptive immune response, as the production of specific antibodies requires the stimulation of B cells by a specific antigen; however 'natural' antibodies also exist and are found in the serum of healthy vertebrates. These latter antibodies are produced in the absence of a specific antigen stimulant and without gene rearrangement (Magnadóttir, 2006). They are a well known characteristic of teleosts, and in mammals they are thought to play a role in immune regulation and the rapid innate immune defence to a variety of pathogens (Logtenberg *et al.*, 1992). Atlantic cod are well known for their relatively high levels of non-specific antibodies and for a correspondingly low or absent specific antibody response to immunisation or infection (Espelid *et al*., 1991; Pilstrőm & Petersson, 1991; Magnadóttir *et al*., 2001; Magnadóttir *et al*., 2002). This will be discussed further later.

Lysozyme and chitinase

Lysozyme is an enzyme that hydrolyses the peptidoglycan layer of bacterial cell walls causing lysis (Ellis, 1999); primarily lysing Gram-positive bacteria, although it also has the capacity to lyse Gram-negative bacteria (Jolles & Jolles, 1984; Grinde, 1989). Therefore, lysozyme may have a limited role in the defence against parasitic copepod infections, such as *L. branchialis,* due to its substrate (Ingram, 1980). Ross *et al*. (2000) found lysozyme levels of Atlantic salmon (*Salmo salar*; Linnaeus, 1758) not to differ between control and *Lepeophtheirus salmonis* (salmon louse) infected fish. Fast *et al*. (2002a) found mucus lysozyme activities of *L. salmonis* infected Atlantic salmon and rainbow trout to increase at 1 d.p.i., and in rainbow trout to remain significantly higher than in control fish for the entire study. However, lysozyme is thought to not play a major role in innate immunity to salmon lice. This is supported by the fact that rainbow trout have higher constitutive levels of both

plasma and mucus lysozyme than more resistant species of salmonid, such as coho salmon (*Oncorhynchus kisutch*; Walbaum, 1792; Fast *et al*., 2002b).

Bergsson *et al*. (2005) found found no lysozyme activity in the mucus extract of healthy Atlantic cod. Magnadóttir (2006) also pointed out that several marine species, such as cod, haddock, pollack and wolffish possess very limited or no tissue and bodily fluid lysozyme activity. These species however, possess high levels of chitinase activity suggested to play an important role in preventing bacterial and fungal invasion (Fange *et al*., 1976; Manson *et al*., 1992; Lindsay & Gooday, 1985; Magnadóttir, 2006). Chitinase is an enzyme, which lacks the muramidase activity of lysozyme but is more effective in hydrolysing chitin. Ingram (1980) pointed out that chitinase may have a protective role against natural infections with chitin containing fungi and invertebrate parasites. The fact that this enzyme hydrolyses chitin and that chitinase activity is relatively high in cod may make it a key player in the innate response to infection by *L. branchialis*.

In summary, the investigations above have highlighted the importance of the innate immune response against parasites, especially demonstrating the importance of complement mediated immunity. Many studies have concluded that the species, strains or individuals proving most resistant to parasitic infections are those which are more capable of initiating an effective innate immune response against the parasite, and of recuperating their immune activity post-infection.

1.5.1.3 Cellular immune response

The cellular immune response of cod to infection with *L. branchialis* has only received limited attention with a few studies on the histopathology of the cellular

reaction at the site of attachment and feeding of the metamorphosing adult female. The harmful effects exerted on the host by a pennellid parasite, such as *Lernaeocera* can be attributed to three causal components; its initial attachment, host entry / migration and feeding activity. Schuurmans Stekhoven (1936) observed the *bulbus arteriosus* to form a connective tissue capsule around the cephalothorax of fully metamorphosed *L. branchialis*. He described this capsule to be composed of fibroblasts near the parasite's surface which were formed from juvenile mesenchyma cells leading to a network of fine elastic fibres, ensued by lymphocyte infiltration. Tissue in the direct vicinity of the parasite showed signs of damage with some necrosis, whilst other parts were highly vascularised showing "reconstruction" and "regeneration" (Schuurmans Stekhoven, 1936 cited in Kabata, 1970). Capart (1947) observed the cephalothorax of *L. branchialis* to be encysted within a "granulous and crumbly" substance; however he did not describe the composition of the cyst further. Smith *et al* (2007) studied the pathology associated with the early stages of *L. branchialis* infection in juvenile Atlantic cod up to 33 days post-infection. They observed initial infection to cause sub-epithelial haemorrhaging of the gill filaments, with thrombus formation associated with the parasite, surrounded by necrotic hyperplasic connective tissue. Following cephalothorax metamorphosis within the ventral aorta they observed an organised thrombus surrounding the parasite and local neo-vascularisation. Lymphocytic and haemorrhagic infiltration and moderate diffuse pericarditis were associated with the migration of the cephalothorax to the *bulbus arteriosus* (Smith *et al*., 2007). Epithelial hyperplasia and granulation tissue at the site of parasite entry was present and described as becoming increasingly necrotic with the formation of fibrous tissue (Smith *et al*., 2007). Shariff & Roberts (1989) found the penetration site of *Lernaea polymorpha* in naïve *Aristichthys nobilis* (bighead carp) to be plugged with a collar of epidermal

cells which developed into a nodule of inflammatory exudate as the parasite grew and migrated further, followed by fibroplasia. This highlights the importance of rapid wound-sealing after parasite penetration to prevent host osmotic imbalance. Ellis (1981) stated that if an inflammatory response fails to destroy or reject a pathogen, the reaction subsides and encapsulation with connective tissue follows, which may become calcified. Elarifi (1982) described the complete encapsulation of nematode larvae within a fibrous capsule in the liver of its gadoid host, whiting, which did not calcify or contain eosinophils. He described initial larvae penetration to be associated with a fibrin clot to limit haemorrhaging, ensued by neutrophil infiltration, necrosis and fibroblast aggregation forming a capsule and followed by 'clearing' of necrotic debris. Another pennellid parasite, *Phrixocephalus cincinnatus*, which burrows into the choroid layer of it's host's eye to feed from host blood, has been descibed to be surrounded by a tough fibrous cyst and its presence to result in a large haematoma surrounding the holdfast bound together by parallel strands of connective fibres (Kabata, 1969). Perkins (1994) described the frontal processes of this parasite to induce neo-vascularisation and diapedesis greatly thickening the capillary bed and quantity of extra-vascular erythrocytes within the choroid layer. This would greatly advantage the feeding of the parasite within the choroid layer increasing the abundance of nutrients. These histopathological responses to copepod parasites seem to allow 'anchorage' within their host and a sustained supply of nutrients via neovascularisation and extravasation of blood cells.

Components of the cellular immune response

The main cellular components of the innate immune system of teleosts include granulocytes, monocytes / macrophages, non-specific cytotoxic cells (Dalmo *et al*., 1997), and dendritic-like cells (Lovy *et al.*, 2006; 2009). During the inflammatory

response of teleost fish there is a biphasic cellular contribution; initially there is an influx of neutrophils pursued by monocytes / macrophages to the site of inflammation (Reite & Evensen, 2006).

Monocytes / macrophages

Monocytes in fish are thought to originate from stem cells in the haematopoietic areas of kidney, and are regarded as immature cells reaching their full maturity when they leave the circulation and enter tissues where they develop into macrophages. Ellis (1977) reported that phagocytic macrophages occur in large numbers in the head kidney, spleen, thymus and non-lymphoid organs such as the heart and gills. Teleost macrophages may be activated at the site of inflammation showing enhanced phagocytic and anti-microbial activity (Rowley *et al*., 1988). Buchmann & Bresciani (1999) reported the attack of *Gyrodactylus derjavini* by macrophages from rainbow trout, particularly on the mannose-rich locations in the cephalic ducts, upon incubation *in vitro*. Parasites opsonised by fresh trout serum resulted in enhanced attack by macrophages on all body parts, whereas heat inactivated serum had no effect (Buchmann & Bresciani, 1999). These authors concluded that a heat labile component released by trout leukocytes, probably C3, was accountable for the opsonisation of the parasite to activated macrophages.

Granulocytes

Mammalian granulocytes comprise three cell types: neutrophils, eosinophils, and basophils, however, the story for teleost granulocytes is less well defined and controversy over the presence of eosinophils and basophils persists (Reite & Evensen, 2006). Neutrophils have been reported in numerous studies to infiltrate injured tissue in the primary stages of inflammation, migrating from the blood into

tissue, where they are suggested to destroy pathogens by phagocytosis and / or cytotoxic-like activity (Reite & Evensen, 2006). Reports of eosinophils and basophils are rare and varied within fish blood, especially for the latter (Ellis, 1977). The controversy surrounding the presence and function of these cells in teleosts is also true of other cells such as mast cells / eosinophilic granule cells (MC/EGCs), and rodlet cells. MC/EGCs of teleosts are thought to be similar to their mammalian mast cell counterparts, except fish MC/EGCs are suggested to elicit different chemical effector agents to those such as histamine in mammalian mast cells. MC/EGCs undergo degranulation at sites of acute tissue damage and inflammation, releasing inflammation mediators. The number of these cells usually increases at sites of chronic inflammation (Reite & Evensen, 2006).

Various studies have demonstrated the importance of the cellular response of fish in their interactions with parasites and possible resistance towards them. Buchmann (1999) and Buchmann and Bresciani (1999) found *G. derjavini* infection of rainbow trout to result in epithelial cells and macrophages of the skin becoming activated at the site of attachment, with the release of C3 and interleukin-1 (IL-1). Intact helminths or their extracts have been shown to activate a chemotactic response in fish leukocytes *in vitro* (Hamers *et al*., 1992: Sharp *et al*., 1991). Faisal *et al*. (1990) showed that leukocytes from anterior head kidneys of *Atheresthes stomias* (Pacific arrowtooth flounder) infected with *Phrixocephalus cincinnatus* had lower non-specific cytotoxicity to murine cells than those of uninfected flounder. This reduction in innate cell-mediated immunity was further evident in flounder infected with parasites in both eyes. Dezfuli *et al*. (2003) found extensive gill tissue damage at the site of attachment and feeding of *Ergasilus sieboldi* (copepod ectoparasite) on *Abramis brama* (bream). They also observed an intense host cellular reaction with a

high infiltration of inflammatory cells, such as eosinophilic granular cells and rodlet cells, at the site of attachment. Silva-Souza *et al*. (2000) reported intense lymphocytopaenia and neutrophilia in a Brazilian freshwater fish species, *Schizodon intermedius*, infected with the copepod *Lernaea cyprinacea*. These authors also observed an increase in monocytes and immature leukocytes in infected fish compared to uninfected ones.

Active host rejection involving cellular responses has been reported in some parasitic copepods (Shariff & Roberts, 1989; Woo & Shariff, 1990). Shariff & Roberts (1989) infected naïve *A. nobilis* with *Lernaea polymorpha* in order to compare the site of infection with that of naturally infected immune fish. Susceptible naïve fish showed “extensive tissue disruption, necrosis and haemorrhage along it’s path of entry, followed by an acute inflammatory response succeeded by a highly vascular chronic granulomatous fibrosis, whereby collagen fibres encapsulated the horns of the parasite” (Shariff & Roberts, 1989; p405). In comparison, resistant fish with previous exposure to the parasite were found to principally suffer extensive haemorrhagic ulcerative lesions rarely followed by penetration through the epidermis. They found resistance to infection to be associated with very large numbers of eosinophilic granule cells (EGCs), lymphocytes, and club cells.

1.5.2 The acquired immune response

There are only a few studies undertaken on *L. branchialis* infection, which have either dismissed or suggested an acquired immune response in cod and whiting to infection. Van Damme & Hamerlynck (1992) found some whiting in the Oosterschelde infected by *L. branchialis* to reject their parasites at the late penella sub-stage in June and July suggesting that immunological factors probably play an

important role in the summer in determining the abundance of *L. branchialis* in whiting. Lysne & Skorping (2002) measured infection rates of *L. branchialis* on caged cod, where they reported that 79% of the cod investigated remained uninfected. They found the caged cod to be composed of two groups; one inherently susceptible group of cod which were infected and re-infected upon losing the initial parasite, and a second larger group of resistant cod with low infection rates. However, the immunological differences between these 'resistant' and 'susceptible' groups were not studied. Khan (1988) found evidence of a decrease in the intensity of parasitism with time in cod experimentally infected with *L. branchialis* due to shedding of the parasite. He described open lesions left after parasite shedding as either becoming necrotic, with caseous exudates or healing completely, leaving an area that was slightly elevated. Healing occurred more rapidly in adults that had shed parasites than in juveniles. However, only 4% of fish became parasite free by shedding, as many of the parasites were shed when the fish possessed multiple infections reducing the number of parasites to 1-2 parasites. Khan (1988) found cod that had been infected the previous year to possess similar infection rates as cod without an infection history. However, he did not look at parasite survival over time as parasites may have been shed quicker on previously infected fish. Khan *et al*. (1990) followed the infection intensity of *L. branchialis* in wild caught cod in sea cages, in order to observe the effect of cod ranching on infection dynamics. They observed some fish to show a tendency to shed one or more parasites, particularly adult cod (50-74cm), with approximately 50% of 86 tagged adults becoming parasite free 4 months after initial infection with 1-2 parasites. However, the juveniles showed little evidence of shedding parasites (Khan *et al*., 1990), and Sundnes (1970) and Khan (1988) reported higher *Lernaeocera* - induced mortality rates in juvenile cod compared to mature cod.

Jones and Taggart (1998) suggested that the immune response is affected by fish age and possibly its infection history. This may be corroborated by Magnadóttir *et al*. (1999b) who found serum IgM levels to increase with fish length in Atlantic cod. Israelsson *et al*. (1991) found no obvious effect of *L. branchialis* infection on wild cod serum Ig levels and total serum protein, however due to the fact that this was not the main direction of the study the stage of the parasites was not recorded and the age of cod was not determined with the fish length ranging from 29-72cm. The studies to date suggest that shedding of *L. branchialis* seems to be due to innate rather than acquired immune responses, and that when multiple infections are present space / nutrient competition may occur between parasites resulting in some being shed. However, these studies are based on field studies and wild cod making it difficult to conclude anything regarding the importance of an adaptive immune response in gadoids to *L. branchialis*-infection. It also seems that adult cod are more capable of shedding their parasites than their juvenile counterparts (Khan *et al.*, 1990).

The evidence for acquired immunity in fish to other parasites is also contradictory. There is little evidence of acquired immunity to salmon lice within salmonids (Bricknell *et al*., 2003). However, Grayson *et al*. (1991) sporadically observed the production of serum antibodies against the salmon louse in farmed Atlantic salmon. The significance of this antibody response is not documented, and Grayson *et al*. (1995) observed no such antibody response in lab-infected Atlantic salmon. Grayson *et al*. (1995) immunised naïve Atlantic salmon with purified *Lepeophtheirus salmonis* extract but found it does not protect the host from re-infection, as there was no significant difference in the number of lice infecting the host. However, the number of ovigerous females infecting immunised salmon and their fecundity was

reduced compared to those infecting naïve salmon (Grayson *et al*., 1995). Fast *et al*. (2006) found that primary infection of Atlantic salmon with a low dose of *L. salmonis* does not appear to provide them with protection from re-infection. However, Fast *et al*. (2006) stated that the two-week period between initial and repeat infection would not have been long enough for Atlantic salmon to produce a significant antibody response against re-infection.

Thoney & Burreson (1988) investigated *Leiostomus xanthurus* (Spot croaker) naturally infected with *Lernaea radiatus* and found no detectable antibody response. Woo and Shariff (1990) found *Lernaea cyprinacea* not to mature to the adult stage on previously infected *Helostoma temmincki* (kissing gourami) when challenged at low doses, and at high challenge doses previously infected fish rejected the adult stages faster than the naïve fish. The adult female *L. cyprinacea* that grew on previously infected *H. temminicki* were also found to lose more egg sacs and to produce fewer viable eggs than those on naïve hosts. Woo and Shariff (1990) suggested that parasite rejection by *H. temminicki* was due to an acquired immune response even though they did not measure any acquired immune parameters. Karvonen *et al*. (2005) found rainbow trout previously infected with *Diplostomum spathaceum* to show acquired resistance with 85 – 89.1% lower parasite establishment than naïve rainbow trout, and stated that the innate immune response provided modest or no protection to infection. Madhavi & Anderson (1985) investigated the susceptibility of guppies (*Poecilia reticulata*) to *Gyrodactylus bullatarudis* infection. They found a susceptible strain and a resistant strain, both of which were refractory immediately following primary challenge. However, within six weeks of the first infection, when a re-infection challenge was carried out, the susceptible strain lost the resistance to re-infection that they had demonstrated

immediately after recovery from the first infection. The resistant strain remained refractory to infection six weeks after the first infection. Madhavi & Anderson (1985) suggested from their results that there was a genetic component to control the resistance / susceptibility traits in guppies.

It is clear from these studies that the innate immune response of some teleosts plays an important role against some parasitic infections. This is probably due to the fact that the adaptive immune system is slower to respond to infection than its innate counterpart. Mustafa *et al*. (2000a) and Nolan *et al*. (2000) both suggested that salmon lice only caused significant innate immunosuppression, increased epithelial damage and osmoregulatory failure in the host once pre-adult stages of the salmon louse appear. Therefore, it is more advantageous for the host to respond quickly to the parasitic invasion via the innate immune system in order to try to prevent the establishment and hence, the moulting of the salmon lice into the pre-adult and adult stages. This is also likely to be true for gadoid hosts of *L. branchialis* as, once the adult female attaches to the gills and penetrates the afferent branchial artery, she will begin demanding large volumes of host blood. However, most studies regarding the adaptive immune response to parasites of teleost hosts to date have concentrated on the production of specific antibodies with very few studies regarding other factors, such as T cell activation, in teleosts post-infection (Alvarez-Pellitero, 2008). This is partly due to the lack of markers available in fish for the components involved, such as MHC II and T cell receptors (TCR). However, with the recent increase in the number of gene sequences available in teleosts, a few studies have looked at the gene expression of TCRs and MHC II post-infection with *G. derjavini* in *O. mykiss* and *Diplectanum aequans* in *Dicentrarchus labrax* (Lindenstrom *et al.*, 2003; Faliex *et al.*, 2008); however, no

clear parasite-related changes in the transcript levels of MHC II or TCR were observed post-infection. The process of parasite removal and / or limitation by the host however, is not as simple as this. Parasites have also evolved corresponding defence strategies to exploit their host's immune system in order to survive in the hostile environment of their host. These strategies will be discussed further in Section 1.7.

1.6 The immune system of *Gadus morhua* (L., Atlantic cod)

Magnadóttir *et al*. (2001) highlighted the importance of studying the immune system of Atlantic cod due to the fact that it is a 'new' species for commercial aquaculture and that studies have shown Atlantic cod to possess unusual immune system characteristics. This includes the apparent inability of the species to elicit a specific antibody response when immunised with haptenised / non-haptenised proteins and bacterial antigens, unlike other teleosts such as salmonids (Espelid *et al*., 1991; Pilstrőm & Petersson, 1991; Magnadóttir *et al*., 2001; Magnadóttir *et al*., 2002). However, 90-100% protection has been shown in cod immersion-vaccinated against the bacterial pathogens *Vibrio anguillarum* and *V. salmonicida* (Espelid *et al*., 1991: Schrøder *et al*., 1992). The specific protection afforded by immunisation with formalin killed *Vibrio* spp. was not correlated to a good specific humoral antibody response, and was principally focused against bacterial lipopolysaccharide (LPS), a T-cell independent antigen (Espelid *et al*., 1991; Magnadóttir *et al*., 2001). Schrøder *et al*. (1998) suggested therefore that the immune system of Atlantic cod was 'driven' by mechanisms other than specific antibody production and B cell memory, which usually plays a predominant role in other teleosts. Teleost Ig is mainly composed of tetrameric IgM, which is usually pentameric in mammals, however recently a H-chain of Ig isotype IgD has been cloned from channel catfish

(*Ictalurus punctatus*; Refinesque, 1818; Wilson *et al*., 1997), Atlantic salmon (Hordvik *et al*., 1999) and Atlantic cod (Stenvik & Jorgensen, 2000). Stenvik *et al*. (2001) suggested that IgD in cod was mainly expressed as a B-cell receptor such as occurs in mammalian IgD. However, little is known about the expression of teleosts IgD, and further studies are required.

The explanation for this unusual trait of Atlantic cod in comparison to other teleosts is uncertain, but many hypotheses have been suggested by a number of researchers. Atlantic cod are well-known for their high levels of natural or non-specific antibodies, with Israelsson *et al*. (1991) reporting IgM to make up 15-20% of total serum proteins of un-immunised wild Atlantic cod, with an average serum concentration of 5.6 ± 0.19 $mg.Ig.ml^{-1}$ detected by ELISA, compared to approximately 2% of serum proteins in Atlantic salmon (Magnadóttir, 1998). Natural antibodies have the capacity to bind polyspecifically to multiple antigen types (Gonzalez *et al*., 1988), and cod natural antibodies are no exception, often described as 'sticky', with high levels of antigen binding in non-immunised control cod (Pilstrom & Petersson, 1991; Widholm *et al*., 1999). Stenvik *et al*. (2000) implied that the high polyspecificity of cod IgM for different antigens was a product of the length of the complementary determining region 3 (CDR3) of the variable domain causing a flat antigen-binding surface. This polyspecificity of cod IgM and the fact that they are present in relatively high concentrations is thought to suppress the formation of 'new' specific antibodies (Espelid *et al*., 1991), or mask any increase in specific antibodies.

It was also thought that the lack of specific antibody response in Atlantic cod was due to them being deficient in terms of generating the variability seen in other

species. However, Bengtén *et al*. (1994) studied the Ig heavy chain locus and found no deficiency in the number of VH genes in Atlantic cod, describing the variability of the Ig heavy chain variable domain within Atlantic cod as being more than sufficient to provide an efficient specific antibody response. Widholm *et al*. (1999) also discounted this hypothesis, describing the variability of Atlantic cod immunoglobulin light chain as similarly sufficient to provide a competent specific antibody response. These authors described how the absence of an antibody response to immunisation in cod could not be explained by the length, variability, or physiochemical properties of the light chain CDRs. Widholm *et al*. (1999) went on to suggest that the high level of 'natural sticky' antibodies in Atlantic cod could be more than sufficient to bind and eliminate invading antigens, diminishing the need for a specific antibody response. Pilstrőm *et al*. (2005) also concluded that there is no evidence so far that the lack of antibody response is associated with the transcriptional control of Ig genes in Atlantic cod.

Dacanay *et al*. (2006) recently reported the quaternary structure of the gadiform Ig (Atlantic cod, haddock and pollack) to be in the form of a tetramer (H8L8) as in other teleost species, however, they found Atlantic cod to only possess a single isoform consisting of a covalently bound trimer (H6L6) with a non-covalently associated H2L2 monomer. In other teleosts of the superorder Protoacanthopterygii, such as Atlantic salmon and rainbow trout, multiple isoforms of tetrameric serum Ig exist (Dacaney *et al*., 2006; Kaattari *et al*., 1998). Dacaney *et al*. (2006, p216) points out that the "differential role, if any, which these isoforms play in the adaptive immune response of fish, is unclear", and conclude that it is also "unknown whether restricted isoform diversity in the Gadiformes, or specifically that lack of a fully disulphide cross-linked tetramer, is either the cause for, effect of,

or unrelated to the extremely limited or absent specific humoral responses reported for members of this order" (Dacanay *et al.*, 2006, p218).

Persson *et al*. (1999) studied the antigen presentation system of Atlantic cod by isolating and characterising genes of the major histocompatibility complex I (MHC I) and β2-microglobulin, observing unusually high numbers of expressed MHC I sequences. From these findings they hypothesised that a gap in the T-cell repertoire of Atlantic cod may be caused by the abundance of expressed MHC I sequences. Pilstrőm *et al*. (2005) went on to suggest that Atlantic cod lack typical, functional MH class II molecules either due to the evolutionary 'loss' of MH class II locus or a MH class II locus with abnormal characteristics making it difficult to detect and functionally ineffective. However, this has not been verified.

The co-localisation of antigens and antibody producing cells in Atlantic cod was investigated by Arnesen *et al*. (2002). They intra-peritoneally injected cod with *V. anguillarum*, localising these antigens within cod tissues by immunohistochemistry. Subsequently the expression of cod IgM heavy chain constant region was tracked within the major lymph organs of cod (spleen, kidney and thymus) which shelter macrophages and lymphocytes, in order to detect any co-localisation with the antigen by *in situ* hybridisation. They observed antigens to accumulate around the small blood vessels of the spleen whilst antibody producing cells were found gathered around the thick walled arteries of the spleen. Schrøder *et al*. (1998) also found immunoglobulin positive plasma cells surrounding the large blood vessels of the haematopoietic tissues and gills, suggesting that they could be related to antigen trapping and lymphocyte stimulation in these areas. However, Arnesen *et al*. (2002) hypothesised from these results that the absence of co-localisation of

antigens and antibody producing cells may contribute towards the lack of specific immune response by Atlantic cod to vaccination.

Magnadóttir *et al*. (2002) studied the host response of Atlantic cod to experimental and natural infection with atypical *Aeromonas salmonicida*. They observed the formation of granulomas in the majority of organs including the spleen, kidney, heart and liver to be composed of a mixture of bacteria and macrophages surrounded by epithelioid cell layers and an outer thin layer of fibroblast cells. They stated how this response to *A. salmonicida* agrees with a previous study in Atlantic cod by Morrison *et al*. (1984) but differs to that seen in other fish species, such as salmonids where no granuloma formation was observed (Gudmundsdottir *et al*., 1997). This led Magnadóttir *et al*. (2002) to suggest that Atlantic cod possess a significantly different immune response to those of previously well studied species like Atlantic salmon and rainbow trout.

The spontaneous haemolytic activity of serum has also been well studied in Atlantic cod compared to the other immune components, playing an important role in both innate and adaptive immune responses. However, Atlantic cod spontaneous haemolytic (SH) activity (usually attributed to the alternative complement pathway (Sakai, 1992; Sunyer *et al*., 1997; Nakao & Yano, 1998)) has been found to be unusually heat-insensitive for teleosts, with an optimal working temperature of 37ºC (Magnadóttir *et al*., 1999a; Magnadóttir *et al*., 1999b; Magnadóttir, 2000). These authors also described the addition of EGTA and EDTA to have no negative effect on SH activity of Atlantic cod, whereas usually EGTA binds Ca^{2+} inhibiting SH activity by the classical pathway, and EDTA binds Ca^{2+} and Mg^{2+} inhibiting activity caused by both the alternative and classical pathways (Magnadóttir, 2000). These

authors actually found EDTA to enhance SH activity in Atlantic cod serum, and the activity to vary considerably between individuals. However, Magnadóttir (2000) showed zymosan and bacterial LPS inhibition of SH activity in Atlantic cod serum suggesting a complement-like nature of cod SH activity. Magnadóttir (2006) however, stated that in the past 4-5 years cultured Icelandic cod sera has shown reduced SH activity compared to that observed in wild and cultured cod in the late 1990s by Magnadóttir *et al*. (1999a & 1999b) and Magnadóttir *et al*. (2001), or no detectable SH activity. This activity was also inhibited by EDTA. The author reported that the basis for this change and the 'true nature' of cod SH activity were unknown. Magnadóttir & Lange (2004) did, however, find a strong hydrophobic association of C3 and apolipoprotein A-I (ApoI) in cod sera possessing no or little SH activity, and no such association in sera with high, unusual SH activity. In humans, ApoI has been found to bind to membranes exposed upon C9 polymerisation, interfering with molecular attack complex (MAC) formation and inhibiting cell lysis (Jenne *et al*., 1991; Hamilton *et al*., 1993 cited in Magnadóttir, 2006). Magnadottir and Lange (2004) went on to speculate that ApoI inhibits the haemolytic activity of cod serum, and could play a regulatory role on cod complement. However, until the haemolytic and complement components of cod have been characterised the true nature of the complement system of cod cannot be concluded.

A few papers have also studied the effect of temperature, size and gender on humoral immune parameters of Atlantic cod. Magnadóttir *et al*. (1999a) described serum immunoglobulin and natural antibody activity to increase with increasing water temperature, whereas increasing temperature was found to have the opposite effect on serum total protein concentration, anti-protease activity, iron concentration

and total iron-binding capacity. Magnadóttir *et al*. (1999b) found serum IgM and total protein concentration to increase with size, and SH activity to be higher in younger cod in spring than in older cod. However, the negative correlation between cod size and SH activity was not observed in cod sampled in autumn leading Magnadóttir *et al*. (1999b) to suggest that the stress of spawning in spring may have led to a decrease in SH activity. These studies highlight the effect of environmental parameters and the biological state of cod on its humoral immune system.

Recently, studies have been undertaken to isolate, cultivate and characterise Atlantic cod head kidney macrophages (Sorensen *et al*., 1997; Steiro *et al*., 1998), these studies finding them to be highly phagocytic and to show enhanced superoxide anion formation upon stimulation. However, Steiro *et al*. (1998) concluded that Atlantic cod macrophages respond less significantly to stimulation when compared to other fish species and mammals. Nikoskelainen *et al*. (2006) investigated this further by comparing the respiratory burst activity of Atlantic cod blood phagocytes with that of rainbow trout. They found cod plasma to possess a reduced opsonisation capacity compared to rainbow trout, cod blood phagocytes to generate respiratory burst activity in the absence of bacteria thought to be due to their phagocytes being more adhesive and possibly directly activated by the microplate walls, and cod blood respiratory burst activity to be at least double that of trout when activated by opsonised and non-opsonised zymosan. Nikoskelainen *et al*. (2006) suggested from their investigations that additional factors to the classical opsonin receptors such as Ig and complement, are involved in the recognition and phagocytic routes of Atlantic cod; and that the respiratory burst of cod is significantly higher than that of trout or that the number of blood phagocytes in cod

is significantly higher than in trout. High proportions of neutrophils have been found in Atlantic cod peripheral blood accounting for approximately 84% of blood leukocytes (Rønneseth *et al*., 2007), compared to 22-36% in Atlantic salmon blood (Pettersen *et al*., 2000 & 2003). This highlights the importance of neutrophils and the innate immune response in cod to infection.

1.7 Host immuno-modulation, and immune evasion by parasitic arthropods

Haematophagous parasites, such as *L. branchialis*, are exposed to the humoral and cellular immune responses of their host's blood, especially the parasite's gut epithelium layer after taking in a blood meal. Therefore, these parasites have evolved mechanisms in order to modulate / evade the immune response of their specific host species, enabling them to successfully feed and reproduce. For instance, the blood-feeding monogenean *Zeuxapta seriolae* has been found to be considerably more resistant to serum killing by its host *Seriola lalandi* (yellowtail kingfish), thought to be due to alternative complement activity, than an epithelial browsing monogenean *Benedenia seriolae* which infects the same host (Leef & Lee, 2008). This highlights the fact that the haematophagous monogenean has evolved mechanisms to survive the hostile environment of host blood in comparison to a monogenean which primarily feeds on host gill mucus and epithelial cells which is still highly susceptible to host blood factors. These mechanisms are well characterised in many terrestrial host–arthropod parasite interactions, however those involving teleost hosts are not as well studied. The prolonged and intimate contact between arthropod parasites, such as *L. branchialis*, and their hosts presents problems for successful feeding, and therefore survival. Parasite feeding could cease due to clotting or blood vessel constriction and / or inflammatory and immune responses of the host. This has led to many haematophagous parasites

evolving ways of modulating their hosts' responses, or evading the inflammatory and immune response. Titus *et al*. (2006) stated that the most striking arthropods studied to date are the terrestrial hard ticks whose saliva displays 18 vaso – and immuno-modulatory effects to date. They also stated the fact that there is great redundancy in the host modulator proteins of tick saliva allowing multiple mechanisms to inhibit or evade the hosts' immune and inflammatory responses at the same time. The secretory products of arthropods have become a growing target of research into vaccines for the prevention of the spread of parasitic diseases by arthropod vectors, which act by means of neutralising arthropod immuno-modulatory molecules. Recently vaccines have been developed against arthropod saliva constituents, such as one developed against an immuno-modulatory saliva protein of the old world sandfly *Lutzomyia longipalpis* (vector of *Leishmania*), called maxadilan (MAX) (Titus *et al*., 2006). Mice vaccinated against MAX were afforded protection from *Leishmania major* when infected by injection with salivary gland extracts to mimic the natural infection via the sandfly vector (Morris *et al*., 2001). Vaccination of the host against MAX led to the production of anti-MAX antibodies rendering the sandfly less efficient at obtaining a blood meal (Morris *et al.*, 2001). Titus *et al*. (2006) stated that these vaccines should however, be used with caution due to the fact that, as with many proteins, arthropod saliva proteins are polymorphic, with different forms at different geographical locations. Therefore, a vaccine that works in one geographical region may not work against the same saliva protein in another.

The immune evasion and immuno-modulation of fish hosts by crustacean parasites have not been as extensively studied to date. However, in the last decade researchers have increased our understanding of the interactions between the

copepod parasite *Lepoephtheirus salmonis* and its salmonid hosts. Ross *et al*. (2000), Firth *et al*. (2000) and Fast *et al* (2002a) found the skin mucus of *L. salmonis* – infected salmonid hosts to possess trypsin-like proteases thought to be derived from *L. salmonis*. Fast *et al.* (2002a) hypothesised that *L. salmonis* secretes these low molecular weight trypsins to aid feeding and / or host immune response evasion. The eicosanoid prostaglandin E_2 has also been identified in the secretions of *L. salmonis* (Fast *et al*., 2004), with inhibitory effects on the expression of several salmonid genes related to the immune system, including IL-1β, cyclooxygenase-2 (COX-2), and major histocompatibility (MH) class I and II, in SHK-1 cells (Fast *et al*., 2005). Fast *et al*. (2006) suggested that PGE_2 released by *L. salmonis* could increase blood flow to the feeding site, and prevent leukocyte recruitment and antigen presentation to host T lymphocytes. Fast *et al*. (2004) observed *L. salmonis* to secrete / excrete PGE_2 at similar levels to several other arthropod ectoparasites, and at even higher levels than those seen in the saliva of *Boophilus microplus* and *Ixodes dammini* (approx. 1-2ng.tick^{-1}; Inokuma *et al*., 1994; Ribeiro *et al*., 1985). The quantity of PGE_2 in the secretory / excretory products (SEPs) decreased with increasing time following lice removal from the host before SEP collection; average SEP PGE_2 levels were measured at 14.5, 0.2, 0.5, and 0ng.louse^{-1} at 3, 12 and 24 h off the host, respectively. Aljamali *et al*. (2002) observed similar variations in saliva prostaglandins from *Amblyomma americanum* and attributed this to the variation in size of ticks and to also depend on the time elapsed since the ticks last fed. Fast *et al*. (2004) only observed PGE_2 in the SEPs of *L. salmonis* and no other prostaglandins, which is in accordance with saliva from ticks except that of *A. americanum* where Aljamali *et al*. (2002) found small quantities of $PGF_{2\alpha}$. Fast *et al*. (2004) stated that PGE_2 released by *L. salmonis* probably acts on a local scale within the host rather than as a systemic immuno-modulator. Eicosanoids are lipid

mediators generated from the oxygenation of C_{20} polyunsaturated fatty acids, with multiple functions in vertebrate and invertebrate organisms. Synthesis of prostaglandins and other eicosanoids have also been reported in a variety of protozoan, trematode, cestode and nematode parasites (Belley & Chadee, 1995), and are increasingly being reported at host-parasite interfaces. The availability of sequenced genomes for parasites, such as *Schistosoma mansoni,* will allow parasite-derived factors at the host-parasite interface, such as prostaglandins, to be investigated more in depth. Prostaglandins present in the saliva of terrestrial haematophagous arthropods are thought to facilitate feeding by increasing the local blood flow and suppressing the host's immune response. They are thought to play a role in penetration, immunosuppression, inflammation or haemostatic modulation in a variety of protozoan and metazoan endoparasite–host interactions. The prostanoid, PGE_2, only acts as an anticoagulant at high doses and $PGF_{2\alpha}$ is vasodilatory (Ribeiro *et al*., 1985). However, PGE_2 also possesses a pro-inflammatory action causing vasodilation, increased vascular permeability and phagocyte attraction; however, it also reduces phagocytosis and inhibits lysosomal enzyme release (Daugschies & Joachim, 2000). Fast *et al.* (2007) also highlighted that other pharmacologically active parasite-derived compounds with a host-modulatory capacity are present in *L. salmonis* secretions, which await identification. Studies on the secretory / excretory products of the haematophagous nematode *Anguillicola crassus* have also identified a detoxifying enzyme, glutathione-s-transferase, which has the capacity to quench host reactive oxygen intermediates (Nielsen & Buchmann, 1997).

Parasite-derived proteases have been shown to facilitate host invasion, degradation and digestion of host tissues, evasion of host immunity, clot dissolution and

increased vascular permeability (McKerrow, 1989; Hotez & Cerami, 1983). Tort *et al*. (1999) concluded that the majority of nematode parasites studied to date release proteases involved in tissue penetration, parasite nutrition from host tissue and evasion of the hosts' defence mechanisms. Polzer and Taraschewski (1993) described a trypsin-like protease in the migratory L3 larval stage of the parasitic nematode *Anguillicola crassus,* thought to be involved in host tissue penetration, and an aspartyl proteinases from the adult blood-feeding stage thought to be involved in digestion. Geldhof *et al*. (2000) stated that proteases secreted by parasitic nematodes are crucial for the maintenance of infection and therefore, may be key targets for anti-parasite vaccines and chemotherapy. They found larvae and adult *Ostertagia ostertagia* (a gastrointestinal nematode of cattle) to release proteases capable of degrading host blood proteins including host IgG *in vitro*. Wang and Nuttall (1995) also detected IgG - binding proteins in the salivary gland extracts and haemolymph of the ixodid tick species *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus*.

Parasites must overcome their hosts' innate immune mechanisms in order to feed successfully and survive, of which the complement system is a major component. Anti-complement activity has been described in a number of parasite species, targeting different points of the complement cascade (Leid, 1988). Therefore, evasion of the host's complement system may take many forms, such as not activating the complement, blocking the cascade before the formation of the membrane attack complex, causing the formation of a membrane attack complex that is unable to lyse organisms, and shedding molecules resulting in fluid-phase depletion of complement components or active destruction of complement proteins by proteolysis, or a combination of the above (Joiner, 1988).

Numerous studies demonstrate the abundance of mechanisms utilised by parasites to modulate and evade their hosts' defence systems. They also highlight the gap in knowledge regarding these mechanisms for aquatic parasites of teleosts, including *L. branchialis*, compared to those of their terrestrial counterparts. Therefore, it is important to investigate the secretions of *L. branchialis* and their effect on various aspects of the humoral and cellular immune system of Atlantic cod. These secretions are likely to originate from exocrine glands of the cephalothorax embedded in the host, especially those in close vicinity of the oral cone. Bell *et al*. (2000) has previously described the location of exocrine gland populations in *L. salmonis* that stained positive with the immunohistochemical stain 3', 3-diaminobenzidine tetrahydrochloride (DAB). Bron (1993) previously described this staining technique which stains endogenous peroxidases and possibly catalases and cytochrome oxidases. These peroxidase enzymes have been reported to possess various roles in animal tissues; the most interesting include free radical neutralisation (Salin & Brown-Peterson, 1993) and prostaglandin production (Bowman *et al*., 1996). Bell *et al*. (2000) suggested that DAB-positive glands of *L. salmonis* and *Caligus elongatus* located in the vicinity of the mouth parts may secrete compounds such as peroxidases, and hypothesised that they may aid in the protection of these parasites from deleterious compounds of their host's immune response and food digestion. If we are able to locate glands within the different adult female stages by DAB staining, histology and electron microscopy, it will aid future investigations of parasite-derived secretory / excretory products (SEPs). It will also be important to investigate the effect of SEPs on host defence mechanisms in order to elucidate how these mechanisms work, and which host factors are targeted. The interactions of host immune factors with the parasite's gut epithelium also require investigation due to its intimate exposure to host factors during blood

feeding, such as C3 and immunoglobulins. For instance, studies by Kopf *et al*. (2000) found the humoral immune response of eels to be targeted more towards the parasite's body wall, with only a few eels found to produce antibodies against antigens of the intestinal wall during the haematophagous stage of the parasite.

1.8 Aims and objectives of study

Even though *L. branchialis* has the potential to cause serious problems for wild and farmed cod stocks no extensive work has been undertaken to further our understanding of this host-parasite interaction. In comparison, the literature concerning the life cycle, pathogenic effects and associated histopathology are far more abundant. Key areas of understanding such as the hosts' immunological defences towards metazoan parasites, and the parasites' mechanisms of host immune evasion and / or immuno-modulation are missing. These could yield initial insights into areas for control and vaccine development in the future, should *L. branchialis* become a pest to gadoid culture as *L. salmonis* has for marine salmonid farming.

The humoral and cellular innate immune system has been demonstrated in teleosts to play a major role in host defence against pathogens, with the complement system and anti-protease activity playing key roles in the fight against parasitic infections, and parasite secretions. A wide variety of mechanisms possessed by parasites to evade or modulate their hosts' immune and haemostatic responses have been studied to date. These mechanisms involve anti-complement activity, proteases, anticoagulants and anti-inflammatory agents from protozoan to metazoan parasites. The immune response of Atlantic cod is also documented as unusual amongst other teleosts, as their specific antibody response is limited or

absent in comparison (Espelid *et al*., 1991; Pilstrom & Petersson, 1991). Therefore, this host - parasite interaction is an intriguing one, as the immune system of Atlantic cod has not been extensively investigated in comparison to salmonids, especially towards parasitic infections, and the adult female *L. branchialis* is intimately exposed to its host's immune mechanisms for an extended period of time.

Therefore, the aim of this thesis was to investigate the host–parasite interactions, in terms of the systemic and local immune responses of Atlantic cod to *Lernaeocera branchialis* infection, and the immuno-modulation of Atlantic cod by *L. branchialis* – derived secretory / excretory products. The specific objectives were:

1) To investigate the systemic and local immune response of naïve hatchery-reared *Gadus morhua*, and wild-infected gadoids, in terms of humoral and cellular immune factors, to laboratory infection with *L. branchialis*.

2) To map and investigate the exocrine glands of *L. branchialis* to allow future studies of the production sites and secretion kinetics of parasite – derived host–modulating products, as well as product identification.

3) To investigate the effect of *L. branchialis* – derived secretory / excretory products (SEPs) on innate immune responses of naïve Atlantic cod. To help address the question: are *L. branchialis* producing factors capable of modulating their host environment? This was achieved via *in vitro* assays with dopamine-induced SEPs and investigation of the local attachment / feeding area of *L. branchialis*.

Chapter 2 General materials and methods

This chapter outlines the general materials and methods used throughout this thesis, however further details of specific techniques applicable to a particular study will be given in the corresponding chapter.

2.1 *Lernaeocera branchialis* supply and maintenance

2.1.1 Source of free-swimming females

Wild flounder (*Pleuronectes flesus*) naturally infected with *L. branchialis* were collected from Longannet power station cooling water screens on the Firth of Forth, Scotland (56.05°N; 3.70°W). They were kept in an isolated fibre glass tank with 300L natural seawater at 13°C ± 1.0 with aeration and an external biofilter canister (Fluval FX5). A filter pan with a 70µm mesh was placed over the biofilter water inlet pipe in order to prevent parasites entering the biofilter. Flounder were fed frozen ragworm (*Neris* spp.; Seabait Ltd) *ad. lib*.. Tank water was filtered daily by siphoning it through a 70µm mesh to isolate free-swimming females. These females were kept in 0.2µm filtered natural seawater with aeration at 12°C for a maximum of 3 days prior to use in infections, and fixed or stained immediately upon collection when used for microscopy.

2.1.2 Source of metamorphosing females

Wild gadoids (*Melanogrammus aeglefinus*, *Gadus morhua* and *Merlangius merlangus*), naturally infected with *L. branchialis,* were collected by trawling in the North sea and Northeast Atlantic on two fish surveys upon the Fisheries Research Services (Aberdeen) Vessel, HMRV Scotia:

Survey 1: Quarter 4 west coast survey in November 2006

Survey 2: Wild fish disease survey in May 2007

L. branchialis females at various sub-stages of metamorphosis were dissected free from host tissue and used for secretory / excretory product collection or fixed for microscopy immediately.

2.2 Naïve host supply (*Gadus morhua*) and maintenance

Juvenile naïve Atlantic cod were supplied by Machrihanish Marine farm Ltd. hatchery (Machrihanish, Scotland) for the first preliminary laboratory infection and Viking Fish Farms Ltd. (Ardtoe, Scotland) for all other experiments. They were acclimatised for at least 2 weeks in 300L tanks on a natural seawater re-circulation system at 13°C ± 0.5 (University of Stirling). Stock and experimental fish were fed cod sinking pellet (Skretting, Stavanger, Norway) at approximately 1.5% body weight once daily, however, being starved for 24 h prior to sampling and infection events.

2.3 Infection of naïve *Gadus morhua*

Naïve Atlantic cod were acclimatised for a minimum of 2 weeks in four 500L tanks (duplicate infection and duplicate sham control tanks). Infections took place in a temperature controlled room with a mean seawater temperature of 13°C, and light:dark cycle of 12h:12h. Tanks were filtered by external biofilter canisters (Fluval FX5) matured using a nitrifying pack (ABIL) with a flow through rate of 2000L/h, and the water quality monitored daily for ammonia, nitrite and pH levels. For infections the water level was dropped from ~500L to ~150L with continuous aeration. Ten free-swimming females were added to the two infection tanks per fish and oxygen monitored for 6 h using a YSI 200-4

dissolved oxygen probe. The same treatment was applied to the sham control tanks except no parasites were added in order to discriminate between effects resulting from the protocol and those due to infection. After 6 h the water volumes were gradually restored to ~500L with seawater, and the external biofilter canisters reconnected.

2.4 *Gadus morhua* anaesthesia

Atlantic cod were anaesthetised using 2-phenoxyethanol (Sigma, Poole, UK) 1:10,000 in seawater. After anaesthesia fish were monitored whilst recovering in an aerated tank prior to being placed back in the experimental tank. Euthanasia was achieved by overdose of anaesthesia using 2-phenoxyethanol 1:1000 in seawater, followed by destruction of the brain.

2.5 Panjet marking

Atlantic cod were anaesthetised as described above, and PanJet™ (Wright Dental Health, Dundee) marked on the belly with alcian blue (Sigma) in order to identify certain fish. The fish were then allowed to recover in seawater with aeration prior to being placed back in the tank. Fish were marked at least 1 week prior to any procedures or sampling.

2.6 Host blood collection

Blood was collected after an overdose of anaesthesia from the caudal vein using a sterile needle and 1ml syringe.

2.6.1 Serum collection

Blood was allowed to clot at room temperature for 1 h and was then kept overnight at 4°C for clot retraction. This was followed by centrifugation at 3000 xg for 7 min. Serum was collected and stored in 60µl aliquots at -70°C.

2.6.2 Heparinised whole blood collection

Blood was collected with a sterile needle and syringe pre-flushed with heparin (Sigma) 2500IU.ml^{-1} Leibovitz–15 (L-15) media (Sigma) to prevent clotting, and temporarily stored on ice until use.

2.6.3 Sodium citrated plasma collection

Blood was collected with a sterile needle and syringe pre-flushed with 3.2% (w/v) sodium citrate in 0.15M saline, followed by centrifugation at 3000 xg for 7 min. Plasma was collected and stored temporarily on ice prior to use in a re-calcification clotting assay.

2.7 Total protein quantification

Total protein content of samples was quantified using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) following manufacturers' instructions for the microplate assay, using bovine serum albumin standards (Pierce) or pig γ–globulin standards (Sigma) for purified immunoglobulin sample quantification.

2.8 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was utilised to determine sample protein profiles and molecular weights. Electrophoresis was carried out according to Laemmli (1970). Protein samples were reduced and denatured by diluting 1:1 in 2x sample buffer (4%

(w/v) sodium dodecyl sulphate; 125mM Tris-HCl, pH6.8; 20% (v/v) glycerol) with 100mM DL-dithiothreitol (DTT; Sigma) and boiling for 5 min. If proteins were to be denatured but not reduced DTT was omitted from the sample buffer. Equal amounts of protein per sample were loaded onto 12% polyacrylamide separating gels with a 4% stacking gel. Electrophoresis was carried out at 150V for 75 min. Gels were either stained with 0.25% Coomassie blue R-250 (40% v/v methanol, 10% v/v acetic acid) or silver stained according to Swain and Ross (1995). Broad range molecular weight markers (New England BioLabs, Ipswich, MA, U.S.A.) were used for calibration of protein molecular weights.

2.9 Leukocyte isolation

Leukocyte isolation was performed using a modified protocol based on Sorensen *et al*. (1997) and Steiro *et al.* (1998) as detailed below. All cellular work was performed aseptically in a laminar airflow cabinet (Gelaire, Sydney, Australia).

2.9.1 Media

L-15 media (Sigma) was used for all cell culture and cellular assay experiments supplemented with antibiotics (100IU.ml^{-1} penicillin; 100ug.ml^{-1} streptomycin (Sigma)) and 2mM L - glutamine (Sigma).

2.9.2 Head kidney

Head kidneys were dissected aseptically into 5 ml L-15 media supplemented with 15IU.ml^{-1} heparin (Sigma), prior to homogenising through a 100µm sterile nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). A maximum of 2.5ml cell suspension was layered on top of each density gradient composed

of 5ml 51% Percoll™ (Sigma) and 5ml 34% Percoll™ in phosphate buffered saline (PBS). The layered gradients were centrifuged at 400 xg for 45 min at 4°C. The cells at the 34 – 51% interface were collected and washed twice in L-15 at 150 xg for 15 min at 4°C, to remove Percoll™. The cells were re-suspended to 1 x 10^6 cells.ml^{-1} L-15 media with 1% (v/v) foetal calf serum (Sigma), and used for flow cytometry or macrophage enrichment.

2.9.3 Blood

Heparinised blood was diluted 1:5 in L-15 media supplemented with 15IU.ml^{-1} heparin and layered on to two 5ml 51% Percoll™ in PBS. They were centrifuged at 500 xg for 30 min, and the cells at the media – Percoll™ interface removed and washed twice in L-15 media. These cells were used for flow cytometry.

2.9.4 Cell counts

Cell counts were performed using a Neubauer haemocytometer counting chamber, and cell viability determined by flow cytometry as detailed below.

2.10 Flow cytometry

Flow cytometry experiments were carried out on a FACScalibur (Becton Dickinson) using Cell quest version 3.3 software (Becton Dickinson).

2.10.1 Cell viability

Cell viability was determined on the flow cytometer using the nucleic acid stain propidium iodide (PI; Sigma). PI is impermeable to cell membranes and therefore only enters cells with damaged cell membranes or impaired

metabolisms *i.e.* non-viable cells. After entering the non-viable cell PI binds to the nucleotides enhancing its fluorescence by 20-30-fold.

PI (Sigma) was added to cell suspensions in L-15 media at a final concentration of 10µg.ml^{-1} for 10 min at 22ºC. Autofluorescence controls of cell suspensions without PI were also included. Phagocytes (10000 per sample) were collected on a FACScalibur, with the phagocyte population selected by gating on the forward scatter channel (FSC) vs side scatter channel (SSC) scattergram due to their larger size and granularity *i.e.* higher FSC and SSC compared to other leukocytes. FL-2 fluorescence, *i.e.* fluorescence quantified on channel 2, of the gated population was measured, and the photomultiplier gain adjusted so that the fluorescence of the controls was confined to the first decade of the FL-2 histogram. The percent fluorescent cells *i.e*. non-viable cells was recorded and taken away from 100 to give the percent population viability.

2.10.2 Respiratory burst activity

Respiratory burst activity of head kidney - or blood – derived phagocytes was measured following a modification of the flow cytometry method of Richardson *et al*. (1998) suggested by Moritomo *et al*. (2003). This assay utilises a non-fluorescent dye called dihydrorhodamine-123 (DHR-123), which is oxidised by hydrogen peroxide (H_2O_2) production intracellularly during respiratory burst to fluorescent rhodamine.

Nine hundred µl head kidney or blood leukocytes (1 x 10^6 cells.ml^{-1} L-15 media) were incubated at 6°C for 1 h in sterile flow cytometry tubes (BD biosciences). Leukocytes were pre-incubated with DHR-123 (Sigma) at a final concentration

of 10µg.ml^{-1} for 5 min at 22°C, followed by activation with phorbol 12-myristate 13-acetate (PMA; Sigma) at a final concentration of 0.1µg.ml^{-1} for 30 min at 6°C. Controls included leukocytes with only PMA or DHR. Phagocytes (10000 per sample) were collected on a FACScalibur, with the phagocyte population selected by gating on the FSC vs SSC scattergram. Rhodamine fluorescence of the gated population was measured on the FL-1 green channel, and the photomultiplier gain adjusted so that the fluorescence of the controls was confined to the first decade of the FL-1 histogram. Mean fluorescence intensity (FL-1) of the gated phagocyte population, which is proportional to the quantity of oxidised DHR-123 *i.e*. H_2O_2, was recorded.

2.11 Light microscopy

2.11.1 Fixation

Different fixatives were used in different circumstances in order to preserve certain characteristics of the specimens.

2.11.1.1 10% (v/v) Neutral buffered formalin

Host tissue / parasites were placed in 10% neutral buffered formalin for 48 hours prior to processing for paraffin wax embedding and routine histology, or parasite counting after laboratory infection.

2.11.1.2 4% Paraformaldehyde

Host tissue / parasites were dissected and fixed immediately in 4% (w/v) paraformaldehyde in phosphate buffered saline, pH7.4. After 24 h specimens were transferred into 70% (v/v) ethanol in nanopure water prior to processing later that day for paraffin wax embedding. This fixative was used in order to

preserve tissue architecture without ‘masking’ epitopes or mRNA for use in immunohistochemistry and *in situ* expression hybridisation.

2.11.1.3 Davidson’s AFA

L. branchialis females were fixed immediately upon collection in Davidson’s alcohol / formaldehyde / acid (AFA) fixative in seawater for 24 h. Specimens were transferred into 70% (v/v) ethanol until processing for Techovit® 7100 resin embedding. This fixative was used due to its capacity to penetrate the thick cuticle of this parasite, hence providing superior fixing of the internal structures.

2.11.2 Paraffin wax processing

Specimens fixed in 10% buffered formalin or 4% paraformaldehyde were routinely processed for embedding in paraffin wax using standard laboratory protocols. Five µm thick paraffin sections were cut using disposable metal knives and a Shandon Finesse® microtome (Thermo Scientific, Waltham, MA, U.S.A.). Sections were dried on slides (Surgipath, Richmond, U.S.A.) at 60°C for 1 h prior to staining.

2.11.3 Technovit® 7100 resin

L. branchialis females fixed in Davidson’s fixative were dehydrated:

1. 2 x 70% ethanol 30 min each

2. 2 x 100% ethanol 30 min each

After dehydration specimens were pre-infiltrated with 1:1 100% ethanol:base liquid Technovit® 7100 (Heraeus Kulzer, Germany) for 2 h. Specimens were transferred into infiltration solution (1g hardener in 100ml base liquid Technovit® 7100) for 12 h, prior to embedding in Technovit® 7100 resin. Resin curing took approximately 2 h at 21°C.

2.11.4 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining of 5µm thick paraffin wax sections was performed using a standard laboratory protocol from the Institute of Aquaculture histopathology laboratory. Briefly, sections were deparaffinised with xylene (BDH, U.K.), brought to water, stained in Mayer's haematoxylin and eosin, dehydrated in an ethanol series, cleared in xylene and coverslipped with Pertex (Cellpath).

2.11.5 Photography

Light microscope images were taken using an Olympus BX51TF light microscope with a Zeiss AxioCam MRc colour digital camera. MRGrab version 1.0 (Zeiss) software was used to capture and save images. Image J software and a slide graticule allowed calibration of scale bars on images.

2.12 Statistical analysis

All statistical analysis were carried out using the program Minitab version 13.32 (Coventry, U.K.).

2.12.1 Homogeneity of variance

Homogeneity of variance was tested using the Bartlett's Test for continuous, normally distributed data or Levene's Test for non-normally distributed continuous data.

2.12.2 Normality

Normal distribution of data is an assumption of parametric tests; therefore the Anderson-Darling test was used to determine if the data deviated significantly from a normal distribution ($p < 0.05$). Transformation of non-normal data will be discussed in the appropriate chapters.

2.12.3 General linear model (GLM) with *post-hoc* Tukey's test

A GLM was used to compare multiple groups due to the disparity in sample sizes between groups *i.e.* unbalanced data. When significant differences were found between groups a *post-hoc* pair-wise comparisons Tukey's test was performed. All other statistical tests utilised within this thesis will be discussed within the appropriate chapter.

Chapter 3 Systemic immune response of gadoid host to *Lernaeocera branchialis* infection.

3.1 Introduction

The study of aspects of host-parasite interactions, including the mechanisms allowing parasite establishment and survival, and the host's immune response to infection, can assist in the development of control measures against the parasite, such as vaccines, which can help to prevent parasite establishment within a fish population. However, the number of publications relating to the immune response of fish to parasites is very limited in comparison to those regarding bacteria and viruses (Alvarez-Pellitero, 2008). Many of these studies have involved the investigation of the immune response to parasites *in vivo* post-exposure, for example, parasitic copepods, nematodes, ciliates, flagellates, monogeneans and myxozoa (Fast *et al*., 2002a, Fast *et al*., 2006; Knopf *et al*., 2000; Sigh *et al*., 2004a and b; Saeij *et al*., 2003; Lindenstrom *et al*., 2003; Lindenstrom *et al*., 2004; Cuesta *et al*., 2006a & b; Munoz *et al*., 2007; Sitja-Bobadilla *et al*., 2006). Within the studies examining the host's immune response to parasitic infections, very few deal with copepod parasites, with the majority of the studies focusing on the economically important *Lepeophtheirus salmonis* (Fast *et al*., 2002a, 2006). These studies have, however, highlighted the importance of the rapid innate immune response to parasitic infection, as in the case of coho salmon (*Oncorhynchus kisutch*), which are able to initiate a more effective innate immune response against *L. salmonis* than more susceptible hosts, such as Atlantic salmon (*Salmo salar*). Fast *et al*. (2002a) observed coho salmon to lose their salmon lice 7-14 days post-infection with copepodids in the laboratory. They suggested that due to the rate of reduction in salmon louse intensity in these coho salmon, the resistance observed must be a result of innate host defences (Fast *et al*., 2002a). This is supported by observations made by Johnson and Albright (1992), where coho salmon were able to initiate an acute inflammatory response one day post *L. salmonis* - infection

(d.p.i.), and epithelial hyperplasia at 10 d.p.i.. The innate immune response is also likely to be of great importance in Atlantic cod in the elimination of a parasitic infection, such as *L. branchialis.* Such a rapid response is likely to be particularly important where development of the parasite to its most pathogenic state occurs over a short period of time, as is the case for the female *L. branchialis,* which metamorphoses rapidly, becoming fully formed approximately 8 weeks post-infection (Khan, 1988).

Several studies have highlighted the importance of host complement activity, particularly through the alternative pathway, in the immune response against a diverse array of fish parasites, including monogeneans and kinetoplastids (Wehnert & Woo, 1980; Forward & Woo, 1996; Buchmann, 1998; Harris *et al*., 1998; Rubio-Godoy *et al*., 2004). However, other host innate immune factors, for instance, anti-protease activity (Zuo & Woo, 1997a), leukocyte phagocytosis and respiratory burst activity (Fast *et al*., 2002a; Sitja Bobadilla *et al*., 2006; Alvarez-Pellitero *et al.,* 2008; Sitja Bobadilla *et al*., 2008), and non-specific cytotoxic cell activity (Graves *et al*., 1985; Jaso-Freidmann *et al*., 2000; Cuesta *et al*., 2006a) have also been shown to be important during infections with parasitic copepods, kinetoplastids and myxozoans. The situation, however, is further complicated by the fact that parasites of various groups have the capacity to evade their host's immune response by several mechanisms, such as immunosuppression, or prevention of parasite recognition by the host (see the recent review by Sitjà-Bobadilla, 2008). For example, the phagocytic capacity and / or respiratory burst activity of leukocytes from a variety of hosts are suppressed during infection by the parasites *L. salmonis* (Mustafa *et al*., 2000a; Fast *et al*., 2002a), *Polysporoplasma sparis* (Karagouni *et al*., 2005), *Schistocephalus solidus* (Scharsack *et al*., 2004) and *Uronema marinum*

(Kwon *et al*., 2002). *L. salmonis*-infection suppressed the phagocytic capacity and respiratory burst activity of Atlantic salmon head kidney macrophages in the absence of an elevated plasma cortisol response *i.e*. stress response, which correlated with moulting of the parasite into later chalimus and pre-adult stages (Fast *et al*., 2002a). The authors hypothesised that this immunosuppression was therefore due to either increased damage by the parasite or the release of parasite-derived secretions at this stage of the infection. For instance, the secretory / excretory products of the haematophagous nematode *Anguillicola crassus*, which infects the swimbladder of the European eel, have been found to contain a detoxifying enzyme glutathione-s-transferase (Nielsen & Buchmann, 1997). Fast *et al*. (2002a; p66) suggested that "once *L. salmonis* biomass increases to a critical level on a fish or *L. salmonis* reaches the pre-adult stage" these parasite-derived secretions could have an immunosuppressive effect that could be observed at a systemic level, such as the detected reduction in macrophage activity. Therefore, when Atlantic cod are infected by multiple *L. branchialis,* or mature fully-metamorphosed females, a systemic immunosuppression may result.

The descriptions of host-parasite relationships between meso-parasitic copepods and their teleost hosts to date have mainly considered the histopathology due to infection, such as in *L. branchialis* (Schuurmans Stekhoven, 1936; Capart, 1947; Smith *et al*., 2007), *Phrixocephalus cincinnatus* (Kabata, 1969; Perkins, 1994), *Lernaea piscinae* (Shariff, 1981) and *Lernaea polymorpha* (Shariff & Roberts, 1989). Studies regarding the key immune mechanisms operating during meso-parasitic copepod infection of the host are still lacking. The aims of this study therefore, were to investigate the systemic immune response of naturally infected

wild and experimentally challenged naïve hatchery-reared Atlantic cod, in terms of their humoral and cellular innate immune factors, to infection with *L. branchialis*.

3.2 Materials and methods

3.2.1 Collection of wild gadoids

Wild *Melanogrammus aeglefinus* (haddock) were collected by trawling in the North sea and Northeast Atlantic on the Quarter 4 west coast survey in November 2006 upon the Fisheries Research Services (Aberdeen) Vessel, HMRV Scotia. Haddock were sampled at 11 trawl sites (Figure 3.1). Twenty haddock (fork length 22-43 cm) per trawl site were sampled (*i.e.* 10 uninfected and 10 infected haddock), and the infection intensity and stage of *L. branchialis* recorded. Any externally visual parasites on the skin, fins, gills and mouth were recorded, however internal infections were not monitored. Originally samples from *L. branchialis*-infected and uninfected Atlantic cod (*Gadus morhua*) were the target species for sampling; however the number of Atlantic cod caught was too low to achieve sufficient sample sizes.

3.2.1.1 Blood collection

Blood was collected from the caudal vein for serum, sodium citrated plasma and whole heparinised blood as detailed in the protocols outlined in Chapter 2.

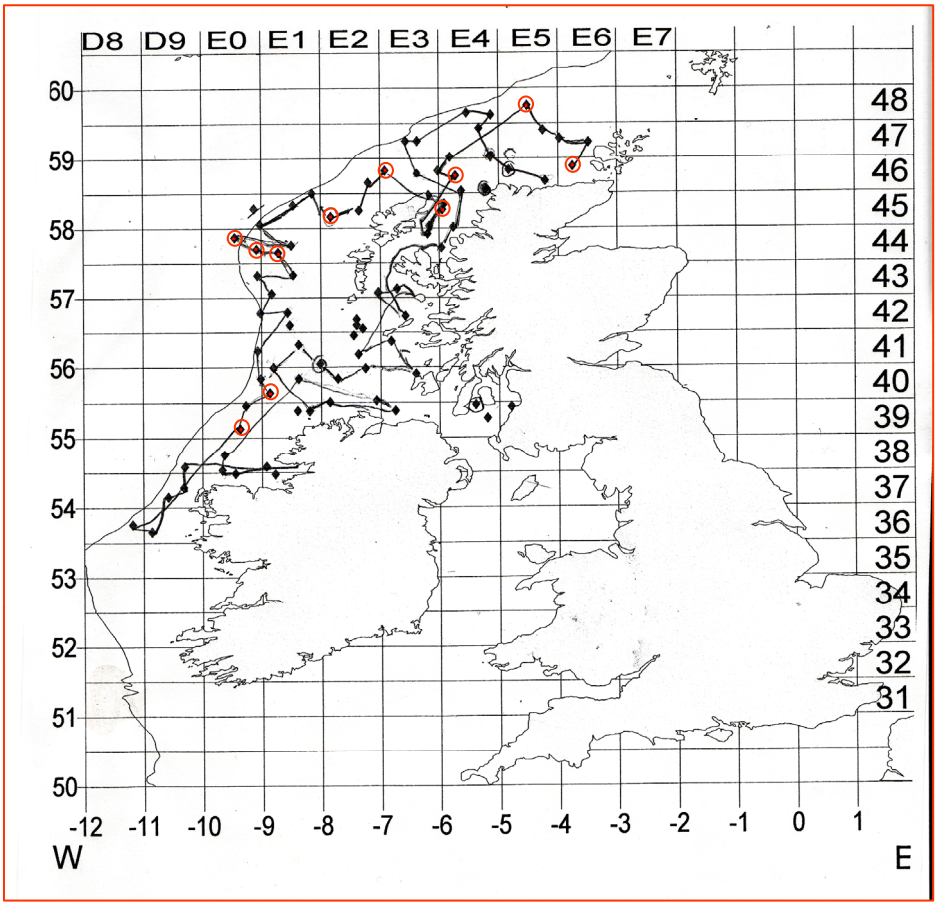

Figure 3.1 Map of Northeast Atlantic ocean and North Sea illustrating the trawl sites at which haddock were sampled (black line = survey route; black diamonds with red circles = trawl sites where 20 haddock were sampled).

3.2.1.2 Blood differential leukocyte and thrombocyte proportions

Five µl whole heparinised blood was dropped onto a glass slide (Surgipath, Richmond, U.S.A.) and smeared using the edge of another slide. Duplicate blood smears per fish were air dried, fixed in methanol and stained with Rapid Romanowsky stain (Raymond A Lamb, U.K.) following manufacturers' instructions.

Differential leukocyte proportions were calculated by counting and identifying 200 leukocytes excluding thrombocytes per blood smear (Thuvander, 1989). Thrombocytes were counted separately, and the number per 100 leukocytes

counted (Alcorn *et al*., 2002). Thrombocytes were differentiated from lymphocytes by the colour of their cytoplasm which was clear/grey in thrombocytes and dark blue for lymphocytes. The shape of the thrombocytes was also often oval or spindle-shaped compared to the often round-shape of lymphocytes.

3.2.1.3 Total protein quantification of serum

Serum total protein was quantified using a BCA protein assay kit (Pierce) as detailed in Chapter 2 (Section 2.7).

3.2.1.4 Anti-trypsin activity of serum

A method originally described by Ellis (1990) and modified by Magnadóttir *et al.* (1999a & b) for Atlantic cod was used to determine serum anti-trypsin activity. This method employs the protease substrate azocasein, which is hydrolysed into azo dye by trypsin. Briefly, in duplicates 20µl serum was pre-incubated with 20µl 5 $mg.ml^{-1}$ trypsin (Sigma; 1420 BAEE $U.mg^{-1}$) in 0.1M sodium phosphate buffer, pH 7.0 for 10 min at 22°C. Two hundred µl 0.1M sodium phosphate buffer, pH 7.0 (PB) and 250µl 2% (w/v) azocasein (Sigma) in PB were added, mixed and incubated for a further 1 h at 22°C. This was followed by the addition of 500µl 10% (w/v) trichloro acetic acid (Sigma) and incubation for 30 min at 22°C in order to precipitate un-hydrolysed azocasein. The tubes were centrifuged at 6000 xg for 5 min at room temperature to pellet the precipitated azocasein, and 100µl of supernatant transferred to a 96 well non-absorbent microplate (Sterilin Ltd, Caerphilly, UK) containing $100µl.well^{-1}$ 1N sodium hydroxide. The reference samples contained 20µl PB instead of serum, and the blanks contained 40µl PB instead of serum and trypsin. The O.D. was read at 450 nm on an MRX microplate reader (Dynex technologies). The percentage inhibition of trypsin activity of serum samples compared to the reference sample was calculated.

3.2.1.5 Percent spontaneous haemolytic activity of serum

This assay is based on the spontaneous haemolysis (SH) of sheep erythrocytes (SRBC), used as targets for the alternative complement pathway (ACP), therefore giving a measure of fish serum alternative complement activity (ACA). The assay used was adapted by Langston *et al*. (2001) from Yano (1992) for microtitre plates, and data analysis of the percent SH followed Magnadottir *et al*. (1999a, b). Briefly, SRBCs diluted in Alsever's solution (Oxoid, U.K.) were washed 3 times in 0.85% (w/v) saline solution. The cells were re-suspended to 2 x 10^8 cells.ml^{-1} in complement fixation test diluent (Oxoid) with 0.1% gelatine with 10mM EGTA (EGTA-CFT-G buffer). Haddock serum was diluted in EGTA-CFT-G buffer to give a final volume of 25µl per well and a final dilution of 1/50 in a 96 U well microtitre plate (Sterilin) in quadruplicates. Two replicates were treated with 10µl of SRBC solution in each well. The other 2 replicates were used as sample blanks where SRBC solution was replaced with EGTA-CFT-G buffer. Positive controls (100% lysis) containing 10µl SRBC and distilled water, negative controls (0% lysis) of 25µl EGTA-CFT-G buffer and 10µl SRBC suspension and blanks of EGTA-CFT-G buffer only were processed in the same plate. All of the microtitre plates were incubated at 22°C for 90 min and the reaction terminated by the addition of 140µl CFT-G buffer with 20mM EDTA, followed by centrifugation at 700 xg for 10 min. After centrifugation 50µl of the supernatant from each well were transferred to a new flat-bottomed 96-well non-absorbent microtitre plate (Sterilin) and the absorbance read at 415 nm using a Power Wave X microtitre plate reader (Bio-Tek Instruments Inc., U.S.A.). The sample absorbance values were corrected by subtraction of the absorbance of the sample blank control. The percentage haemolysis for each sample was calculated from the O.D. value of the 100% lysis control.

3.2.1.6 Total serum IgM by competitive ELISA

Serum total IgM concentration was quantified using a competitive ELISA following a modified method of Magnadóttir and Guðmundsdóttir (1992). This assay employed the use of a commercial monoclonal anti-cod/haddock IgM antibody (ADL, Stirling, UK) and sodium sulphate precipitated haddock serum proteins. An attempt was made to purify haddock IgM from wild haddock serum using a HiTrap IgM purification column (GE Healthcare) following manufacturers' instructions. However, the purified IgM was found to be non-reactive with the commercial monoclonal anti-cod/haddock IgM antibody (ADL, Stirling) in ELISA, and therefore sodium sulphate precipitated haddock serum (see Appendix) was utilized instead. Although the use of precipitated serum in the ELISA would cause an over-estimation of the serum IgM it would be standardised throughout the experiment allowing for relative comparisons between samples to be made.

Briefly, 96-well Immulon™ 4HBX plates (ThermoScientific, Maine, USA) were coated with haddock precipitated serum proteins by incubating 50µl 10µg.ml^{-1} proteins in carbonate-bicarbonate buffer, pH 9.6 (Sigma) overnight at 4°C. Plates were washed three times with low salt wash buffer and blocked with 250µl.well^{-1} 5% (w/v) casein in phosphate buffered saline (PBS) overnight at 4°C (Sigma). Plates were again washed three times with low salt wash buffer. Serum samples (100µl) diluted 1/100, 1/500 and 1/1000 in PBS with 0.05% (v/v) Tween-20 (Sigma; PBS-T) with 100µl 2µg.ml^{-1} anti-cod IgM MAb (ADL) diluted in PBS-T in triplicates were added per well and incubated for 2 h at 37°C. Plates were washed five times with high salt wash buffer. Bound mouse anti-cod IgM was detected by incubating 100µl.well^{-1} peroxidase-labelled goat anti-mouse IgG antibody (Sigma) diluted 1/4000 in 1% (w/v) bovine serum albumin (Sigma) in low salt wash buffer for 1 h at

room temperature. Plates were washed five times with high salt wash buffer and developed with 100µl 42µM 3'3'5'5'-tetramethylbenzidine dihydrochloride (TMB; Sigma) in substrate buffer with 0.1% hydrogen peroxide (Sigma) per well for 10 min at room temperature. The assay was stopped by the addition of 50µl 2M sulphuric acid per well. A standard curve was produced by incubating 100µl precipitated haddock serum proteins ranging from 0 – 320µg.ml^{-1} in two-fold serial dilutions (instead of serum) with 2µg.ml^{-1} mouse anti-cod IgM antibody. Positive controls consisted of wells without serum incubated with the mouse anti-cod IgM. Negative controls omitted the mouse anti-cod IgM antibody or coating of the well with precipitated haddock serum protein. The O.D. was read at 450 nm on an MRX microplate reader (Dynex technologies), and the unknown IgM concentrations of serum samples extrapolated from the straight portion of the standard curve graph of IgM against O.D.450 nm.

3.2.1.7 Plasma re-calcification clotting time

Plasma re-calcification clotting times were evaluated following a modified version of Horn *et al*. (2000). This method allows the clotting time of fish plasma to be measured at a later time when collecting samples in the field, and involves the collection of sodium citrated plasma which removes calcium, preventing the plasma from clotting. Later when calcium is added, the plasma will start to clot and the clotting time is measured. Briefly, 300µl sodium citrated plasma was incubated with 825µl 0.85% (w/v) saline for 10 min at 22°C. Followed by the addition of 375µl 40mM calcium chloride, agitation and read immediately at absorbance 600nm at 11 second intervals on a WPA Biowave CO 8000 cell density reader (Biochrom Ltd., Cambridge, UK). A graph of time (seconds) against OD_{600} was plotted and the re-

calcification clotting time taken as the time OD_{600} took to plateau *i.e.* no longer increasing.

3.2.1.8 Fixation of *L. branchialis in situ* and parasite quantification

Initial counts of *L. branchialis*, present on sampled fish, were made on board the Scotia, and the stage of infection determined as:

1. old infection *i.e.* presence of residual attachment organ of dead parasite.

2. immature *i.e*. not fully metamorphosed P-V sub-stages (Chapter 1, Table 1.1).

3. mature / gravid *i.e*. fully metamorphosed W stage or fully metamorphosed female with egg sacs X stage (Chapter 1, Table 1.1).

The gills and hearts of sampled haddock were fixed in 10% (v/v) neutral buffered formalin for further examination with a stereomicroscope back at the laboratory. This was performed to ensure that haddock deemed uninfected did not carry immature infections that were not visible by eye. Haddock infected by more than one gravid *L. branchialis* and other sub-stages of parasites were categorised within the >1 gravid *L. branchialis* group.

3.2.2 Laboratory infection of naïve *Gadus morhua*

Laboratory infections of naïve Atlantic cod were performed as stated in Chapter 2 (Section 2.3). A preliminary infection involved 81 naïve Atlantic cod (mean weight 141.3 ± 50.5g) from Machrihanish Marine farm Ltd. hatchery (Machrihanish, Scotland). Fish were sampled at 0 days post infection (d.p.i.; n=9), half the

remaining fish were infected, and 9 fish sampled from each group of sham control and infected fish in duplicate tanks at 1, 3, 7 and 14 d.p.i..

A second longer infection study was carried out in order to follow the infection as the female *L. branchialis* metamorphosed. This involved 100 naïve Atlantic cod (mean weight 144.6 ± 30.0g) from Viking Fish Farms Ltd. (Ardtoe, Scotland). Fish were sampled at 0 d.p.i. (n=10), half the remaining fish were infected and 9 fish sampled from each group of sham control and infected tanks at 3, 7, 14, 28 and 56 d.p.i..

3.2.2.1 Blood collection

Blood was collected from the caudal vein for serum and whole heparinised blood as described in Chapter 2 (Section 2.6).

3.2.2.2 Blood erythrocyte counts

Whole heparinised blood was diluted 1 in 200 in L-15 medium and erythrocytes counted in a Neubauer haemocytometer counting chamber. The number of erythrocytes per ml counted was multiplied by the dilution factor to give the total number of erythrocytes per ml of blood.

3.2.2.3 Haematocrit

Whole heparinised blood was placed in a heparinised haematocrit tube and centrifuged for 5 min in a haemofuge (Hawksley, Lancing, England). The erythrocyte packed cell volume was expressed as a percentage of the total volume of cells and plasma.

3.2.2.4 Partial differential leukocyte counts using flow cytometry

A flow cytometry method described by Inoue *et al*. (2002) was used to determine partial differential leukocyte counts. The green fluorescent, cell-permeant dye 3, 3-dihexyloxacarbocyanine ($DiOC_6(3)$) is lipophilic and stains lipid bilayers such as plasma membranes and cell organelle membranes like that of mitochondria and endoplasmic reticulum (Terasaki & Fujiwara, 1992). The assay therefore relies on the fact that $DiOC_6(3)$ stains erythrocytes less than leukocytes due to the higher occurrence of cell organelles in the latter cell type. Therefore, leukocytes and erythrocytes from a whole blood sample can be discriminated by their fluorescence intensity.

Ten µl of whole heparinised blood diluted 1/10 in L-15 medium was incubated with $DiOC_6(3)$ at a final concentration of 1µg.ml^{-1} L-15. The sample was mixed gently and incubated at 22ºC for 10 min in the dark.

For absolute cell counting, CytoCount™ 5.2µM polystyrene fluorospheres (DakoCytomation) were added to the blood cell suspension at a final concentration of 100 particles.µl^{-1} sample in a total volume of 2ml just prior to flow cytometry analysis. Cytocount™ fluorospheres were gated on a forward scatter (FSC) versus side scatter (SSC) scattergram and all events counted and recorded until 5000 fluorospheres had been acquired. The total leukocytes were distinguished and gated on an FL-1 versus SSC scattergram by their FL-1 intensity. These leukocytes were then plotted on an FSC versus SSC scattergram in order to differentiate between the leukocyte populations. Three leukocyte populations were distinguished based on their size and granularity 1) lymphocytes and thrombocytes, 2) monocytes

and 3) granulocytes. The absolute counts *i.e.* number of cells per µl blood of each population was calculated using the following equation:

Number cells.µl^{-1} blood = (no. of events per population) x 100^{A} / 5000^{B} x 2000^{C}

^{A} concentration of fluorospheres added to sample. ^{B} Number of fluorospheres counted. ^{C} Dilution factor of blood sample.

In order to cross-check leukocyte counts obtained by flow cytometry, heparinised blood from naive cod (n = 10) was used to correlate absolute leukocyte counts made by flow cytometry and those from microscopy counts. Total erythrocyte and total leukocyte plus thrombocyte counts were made using a haemocytometer, and duplicate blood smears were also prepared for differential counts of each leukocyte population counted with lymphocytes and thrombocytes together recorded for 200 leukocytes and thrombocytes. The proportion of each leukocyte population was multiplied by the absolute number of leukocytes per µl blood and divided by 100 to give the number of leukocytes within that population per µl blood. Pearson's correlation was used to determine the correlation between the outcome results of differential leukocyte counts by microscopy and flow cytometry ($p < 0.05$).

3.2.2.5 Blood phagocyte respiratory burst activity

The respiratory burst activity of blood phagocyte cells was determined using a modification of the flow cytometry method of Richardson *et al.* (1998) suggested by Moritomo *et al*. (2003) as described in Chapter 2 (Section 2.10.2).

3.2.2.6 Total protein quantification of serum

Serum total protein concentration was determined as detailed in Section 3.2.1.3.

3.2.2.7 Anti-trypsin activity of serum

Serum anti-trypsin activity was quantified as detailed in Section 3.2.1.4

3.2.2.8 Spontaneous haemolytic activity of serum

Serum SH activity was determined following the protocol of Magnadottir *et al*. (2001) except with the modification of no EDTA being present in the assay. EDTA was excluded from the assay buffer due to the fact that Magnadottir (2006) found that the spontaneous haemolytic activity of cultured-Icelandic cod serum since 2000 is inhibited by EDTA, unlike the unusual feature of this factor in wild and cultured-cod in the late 1990s which was enhanced by the addition of EDTA (Magnadottir *et al.*, 1999a; b; Magnadottir *et al.*, 2001). Briefly, SRBCs 1:1 in Alsevers solution (Oxoid, U.K.) were washed 3 times in 0.85% (w/v) saline solution and diluted in complement fixation test diluent (Oxoid) with 0.1% gelatine (Sigma; CFT-G buffer) until 100µl added to 3.4ml distilled water gave an O.D. of 0.740 at 414 nm. This suspension was then further diluted 1:1 with CFT-G buffer (to give a 0.5% SRBC suspension). Serum was diluted in double serial dilutions in CFT-G and 100µl added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicates. Fifty µl 0.5% SRBC suspension was added to each serum dilution and incubated at 37°C for 1 h. Controls on each plate comprised distilled water replacing serum (100% lysis), CFT-G buffer replacing serum (0% lysis) and serum blanks (duplicate wells of serum dilutions with CFT-G buffer replacing SRBC suspension). After 1 h the plates were centrifuged at 700 xg for 10 min at 4°C, and 125µl supernatant transferred into a non-absorbent flat-bottomed 96-well microplate. The absorbance was read at 405 nm using a MRX microplate reader (Dynex technologies). The sample absorbance values were corrected by subtraction of the absorbance of the sample blank control. The percentage haemolysis for each sample and dilution was

calculated from the O.D. value of the 100% lysis control. The reciprocal of the serum dilution giving 50% haemolysis was calculated from a graph of percentage haemolysis against serum dilution.

3.2.2.9 Total serum IgM concentration by competitive ELISA

Serum total IgM concentration was quantified using a competitive ELISA following a modified method of Magnadóttir and Guðmundsdóttir (1992), as described in Section 3.2.1.6. This assay however, employed the use of sodium sulphate precipitated proteins from cod serum instead of purified Atlantic cod IgM, for the reasons previously stated in Section 3.2.1.6.

3.2.2.10 Fixation of *L. branchialis in situ* and parasite counting

The gills and hearts of Atlantic cod were fixed in 10% (v/v) neutral buffered formalin (NBF; as in Chapter 2, Section 2.11.1.1) for examination with a stereomicroscope back at the laboratory in order to count parasites, identify parasite stages and record the site of infection.

3.2.2.11 Light microscopy

Gills and *bulbus arteriosus* fixed with 10% NBF from infected and control fish were processed for wax embedding. Five µm thick paraffin wax sections were cut and routinely stained with H&E.

3.2.3 Statistical analysis

A GLM with the factors infection status and trawl site was performed in order to test their influence on wild haddock immune parameters ($p < 0.05$). A GLM was also used to determine if fish from different trawl sites possessed significantly different lengths. If no significant differences existed between immune data collected from

different trawl sites within infection status groups, and the length of fish at different trawl sites, the data were pooled for further analysis. The effect of fish length on haddock infection status was determined by GLM ($p < 0.05$), and any correlations between fish length and immune parameters determined by Pearson's correlation ($p < 0.05$). The proportion of monocytes from haddock blood smears was not normally distributed and could not be transformed; therefore a non-parametric Kruskal-Wallis test was performed in order to determine any differences between infection groups.

A GLM with the factors treatment and time post-infection with interactions was performed in order to test their influence on laboratory-infected cod immune parameters ($p < 0.05$). A GLM was also undertaken on cod immune parameter data segregated by infection status (*i.e.* uninfected sham control and infected fish) with the factors time post-infection and tank number with interactions, in order to determine any differences within control or infection tanks over the time series. If no significant differences existed between data collected from different tanks within infection groups, the data were pooled prior to further analysis. Tukey's *post-hoc* pair-wise comparison test was performed in order to determine where significant differences ($p < 0.05$) between groups occurred. Graphs represent means ± 2x standard error (2SE), and mean fish weights are given ± standard deviation.

3.3 Results

3.3.1 Systemic immune response of wild haddock

There were no significant differences observed between the immune parameter data collected from fish sampled at different trawl sites or between the lengths of fish at different trawl sites ($p = 0.151$), therefore all data within each infection group

were pooled prior to further analysis. A positive correlation was found between plasma re-calcification clotting times and fish length (correlation coefficient = 0.403, p = 0.001). However, there were no significant differences in the length of fish from different infection groups *i.e.* infection had no effect on fish length in this study (p = 0.65). No correlations were found between fish length and the other parameters measured.

3.3.1.1 Infection status

Sampled haddock possessed *L. branchialis* female infections at various stages of metamorphosis from immature post-penella (Figure 3.2A) to gravid fully metamorphosed females (Figure 3.2B); and with more than one parasite present. The highest parasite intensity observed was 4 gravid and 2 immature females. The majority of gravid female cephalothoraxes were found to reside within the ventral aorta (Figure 3.2C). When multiple parasites were present, the ventral aorta was severely distorted and increased in size (Figure 3.2E). A small number of previously infected haddock with necrotic dead parasites without any new infections were also sampled when observed in a trawl load; only the cephalothorax and neck of the parasite remained at the site of infection. The entry site of immature parasites, usually at the ventral portion of the gill arch, was found to undergo epithelial hyperplasia with the presence of granulation tissue and necrosis (Figure 3.2A & D).

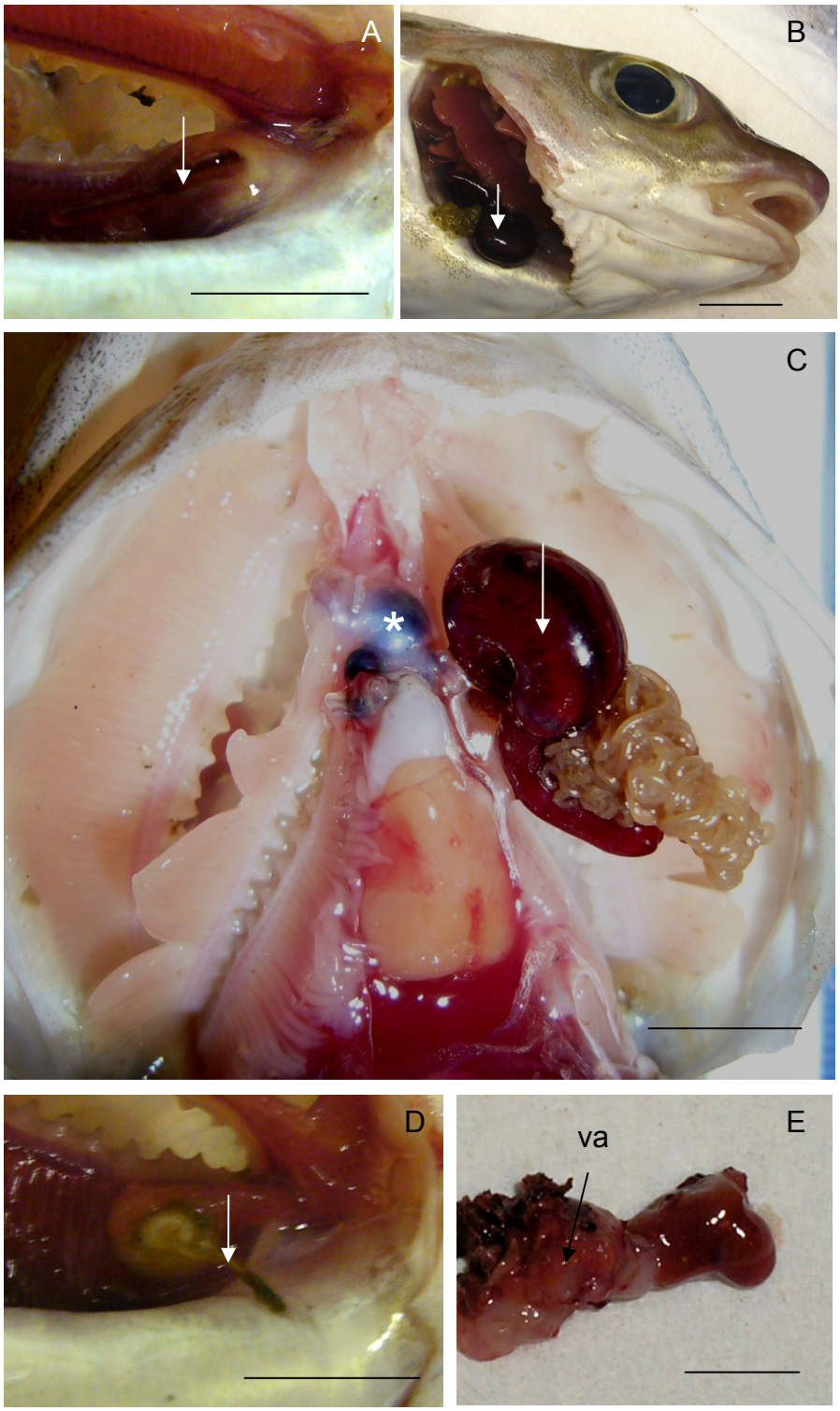

Figure 3.2 Infection site of wild haddock infected with *L. branchialis* (A) immature female, (B) mature gravid female, and (C) the feeding/attachment site in the ventral aorta. D illustrates an immature post-penella female and E shows the distortion of the host's ventral aorta when infected by more than one gravid female. Bar = 1cm. → denotes parasite abdomen and * denotes parasite cephalothorax; va = ventral aorta.

3.3.1.2 Blood differential leukocyte and thrombocyte proportions

The proportion of haddock blood leukocytes made up of lymphocytes did not significantly change with infection status (Table 3.1). The proportion of granulocytes, on the other hand, significantly increased in infected haddock (16.3 ± 1.64%) compared to that of uninfected haddock (13.1 ± 1.66%; $p = 0.020$; Figure 3.3A). However, when the group of infected haddock were further separated into groups by means of the maturation stage of the parasite and parasite intensity, no significant differences in the proportion of granulocytes compared to uninfected haddock could be determined, even though haddock with immature parasites seemed to possess the highest mean blood granulocyte proportions (Figure 3.3B). This is likely to be due to the decreased sample size, especially for the haddock infected by immature *L. branchialis*, as fewer infected haddock with parasites at this maturation stage were caught. The proportion of blood granulocytes of haddock with old infections did not significantly differ from that of uninfected or infected haddock. The proportion of blood leukocytes made up of monocytes however, was significantly lower in haddock infected by more than one mature *L. branchialis* (0.59 ± 0.52%) compared to that of uninfected haddock (2.6 ± 0.98%; $p = 0.034$; Figure 3.3C). The proportion of blood thrombocytes was also significantly increased in infected haddock (78.9 ± 7.6 per 100 leukocytes) compared to their uninfected counterparts (57.5 ± 7.9 per 100 leukocytes; $p < 0.001$) but not to haddock with old infections (63.6 ± 8.8 per 100 leukocytes). When the infected haddock were again separated further into groups by their infection status, only the haddock possessing single or multiple mature parasites had significantly higher proportions of blood thrombocytes compared to uninfected haddock ($p < 0.001$ and $p = 0.006$, respectively; Figure 3.3D). The proportion of thrombocytes possessed by haddock

infected with one mature parasite was just significantly higher than that of the haddock infected by immature parasites ($p = 0.046$), however haddock with multiple mature parasites did not have significantly different proportions of blood thrombocytes to haddock with immature parasites ($p = 0.072$; Figure 3.3D). Infection of wild haddock with *L. branchialis* led to an increase in the proportion of blood leukocytes made up of granulocytes and thrombocytes, and a decrease in monocytes. The proportion of granulocytes appeared to be highest in haddock with immature infections, however no significant differences could be found. The maturation stage and parasite intensity seemed to have an effect on the leukocyte response to infection, as haddock with mature parasites showed increased proportions of thrombocytes, and when multiple mature parasites were present a reduction in circulating monocyte proportions were seen.

Table 3.1 Blood lymphocytes as a proportion of all leukocytes, serum total IgM concentration and total IgM as a proportion of total protein content of uninfected and infected wild haddock with different *L. branchialis* maturation stages and intensities. Values represent mean ± 2x standard error (2SE).

Infection intensity & maturation stage		Immune factor measured		
		Lymphocytes as % blood leukocytes	Serum total IgM ($mg.ml^{-1}$)	Serum total IgM as proportion of total protein (%)
Uninfected		84.4 ± 1.4	5.0 ± 0.3	10.1 ± 0.7
Old infection		83.1 ± 1.6	4.8 ± 0.9	11.5 ± 2.6
Infected	Immature	80.0 ± 7.0	5.0 ± 0.8	10.4 ± 2.0
	Mature	83.1 ± 1.9	4.9 ± 0.4	11.6 ± 1.3
	>1 mature	83.9 ± 3.6	4.8 ± 0.6	10.1 ± 1.5

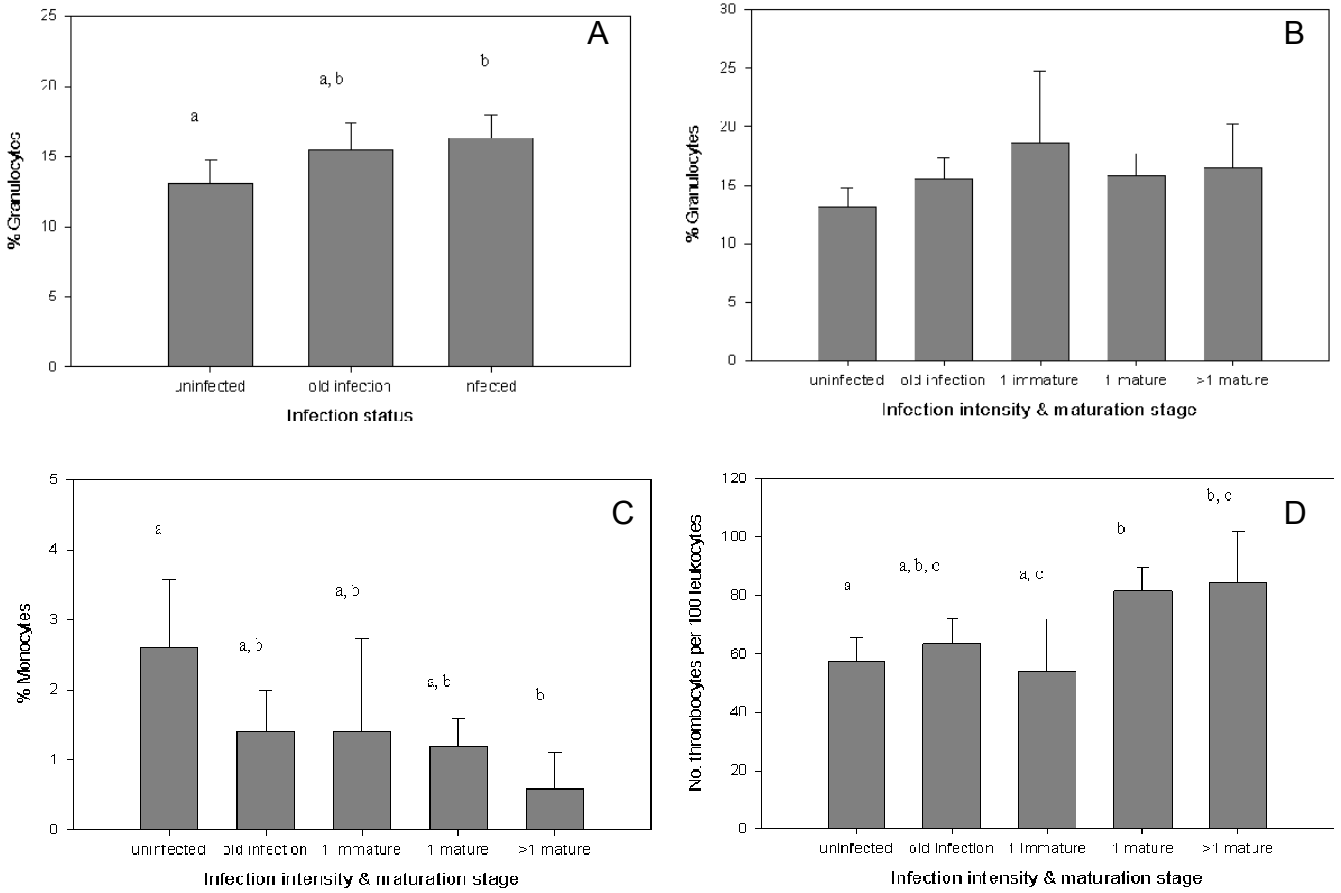

Figure 3.3 (A & B) Blood granulocytes and (C) monocytes as a proportion of all leukocytes, and (D) the number of thrombocytes per 100 blood leukocytes of uninfected and infected wild haddock with different *L. branchialis* maturation stages and intensities. Different letters denote significant differences between groups ($p < 0.05$). Error bars = 2SE, n = 40 uninfected, 40 infected and 10 old infection.

3.3.1.3 Total protein concentration of serum

The total protein concentration of serum was found to be significantly lower in infected haddock (46.5 ± 2.0 mg.ml^{-1}) than in uninfected haddock (52.0 ± 2.2mg.ml^{-1} ; $p < 0.001$). When the infected haddock were further segregated by parasite maturity and intensity, only the serum protein concentration of haddock with single mature parasites was found to be significantly lower than uninfected haddock ($p = 0.002$; Figure 3.4). This is probably due to the smaller sample sizes of wild haddock with immature or multiple mature parasites in comparison to those with single mature parasites. The haddock with old infections did not show any significant difference in serum protein concentration compared to all other groups sampled.

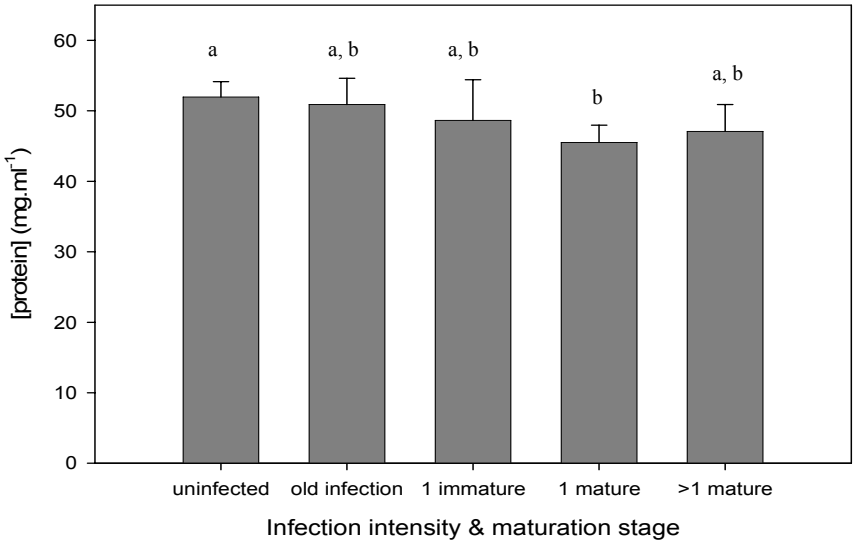

Figure 3.4 Serum protein concentrations of uninfected and infected wild haddock with different *L. branchialis* maturation stages and intensities. Different letters denote significant differences between groups ($p < 0.05$). Error bars = 2SE, n = 70 uninfected, 70 infected and 15 haddock with old infections.

3.3.1.4 Anti-trypsin activity of serum

The haddock with old infections and infected haddock both had significantly higher serum anti-trypsin activities (70.0 ± 5.2% and 67.4 ± 3.5%, respectively) than uninfected haddock (58.2 ± 2.4%; $p = 0.004$ and $p < 0.001$, respectively). After further segregating the infected haddock into groups by parasite maturity and intensity, only the haddock with multiple mature parasites had significantly higher serum anti-trypsin activities compared to uninfected haddock ($p < 0.001$; Figure 3.5). The anti-trypsin activities of haddock with immature and single mature parasites were found to be higher than that of uninfected haddock, however this was not significant ($p = 0.061$ and $p = 0.063$, respectively).

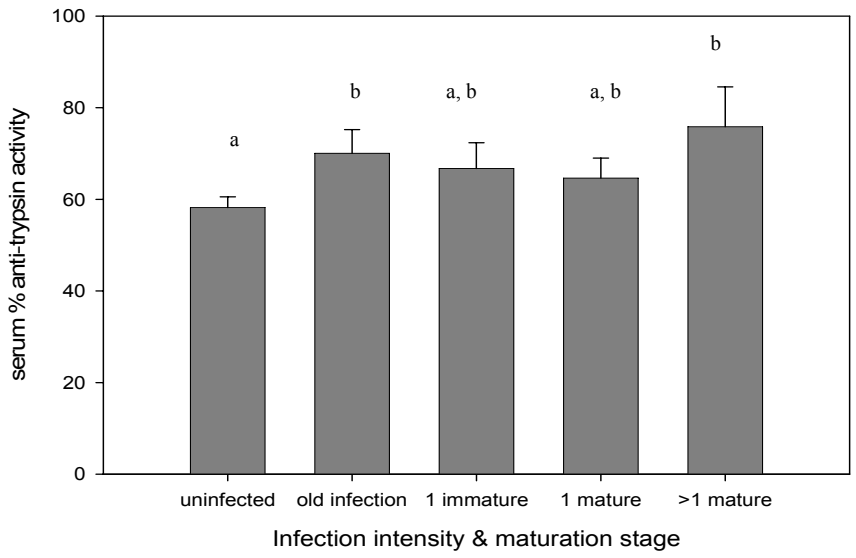

Figure 3.5 Serum anti-trypsin activity of uninfected and infected wild haddock with different *L. branchialis* maturation stages and intensities. Different letters denote significant differences between groups ($p < 0.05$). Error bars = 2SE, n = 70 uninfected, 70 infected and 15 haddock with old infections.

3.3.1.5 Percent spontaneous haemolytic activity of serum

The SH activity of haddock serum was significantly reduced in haddock infected by *L. branchialis* (45.29 ± 8.9%) compared to uninfected haddock (73.9 ± 12.2%; p = 0.002; Figure 3.6A). The SH activity of haddock possessing old infections (60.6 ± 26.8%) however, did not significantly differ from that of uninfected or infected haddock (p = 0.912 and p = 0.264, respectively; Figure 3.6A). Upon further analysis of the infected group of haddock no significant differences between the SH activity of haddock with immature (39.8 ± 12.3%), mature (48.5 ± 12.1%) or multiple mature parasites (43.9 ± 26.2%) and the activity of uninfected haddock could be observed (p = 0.124, p = 0.083 and p = 0.145, respectively; Figure 3.6B). This again is likely due to the large variation between fish for SH activity (Figure 3.6B).

3.3.1.6 Total IgM concentration of serum

The total IgM concentration of haddock serum and the proportion of serum protein made up of IgM did not differ between uninfected haddock and those infected by *L. branchialis* or old infections (Table 3.1).

3.3.1.7 Plasma re-calcification clotting times

The plasma re-calcification clotting times of haddock showed a decreasing trend with infection, especially when single and multiple mature parasites were present (Figure 3.7), however this was not significant.

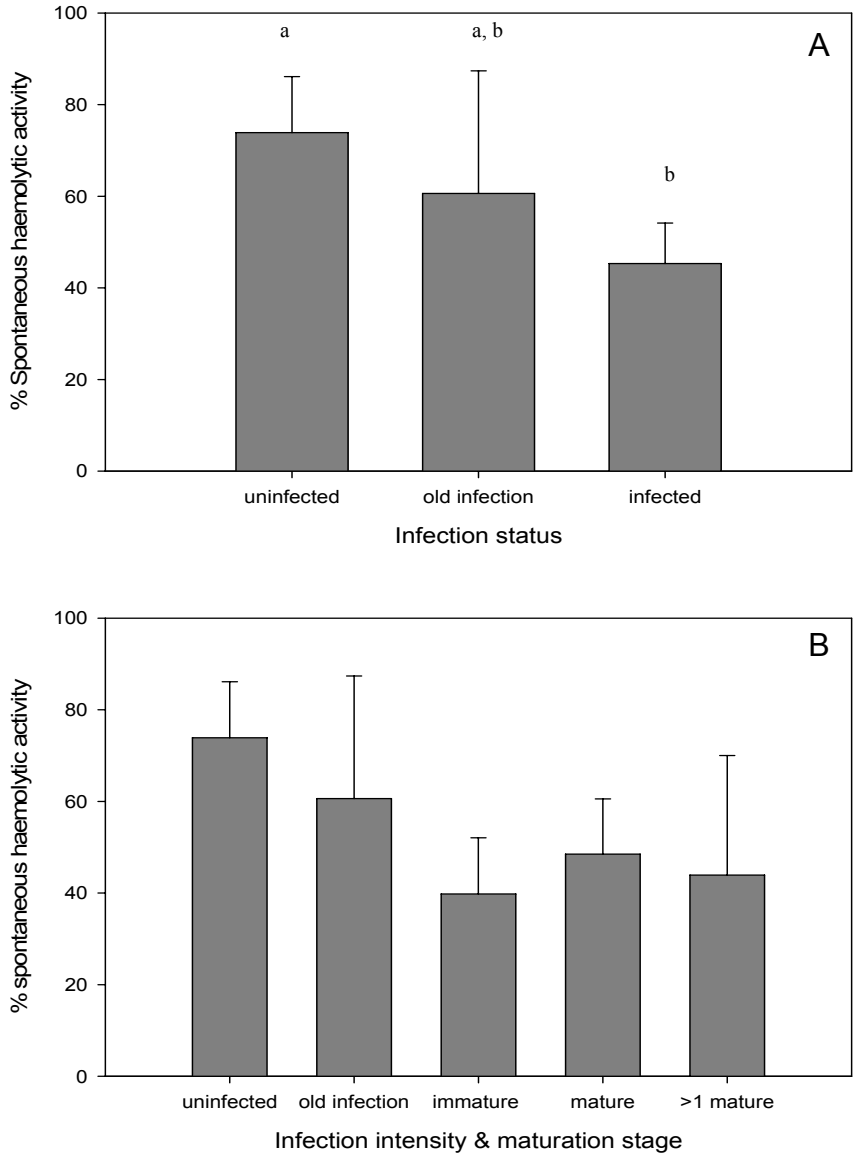

Figure 3.6 (A) Serum SH activity of uninfected wild haddock and those infected with *L. branchialis* or with old infections. (B) The SH activity of infected haddock was further segregated by the maturation stage and intensity of the *L. branchialis* infection. Different letters denote significant differences between groups ($p < 0.05$). Error bars = 2SE, n = 70 uninfected, 70 infected and 15 haddock with old infections.

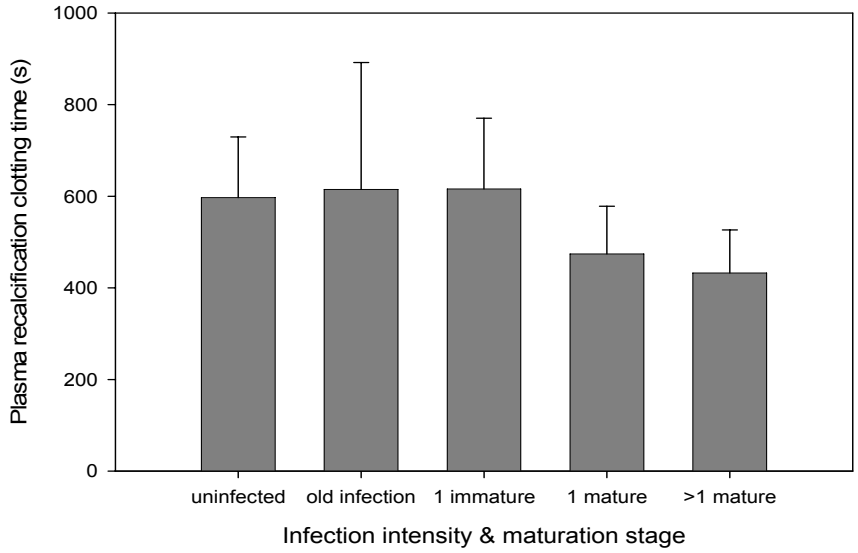

Figure 3.7 Plasma re-calcification clotting time of uninfected and infected wild haddock with different *L. branchialis* maturation stages and intensities. Error bars = 2SE, n = 40 uninfected, 40 infected and 10 with old infections.

3.3.2 Systemic immune response of naïve Atlantic cod laboratory infected with *L. branchialis*

3.3.2.1 Infection status

The preliminary infection study on Machrihanish cultured cod ended 14 d.p.i., concentrating on the immune response immediately post-infection. The infection prevalence was maintained at 88.9% over the entire infection study, however the mean parasite intensity of infected fish decreased by 14 d.p.i. (Table 3.2). The longer infection study on Ardtoe-cultured cod ended 56 d.p.i., in the hope of following the immune response of cod as the parasite metamorphosed fully. Initially, 100% of the fish were infected 3 d.p.i., however the infection prevalence decreased over time post-infection with 5 out of the 9 fish sampled at 28 d.p.i. being infected (Table 3.2). The mean parasite intensity of infected fish did not seem to change much over time, however, the initial parasite intensity was lower in comparison to infected Machrihanish-cultured cod (Table 3.2). The cod within the longer infection

study however, were not sampled at 1 d.p.i. and the infection was carried out at a different time therefore, no conclusion can be drawn as to why the initial parasite intensity was lower immediately post-infection in the longer infection study than in the preliminary infection study.

Table 3.2 *L. branchialis* infection prevalence and mean parasite intensity after bath infection of Machrihanish 0-14d post-infection and Ardtoe-cultured cod 0 to 56d post-infection. Mean parasite intensity ± 2SE.

Days post-infection	Prevalence (%)		Mean parasite intensity	
	0 – 14d	0 – 56d	0-14d	0-56d
0 n = 9	0	0	-	-
1 n = 9	88.9	-	6 4± 2.9	-
3 n = 9	88.9	100	4.2 ± 1.6	1.8 ± 0.7
7 n = 9	88.9	87.5	2 ± 0.5	1.7 ± 0.8
14 n = 9	88.9	77.8	1.4 ± 0.4	1.1 ± 0.3
28 n = 9	-	55.5	-	1 ± 0.0
56 n = 9	-	66.7	-	1.5 ± 0.4

In both infection studies 1 d.p.i. the infective stage was either attached to the base of the gill filament with their antennae and proceeded to burrow into the gill arch, or they attached to the tip of the gill filament and burrowed into the gill filament blood vessels migrating up towards the afferent branchial artery (Figure 3.8A and Figure 3.9A-B). In one case the parasite had entered the gill filament the 'wrong-way' migrating down towards the filament tip (Figure 3.8B). At 3 d.p.i. the parasites had entered the afferent branchial artery, beginning to migrate towards the ventral aorta (Figure 3.8C&D and Figure 3.9C). The entry point of the parasite at the base of the gill arch resulted in haemorrhaging (Figure 3.9D), and the migration up the gill

filament led to haemorrhaging and vessel congestion (Figure 3.8). At 7 d.p.i. the parasites had entered the ventral aorta anterior to the entrance of the *bulbus arteriosus* (Figure 3.9E). No parasites were found to advance into the *bulbus arteriosus*, as they remained within the ventral aorta 14 d.p.i. with the elongated abdomen protruding into the gill cavity, resembling an early penella sub-stage (Chapter 1, Table 1.1). The Ardtoe-cultured cod sampled at 28 and 56 d.p.i., in the longer infection study, possessed parasites which had not completed metamorphosis. These parasites were found to be dead (Figure 3.9F-H).

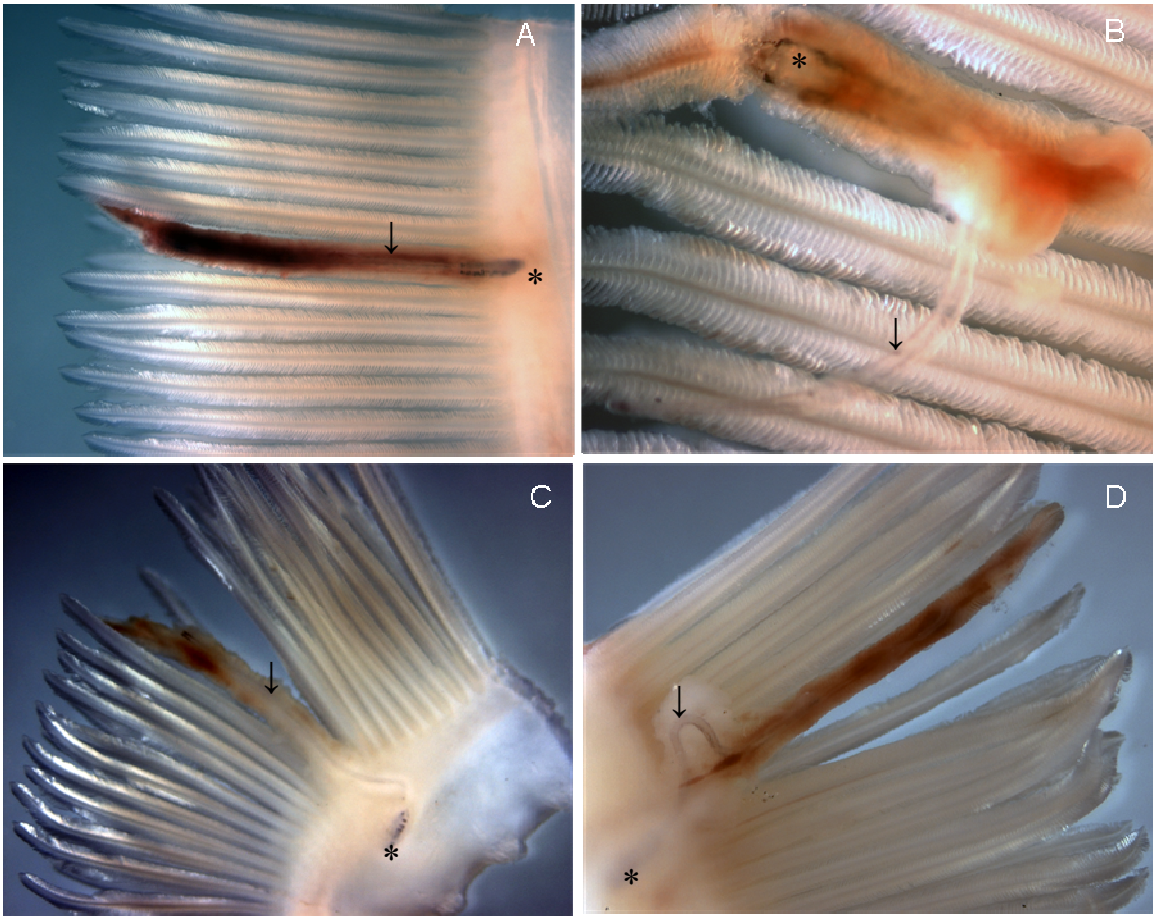

Figure 3.8 Site of infection on the gills of Machrihanish-cultured cod after bath challenge with *L. branchialis* (A) 1 d.p.i. and (B, C & D) 3 d.p.i.. (B) An infective female migrating the 'wrong' direction through the gill filament.* denotes parasite cephalothorax and → denotes abdomen.

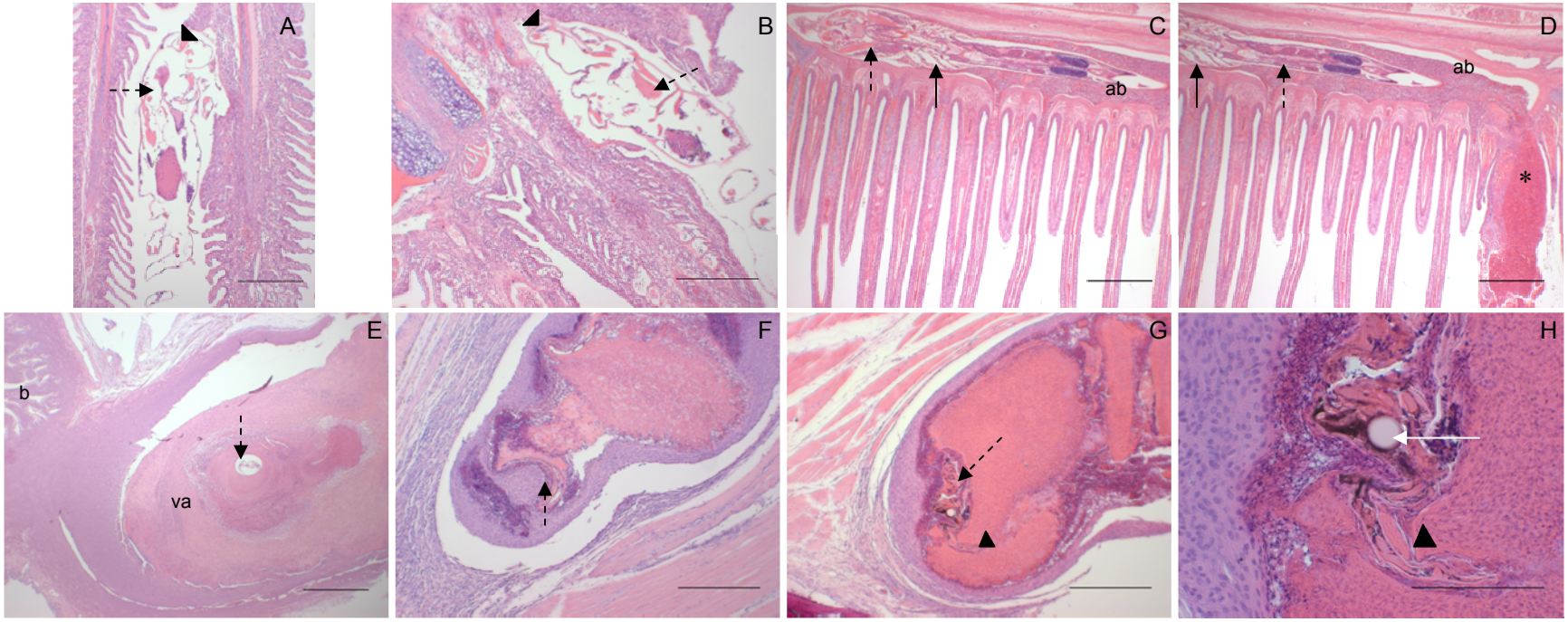

Figure 3.9 Site of infection of *L. branchialis* in laboratory-infected cultured cod from (A-E) Machrihanish and (F-H) Ardtoe over time series. (A) Infective stage attached to base of gill filament with antennae 1 d.p.i.. (B) Infective stage burrowing into gill arch at the base of a filament 1 d.p.i.. (C) Female *L. branchialis* migrating along afferent branchial artery 3 d.p.i.. (D) Female *L. branchialis* migrating along afferent branchial artery 3 d.p.i.; note haemorrhage (asterisk) at point of entry. (E) *L. branchialis* migrated to the ventral aorta by 7 d.p.i.. (F) Dead female *L. branchialis* 56 d.p.i.. (G) Dead female *L. branchialis* 56 d.p.i.. (H) Close-up of cephalothorax in G of dead female *L. branchialis* 56 d.p.i.. ⇢ parasite; black arrow head = parasite antenna; → parasite swimming legs; white arrow = parasite lens; ab, afferent artery; b, *bulbus arteriosus*; va, ventral aorta;. Bar = 250µm (A, B, F, G); Bar = 500µm (C, D, E); Bar = 100µm (H).

No tank effects on any parameter measured in both challenge experiments were found to occur within each treatment group at a given time point. Therefore, data from duplicate treatment tanks were pooled at each time point.

3.3.2.2 Whole blood erythrocyte counts

The number of circulating erythrocytes in the peripheral blood did not change due to infection or the infection protocol in either of the infection studies ($p = 0.529$ (Machrihanish-cultured cod) and $p = 0.530$ (Ardtoe-cultured cod); Table 3.3a and b).

3.3.2.3 Haematocrit

The haematocrit value of cod blood did not change with infection or with time post-infection in both the preliminary and longer infection studies ($p = 0.233$ and $p = 0.491$; Table 3.3a and b, respectively).

3.3.2.4 Blood leukocyte counts

The infection protocol of the preliminary infection study induced an increasing trend in peripheral blood leukocyte and thrombocyte numbers up to 7 d.p.i., as observed in sham control cod (Figure 3.10A). However, the number of leukocytes at 7 d.p.i. was not significantly higher from that at 0d.p.i. ($p = 0.177$), but at 14 d.p.i. the level dropped significantly below that of 7 d.p.i. remaining within pre-infection levels ($p = 0.038$; Figure 3.10A). The number of circulating leukocytes and thrombocytes of infected cod showed the same trend, except that the level at 7 d.p.i. was significantly higher than that at pre-infection ($p = 0.036$), and at 14 d.p.i. the number of leukocytes was not significantly lower than those at 7 d.p.i. ($p = 0.103$), suggesting a slight elevation of the number of these cell types circulating in the blood 7 and 14 d.p.i. with *L. branchialis* (Figure 3.10A). However, no significant

differences between the number of blood leukocytes and thrombocytes in sham control and infected cod at any of the time points were observed.

The infection protocol did not result in any significant changes in blood leukocyte and thrombocyte numbers over time post-infection compared to 0 d.p.i. in the longer infection study of Ardtoe cod (Figure 3.10B). However, the number of leukocytes and thrombocytes in sham control cod showed a decreasing trend at 7 d.p.i. highlighted by a significant reduction 7 d.p.i. compared to levels at 3 d.p.i. ($p = 0.021$). This was followed by a recovery of leukocytes / thrombocytes which were significantly increased 28 d.p.i. in sham control cod above levels at 7 and 14 d.p.i. ($p < 0.001$ and $p = 0.020$, respectively; Figure 3.10B). The infected cod showed a similar trend however, no significant changes in blood leukocyte and thrombocyte numbers over time post-infection, and no significant differences from levels of sham control cod during the experiment were observed (Figure 3.10B).

3.3.2.5 Partial differential leukocyte counts by flow cytometry

The leukocyte counts determined by microscopy and flow cytometry showed good correlation between them ($r = 0.908$, $p = 0.033$; $r = 0.858$, $p = 0.029$; $r = 0.805$, $p = 0.045$; for lymphocytes plus thrombocytes, granulocytes and monocytes, respectively). Therefore, flow cytometry was chosen as the method to determine blood differential leukocyte counts due to time constraints.

Table 3.3a Blood erythrocyte count, haematocrit, serum spontaneous haemolytic activity and serum total IgM concentration of sham control and *L. branchialis* infected Machrihanish cultured cod 0 to 14d post-infection (preliminary infection). Values represent mean ± 2SE.

Days post-infection		Factor measured			
		Erythrocyte No ($x10^8.ml^{-1}$)	Haematocrit (%)	Serum spontaneous haemolytic activity ($SH_{50\%}$)	Serum total IgM ($mg.ml^{-1}$)
0	Uninfected	15.92 ± 1.93	22.3 ± 2.1	1.8 ± 2.6	4.4 ± 1.3
1	Control	16.71 ± 2.56	21.4 ± 1.5	8.8 ± 10.1	3.6 ± 1.2
	Infected	16.61 ± 2.08	22.4 ± 1.9	13.0 ± 13.0	4.0 ± 1.6
3	Control	15.92 ± 1.22	24.4± 1.8	14.0 ± 11.8	4.4 ± 0.9
	Infected	15.41 ± 1.01	23.3± 2.1	13.2 ± 20.0	4.6 ± 1.4
7	Control	18.71 ± 2.36	23.5± 1.8	10.6 ± 11.9	6.3 ± 2.0
	Infected	18.22 ± 2.30	26.0± 3.9	9.1 ± 15.1	5.2 ± 1.4
14	Control	14.46 ± 1.20	23.2± 2.1	3.4 ± 4.0	4.0 ± 0.6
	Infected	14.85 ± 2.68	23.6± 2.4	5.4 ± 7.1	6.0 ± 1.1

Table 3.3b Blood erythrocyte count, haematocrit, serum spontaneous haemolytic activity and serum total IgM concentration of sham control and *L. branchialis* infected Ardtoe cultured cod 0 to 56d post-infection (longer infection study). Values represent mean ± 2SE.

Days post-infection		Factor measured			
		Erythrocyte No ($x10^8.ml^{-1}$)	Haematocrit (%)	Serum spontaneous haemolytic activity ($SH_{50\%}$)	Serum total IgM ($mg.ml^{-1}$)
0	Uninfected	20.44 ± 2.5	26.1 ± 2.4	22.6 ± 11.7	14.4 ± 4.0
3	Control	24.64 ± 6.1	26.6 ± 3.4	12.3 ± 8.3	13.8 ± 3.1
	Infected	26.08 ± 6.9	29.1 ± 1.1	6.6 ± 6.8	14.9 ± 4.7
7	Control	22.95 ± 3.7	26.6 ± 2.6	19.4 ± 16.1	10.5 ± 3.1
	Infected	22.28 ± 3.4	27.6 ± 3.5	26.4 ± 17.4	12.0 ± 2.0
14	Control	22.28 ± 2.2	23.9 ± 2.3	5.0 ± 10.0	6.7 ± 3.7
	Infected	25.74 ± 4.7	25.6 ± 4.7	21.9 ± 8.4	11.6 ± 3.6
28	Control	28.84 ± 5.5	23.6 ± 1.8	25.1 ± 10.4	10.5 ± 2.0
	Infected	30.02 ± 6.2	22.7 ± 1.1	17.6 ± 11.1	11.6 ± 3.5
56	Control	21.18 ± 5.5	28.9 ± 4.9	16.7 ± 14.6	6.5 ± 2.1
	Infected	24.47 ± 2.8	25.7 ± 2.4	37.1 ± 14.4	12.1 ± 3.7

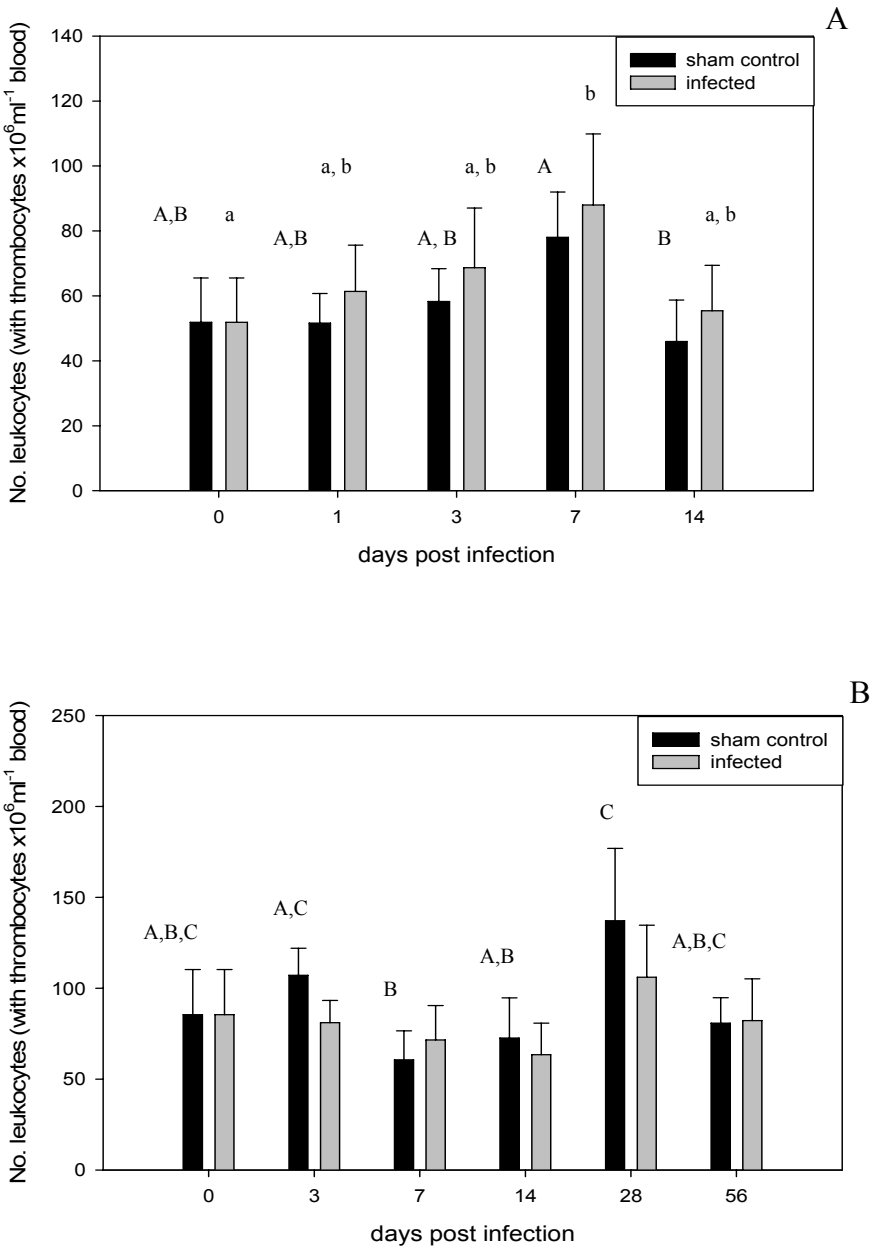

Figure 3.10 Blood leukocyte counts (including thrombocytes) of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

Lymphocyte and thrombocyte counts

An increasing trend in the number of lymphocytes and thrombocytes in the peripheral blood circulation up to 7 d.p.i. was found in the preliminary infection study, decreasing 14 d.p.i.. However, this was not significant and no significant changes with infection or with time post-infection were observed (Figure 3.11A). The infection protocol in the longer infection study of Ardtoe cod however, resulted in a decreasing trend in circulating lymphocyte and thrombocyte numbers, as evidenced by the sham control cod at 14 d.p.i., where they had significantly dropped below that of sham control cod 3 d.p.i. ($p = 0.021$; Figure 3.11B). The number of lymphocytes and thrombocytes recovered 28 d.p.i. in the sham control cod significantly increasing above that at 14 d.p.i. ($p < 0.001$; Figure 3.11B). The infected cod showed a similar trend with a significant reduction in the number of peripheral blood lymphocytes and thrombocytes in infected cod 14 d.p.i. compared to pre-infection levels ($p = 0.004$), and the recovery of cell numbers 28 d.p.i. to pre-infection levels ($p = 0.029$; Figure 3.11B). The lymphocyte and thrombocyte numbers of infected cod 3 d.p.i. however, also significantly dropped below pre-infection levels and sham control levels 3 d.p.i. ($p = 0.039$ and $p = 0.008$, respectively; Figure 3.11B). Therefore, the infection protocol resulted in a depression of peripheral blood lymphocyte and thrombocyte numbers 14 d.p.i., which recovered 28 d.p.i.; however, the infection of cod decreased the number of these cells circulating in the peripheral blood 3 d.p.i..

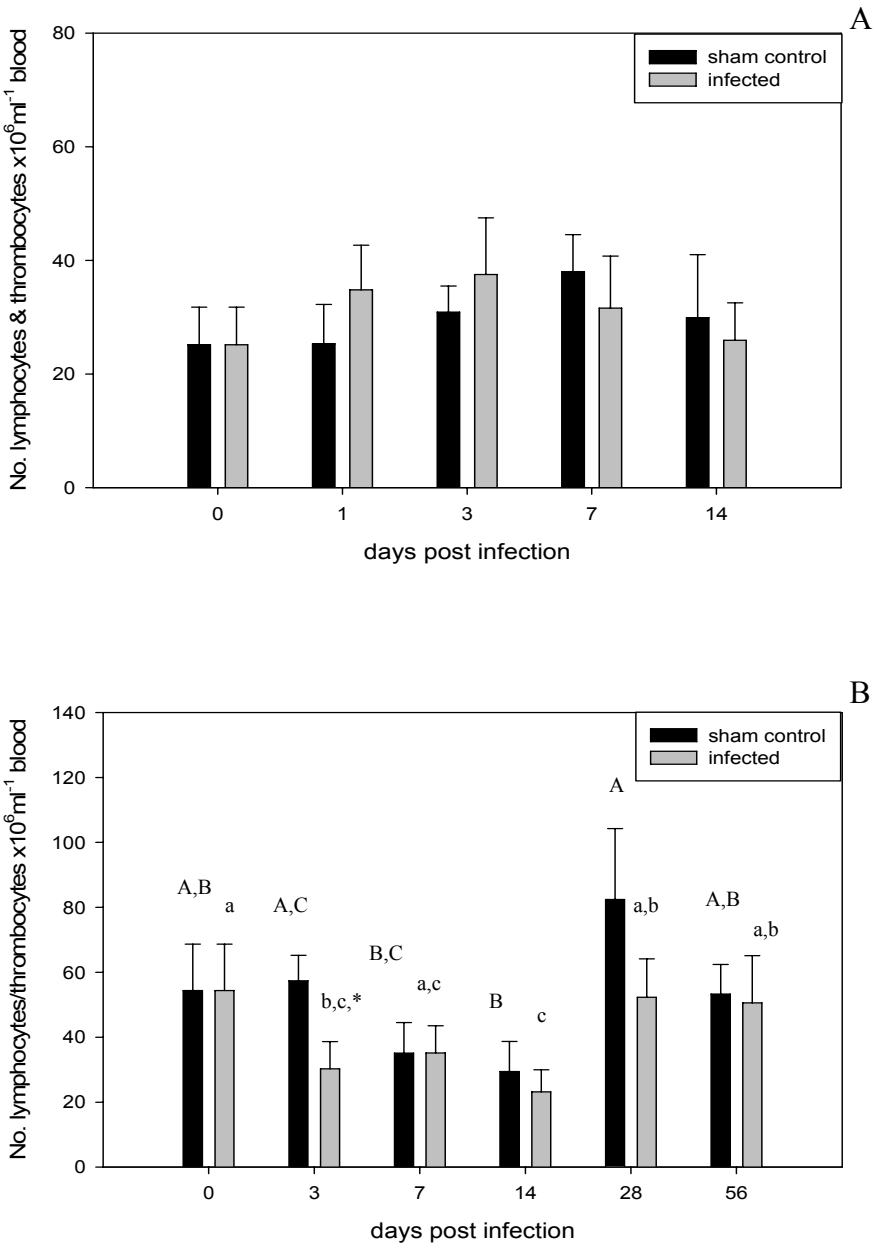

Figure 3.11 Blood lymphocyte and thrombocyte counts of sham control and *L. branchialis* infected (A) Machrihanish (preliminary infection) and (B) Ardtoe (longer infection) cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

Granulocyte counts

The infection protocol resulted in an increasing trend in peripheral blood granulocyte numbers in the preliminary infection study up to 7 d.p.i. (not significantly different to pre-infection levels), which significantly dropped 14 d.p.i. compared to levels at 7 d.p.i. ($p = 0.008$; Figure 3.12A). A similar pattern was observed in infected cod with the granulocyte number increasing significantly above pre-infection levels at 7 d.p.i. ($p = 0.038$; Figure 3.12A). The granulocyte numbers fell back down to within pre-infection levels 14 d.p.i.; however, unlike in sham control cod they were not significantly lower than those at 7 d.p.i. (Figure 3.12A). Even though no significant differences were observed between the number of circulating granulocytes in sham control and infected cod, the infection by *L. branchialis* seemed to increase the number of circulating granulocytes. This is due to an increase in granulocytes at 7 d.p.i. which was significantly higher than pre-infection levels, and a drop in granulocytes at 14 dp.i. that was not significantly different to that at 7 d.p.i, in contrast to sham control cod.

The infection of Ardtoe cod in the longer infection study, on the other hand, resulted in no significant changes in peripheral blood granulocyte numbers (Figure 3.12B). The number of circulating granulocytes showed a cyclical change over time with an increasing number 3 and 28 d.p.i, however this was not found to be significant (Figure 3.12B).

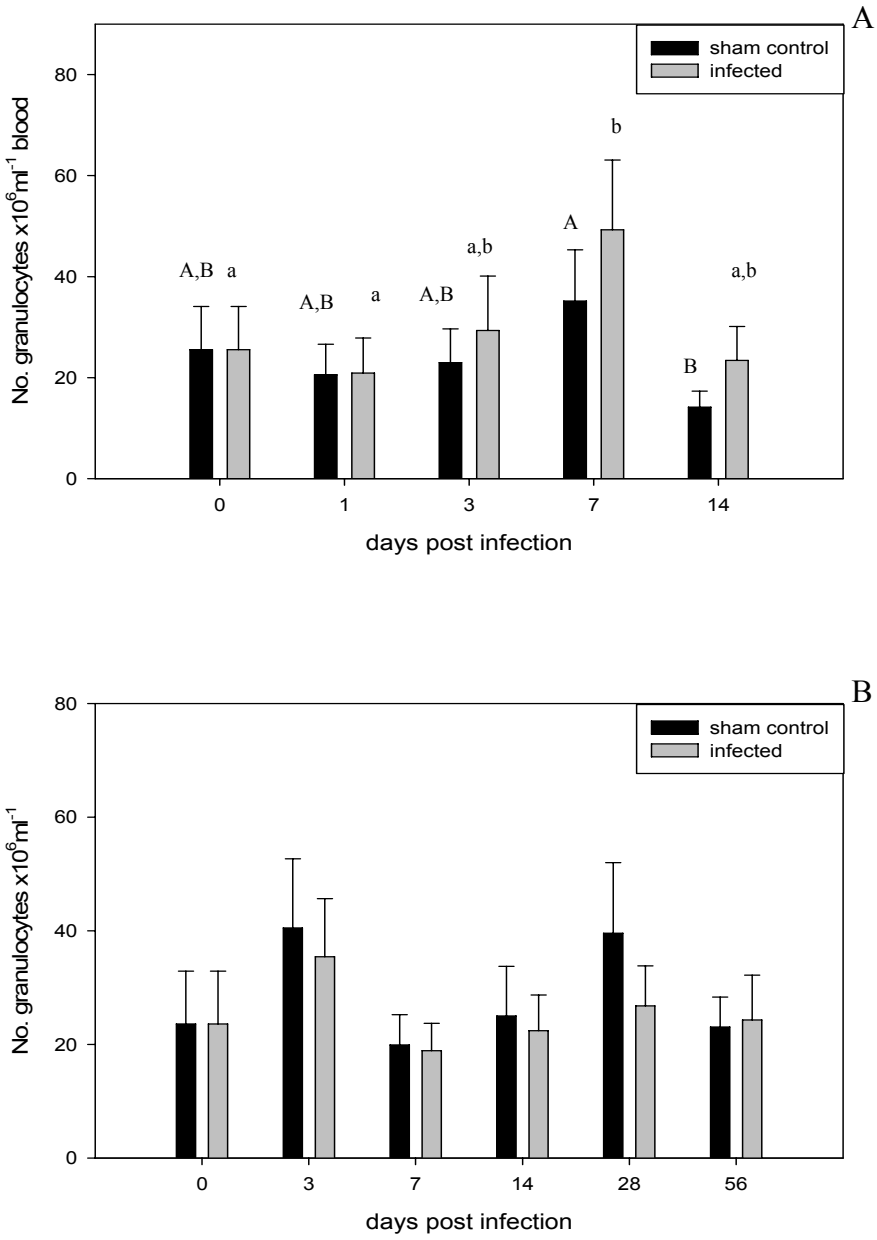

Figure 3.12 Blood granulocyte counts of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

Monocyte counts

The number of circulating monocytes in sham control cod significantly increased 1-7 d.p.i. above pre-infection levels ($p = 0.009$, $p = 0.011$ and $p = 0.002$, respectively; Figure 3.13A), suggesting a stress response to the infection protocol. The number of monocytes reduced 14 d.p.i. back to within pre-infection levels (Figure 3.13A). Infected cod showed a similar trend to sham control cod with an increase in monocyte numbers 1 d.p.i. ($p = 0.014$), and no significant differences with those of sham control cod were observed (Figure 3.13A). However, the number of monocytes fell back down to within pre-infection levels at 3 d.p.i., which was earlier than occurred in sham control cod (Figure 3.13A). Therefore, the level of circulating monocytes appeared to be lower in infected cod at 3 and 7 d.p.i., however due to the high variation between sham control fish this was found to not be significant ($p = 0.178$ & $p = 0.095$, respectively; Figure 3.13A).

The longer infection study of Ardtoe cod showed the infection protocol to elicit an increasing but not significant trend in circulating monocyte numbers up to 28 d.p.i.(Figure 3.13B). A similar and significant increasing trend in monocyte numbers was observed in infected cod, as monocyte numbers had increased significantly above pre-infection levels 14 and 28d.p.i. ($p = 0.018$ and $p = 0.04$, respectively; Figure 3.13B). The blood monocyte numbers had dropped back down to pre-infection levels 56 d.p.i. (Figure 3.13B). However, due to the slight increase in monocyte numbers as a result of the infection protocol no significant differences between infected and sham control cod were observed at any of the sampling time points (Figure 3.13B).

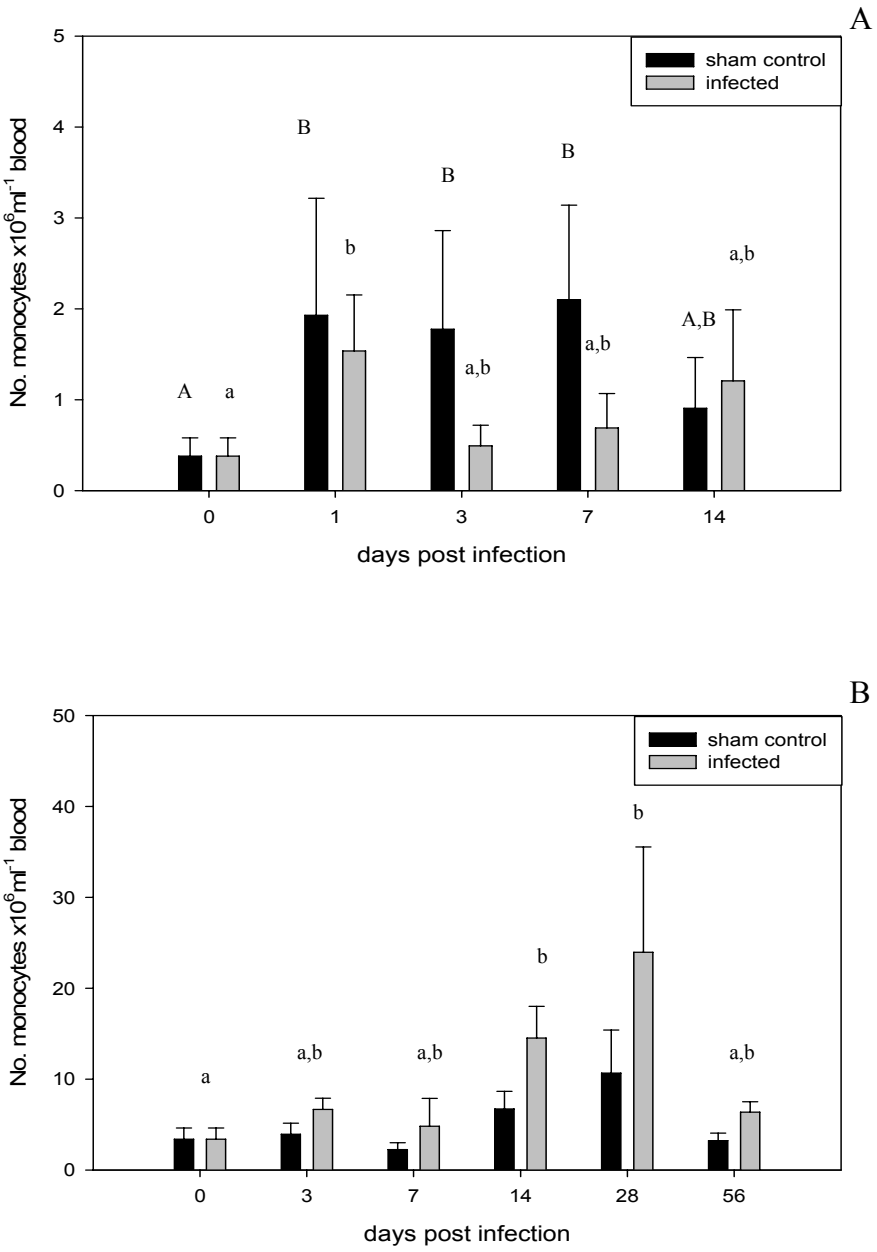

Figure 3.13 Blood monocyte counts of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * Denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

3.3.2.6 Blood phagocyte respiratory burst

The respiratory burst activity of blood phagocytic cells was measured both in terms of the percent of the population undergoing respiratory burst and the mean FI *i.e*. mean intracellular H_2O_2 production of the phagocytic cells. The infection protocol elicited an increase in the proportion of the phagocyte population undergoing respiratory burst in the preliminary infection study, as it was significantly higher in sham control cod 3 d.p.i. than at pre-infection ($p = 0.0001$; Figure 3.14A1). This was followed by a recovery of the number of phagocytes undergoing respiratory burst to pre-infection levels at 7 d.p.i. ($p = 0.0001$). However, 14 d.p.i. the phagocytic leukocyte population were activated again above the pre-infection and 7 d.p.i. level ($p = 0.018$ and $p = 0.017$, respectively; Figure 3.14A1). The mean intracellular production of H_2O_2 by the phagocytic cells however, did not change over time in the sham control cod (Figure 3.14A2). The same trend in the proportion of the phagocyte population undergoing respiratory burst over time was observed in infected cod with a significant increase 3 d.p.i. ($p < 0.0001$); however, once the levels returned to pre-infection levels 7 d.p.i. they remained at this level 14 d.p.i., and hence significantly lower than that of sham control cod 14 d.p.i. ($p = 0.019$; Figure 3.14A1). The mean intracellular production of H_2O_2 by the infected cod phagocytic cells showed a decreasing trend over time post infection, which was significantly lower at 14 d.p.i. than pre-infection levels and that of sham control cod 14 d.p.i. ($p < 0.001$ and $p = 0.019$, respectively; Figure 3.14A2).

The infection protocol also seemed to result in an increase in the proportion of blood phagocytes undergoing respiratory burst at 3 d.p.i. in the longer infection study of Ardtoe cod, as seen by the sham control cod however, this was found not to be significantly different to pre-infection levels ($p = 0.098$; Figure 3.14B1). The

proportion of phagocytes undergoing respiratory burst also seemed to decrease in infected cod 7 d.p.i. but this was not significantly different from 3 d.p.i. ($p = 0.068$; Figure 3.14B1). The mean intracellular production of H_2O_2 by the sham control cod phagocytic cells significantly increased 3 d.p.i. ($p = 0.003$) and then fell back down to pre-infection levels 14 d.p.i. (Figure 3.14B2). This was followed by an approximately 4.4 fold increase in the mean intracellular production of H_2O_2 28 d.p.i. in sham control cod compared to pre-infection levels ($p < 0.0001$; Figure 3.14B2). At 56 d.p.i. this parameter decreased significantly below that at 28 d.p.i. ($p = 0.003$) back down to levels observed at 3 d.p.i. (Figure 3.14B2). A similar trend in the mean intracellular production of H_2O_2 of infected Ardtoe cod phagocytic cells was observed with a significant increase 3 d.p.i. compared to pre-infection levels ($p = 0.018$), followed by a recovery to pre-infection levels 7 d.p.i. which again increased 2.4 fold at 28 d.p.i. compared to pre-infection levels ($p = 0.0001$; Figure 3.14B2). The mean intracellular H_2O_2 production levels of phagocytic cells recovered back to that of pre-infection levels 56 d.p.i., and no significant differences between infected and sham control cod over time were observed for this parameter (Figure 3.14B2).

In conclusion, the preliminary infection of Machrihanish cod led to a significant reduction in the proportion of blood phagocytes undergoing respiratory burst and their intracellular production of H_2O_2 at 14 d.p.i.. The longer infection study of Ardtoe cod however, did not result in any significant differences in the respiratory burst parameters measured to those observed in sham control cod as a result of the stress response of the infection protocol.

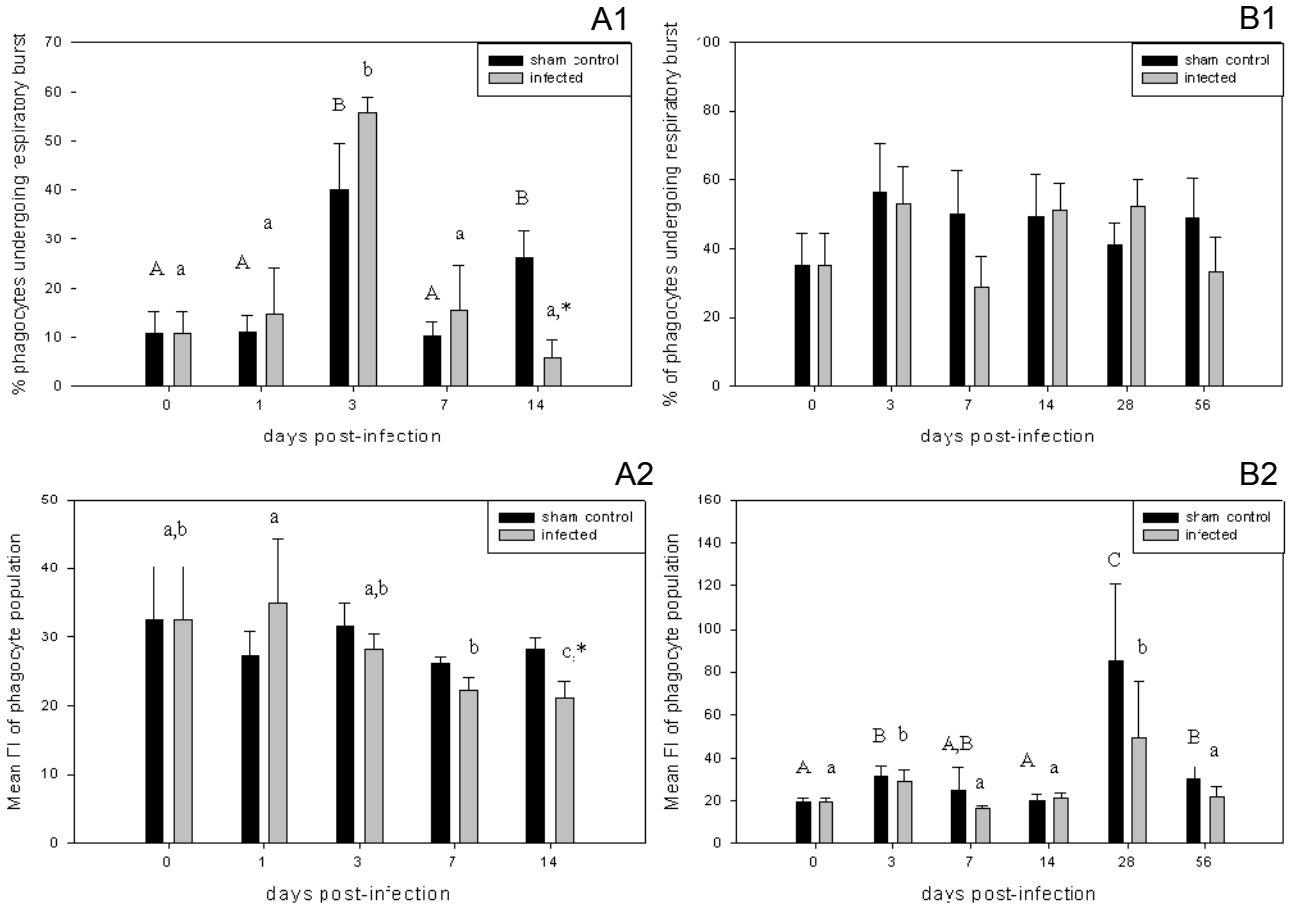

Figure 3.14 Percentage of blood phagocytes (1) undergoing respiratory burst and (2) their mean FI *i.e.* intracellular H_2O_2 production of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

3.3.2.7 Total protein concentration of serum

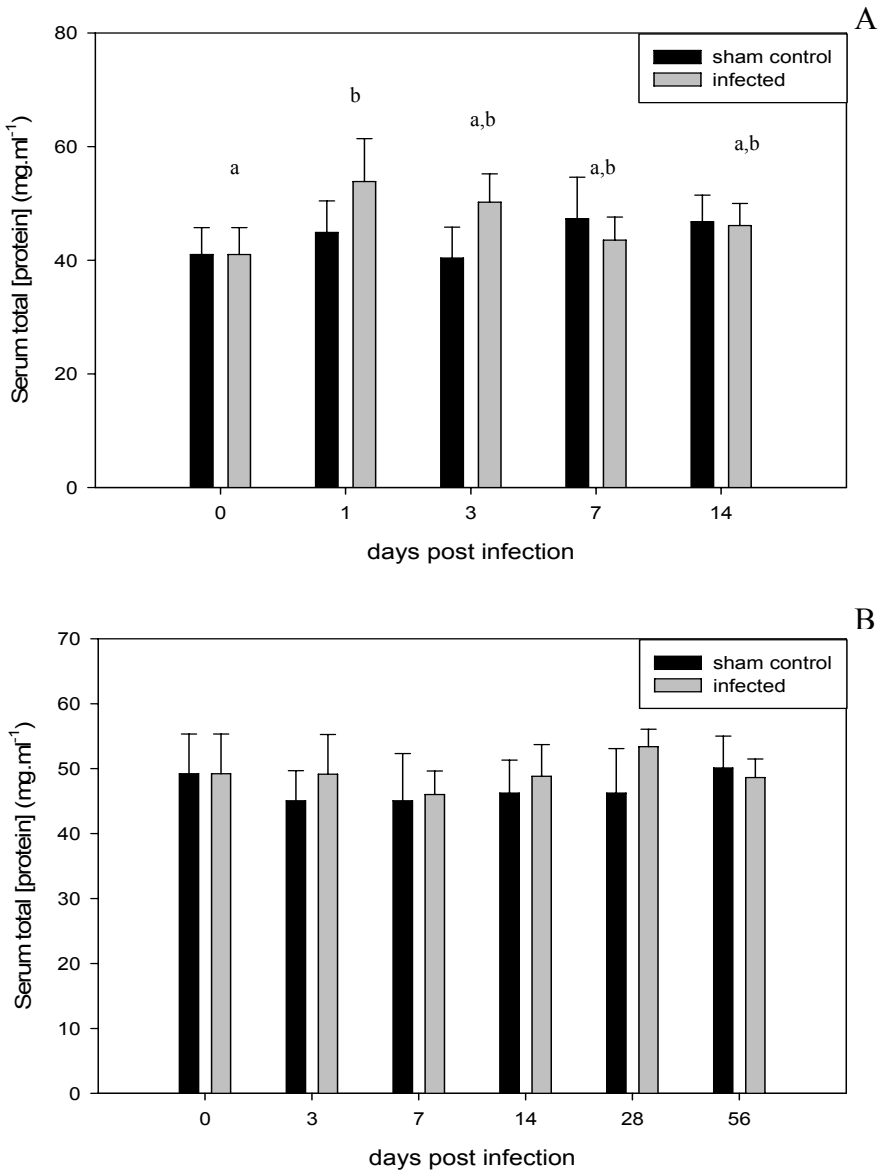

Figure 3.15 Serum protein concentration of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

The total serum protein concentration of sham control cod did not significantly change over time post-infection in either of the infection studies (Figure 3.15A and

B). The preliminary infection study of Machrihanish cod however, showed a significant increase in total protein content 1 d.p.i. in infected cod compared to pre-infection levels ($p = 0.044$; Figure 3.15A). The serum protein content remained within pre-infection levels 3-14 d.p.i. (Figure 3.15A), and no significant differences in serum protein concentration were observed in the longer infection study. However, cod were not sampled at 1 d.p.i. in the latter study and therefore, any increase in protein at 1 d.p.i. would not have been measured.

3.3.2.8 Anti-trypsin activity of serum

The infection protocol was associated with an increasing trend in serum anti-trypsin activity, which was significantly higher in sham control cod 14 d.p.i. than that at 0 d.p.i. ($p < 0.0001$; Figure 3.16A). The infection by *L. branchialis* resulted in an earlier increase in serum activity 1 d.p.i. ($p = 0.0005$) which remained higher than pre-infection levels 3 d.p.i. ($p =0.015$; Figure 3.16A). The anti-trypsin activity fell back down to within pre-infection levels 7 d.p.i., and following sham control cod, significantly increased at 14 d.p.i. above pre-infection levels ($p < 0.0001$; Figure 3.16A). This highlights the fact that even though no significant differences were observed between the serum activities of sham control and infected cod, the infection itself and the infection protocol together was associated with a slight increase in anti-trypsin activity 1 d.p.i., which was sustained 3 d.p.i., as the activity was found to increase above pre-infection levels earlier than that of the sham control cod, which occurred 14d.p.i. in sham control cod. However, no significant differences between sham control and infected cod were found.

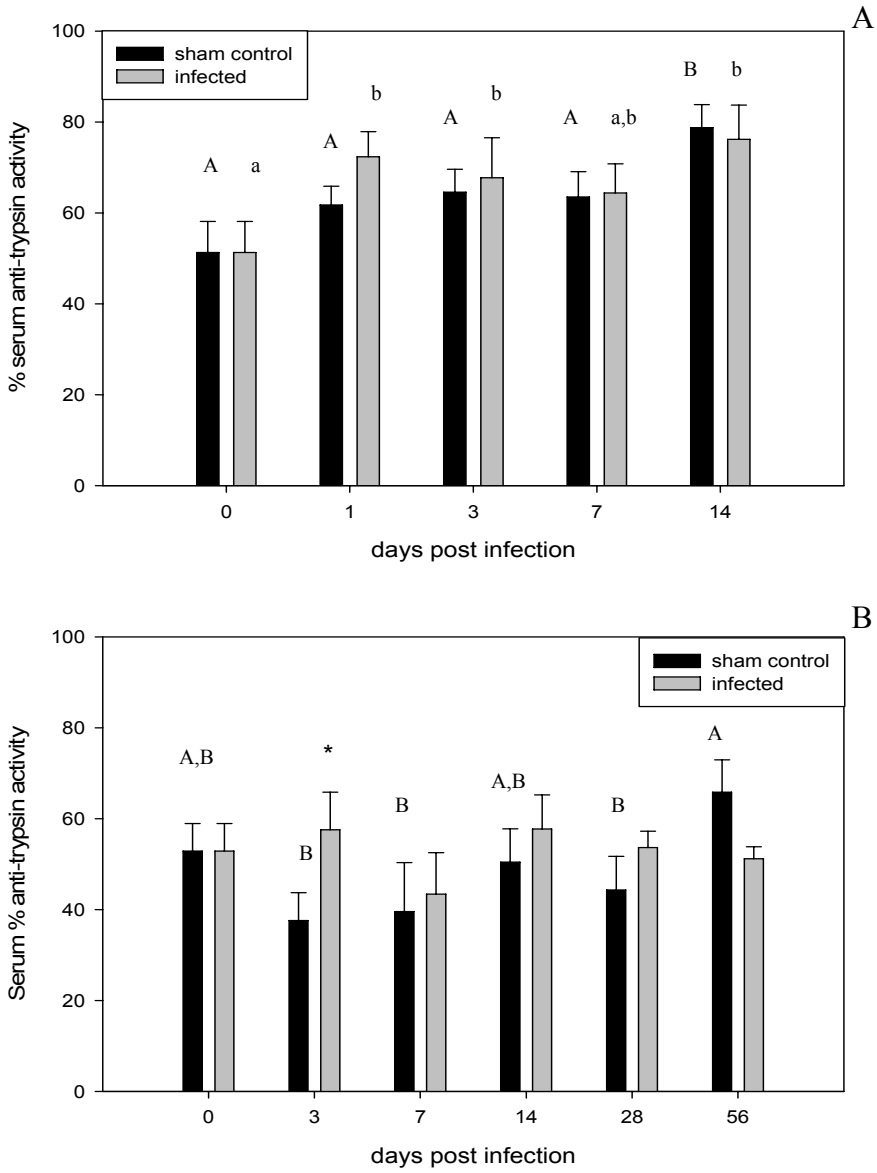

Figure 3.16 Serum anti-trypsin activity of sham control and *L. branchialis* infected (A) Machrihanish and (B) Ardtoe cultured cod 0 to 14 d.p.i. and 0 to 56 d.p.i, respectively. * Denotes significant differences between sham control and infected fish, and different letters denote significant differences ($p < 0.05$) within sham control fish (capital letters) and infected fish (lower case letters) over time. Error bars = 2SE.

The infection protocol of the longer infection study of Ardtoe cultured cod resulted in the reduction of serum anti-trypsin activity 3 d.p.i., however, this was found not to be significant ($p = 0.073$; Figure 3.16B). The serum activity seemed to increase

again becoming significantly higher 56 d.p.i. than at 3, 7 and 28 d.p.i. ($p < 0.0001$, $p < 0.001$ and $p = 0.004$, respectively; Figure 3.16B). The anti-trypsin activity of infected cod on the other hand did not significantly change over time post-infection, but was significantly higher than the activity of sham control cod serum 3 d.p.i. ($p = 0.005$; Figure 3.16B). In conclusion, the infection resulted in an increase in serum anti-trypsin activity 3 d.p.i. which was 'masked' by a reduction in activity resulting from the stress of the infection protocol.

3.3.2.9 Spontaneous haemolytic activity of serum

The serum SH activity of cod from Machrihanish-cultured and Ardtoe-cultured cod did not significantly change over time post-infection or due to infection by *L. branchialis* ($p = 0.703$ and $p = 0.169$; Table 3.3a and b, respectively). The serum SH activity of Machrihanish cod from the preliminary infection and Ardtoe cod from the longer infection study were found to possess large variations between fish, as evidenced by the mean SH activity at 0 d.p.i. $1.8 \pm 3.9_{SH50\%}$ and $22.6 \pm 18.5_{SH50\%}$, respectively.

3.3.2.10 Total IgM concentration of serum

The serum total IgM concentration did not significantly change due to the infection protocol or infection by *L. branchialis* in either of the infection studies performed ($p = 0.258$ (Machrihanish-cultured cod) and $p = 0.549$ (Ardtoe-cultured cod); Table 3.3a and b).

3.4 Discussion

3.4.1 The systemic immune response of naturally infected wild haddock with *L. branchialis*

Fully metamorphosed *L. branchialis* were found to principally reside within the ventral aorta of wild infected haddock, and not the *bulbus arteriosus*, in accordance with Kabata (1957). This is in contrast to infected cod where the principal infection site is the *bulbus arteriosus* (Smith *et al.*, 2007).

3.4.1.1 Circulating leukocyte levels of *L. branchialis*-infected wild haddock

Infection of wild haddock was associated with a slight increase in the proportion of circulating blood granulocytes, which corresponded to a reduction in the proportion of circulating blood monocytes. The increase in granulocytes observed in infected haddock was mainly attributed to fish infected by immature *L. branchialis*, however due to the smaller sample size of this group no significant difference was found with that of the uninfected haddock. Nevertheless, this could highlight an increase in circulating granulocytes responding to damage resulting from the migration and metamorphosis of the earlier stages of infection. However, the increased proportion of blood granulocytes in infected haddock was only marginal, increasing by an average of 3.2% in infected haddock or 5.5% in haddock with immature parasites when compared to uninfected haddock. This is in contrast to other studies showing changes in peripheral leukocyte numbers in response to infection, such as that of Sitja-Bobadilla *et al*. (2006) where an approximately 15 – 30% increase in the proportion of circulating granulocytes was observed in turbot (*Scophthalmus maximus*; Linnaeus,1758) 29-43d post-exposure (d.p.e.) to *Enteromyxum scophthalmi* (a myxozoan parasite). The proportion of monocytes, on the other hand, was found to be significantly reduced in the present study when multiple

mature parasites were present. This could result from the migration of monocytes from the blood circulation to the site of infection. When multiple mature parasites are present, an increased requirement for macrophages at the site of infection may also ensue as the parasites feed, resulting in host tissue damage and preventing the recovery of normal monocyte levels circulating in the blood. These phagocytic leukocytes are the principal cell types observed at parasitic copepod infection sites in fish (Joy & Jones, 1973; Boxshall, 1977; Shields & Goode, 1978; Paperna & Zwerner, 1982; Shariff & Roberts, 1989). They play a key role in inflammatory responses to infection and / or injury, resulting in the phagocytosis and subsequent degradation of pathogens and cellular debris by toxic reactive oxygen species (ROS), for example hydrogen peroxide, within the phagosome, via the respiratory burst. Ellis (1979) described monocytes as mobile cells supplying macrophages to tissues. Macrophages become activated at the site of inflammation with enhanced phagocytic and anti-microbial activity (Rowley *et al*., 1988), helping to scavenge and clear dead cells and pathogens, as well as processing and presenting antigens to lymphocytes (Vallejo *et al*., 1992).

The mature stages of the parasite were also associated with an increase in circulating blood thrombocytes, possibly highlighting increased host damage due to the parasite feeding on host blood. This was accompanied by a decrease in host plasma re-calcification clotting times in haddock infected with mature parasites. This was not however, a significant decrease due to the high variation for clotting times between fish, which may be related to its correlation with fish size. The number of thrombocytes is associated with blood clotting times in teleosts, and therefore an increase in thrombocytes is usually followed by decreased blood clotting times

(Srivastava, 1969). Richards *et al*. (1994a) similarly found an increase in thrombocytes in the spleen of carp (*Cyprinus carpio*; Linnaeus,1758) infected by *Sanguinicola inermis* (a digenean blood fluke), and hypothesised it to be as a result of increased haemorrhaging of damaged host tissue due to infection with adult parasites and eggs within the blood vessels and tissue. A similar scenario may be occurring in the infected haddock as the mature fully metamorphosed *L. branchialis* feeds on host blood. However, the combined stress of the sampling regime by trawling and the infection may have exacerbated the increased thrombocyte numbers and decreased clotting times. Hattingh and Van Pletzen (1974) observed handling stress to result in increased thrombocyte counts in mudfish (*Labeo umbratus*) and Casillas and Smith (1977) found decreased clotting times in stressed rainbow trout (*Oncorhynchus mykiss*). During this study all fish were sampled by the same regime, infected and uninfected alike, however, there is potential for a differential response to stress in the haddock according to their infection status.

3.4.1.2 Serum protein and innate immune parameter levels of *L. branchialis*-infected wild haddock

The anti-trypsin and SH activities of wild haddock serum were found to be similar to those of wild Atlantic cod (Magnadottir *et al*., 1999b; Magnadottir *et al.*, 2001). Infection of haddock with multiple mature *L. branchialis* and old necrotic infections was associated with an increase in anti-trypsin activity. The former could be due to the host trying to counteract *L. branchialis*-derived secretions containing proteases, such as trypsin, which could increase in quantity with multiple parasites. Trypsin-like proteases have been found to be produced by numerous ectoparasites, such as *L. salmonis* which secrete them onto their hosts (Ross *et al*., 2000, Firth *et al*., 2000 and Fast *et al.,* 2002a), and are hypothesised to aid in parasite feeding and / or host

immune response evasion (Fast *et al*., 2002a). Pathogen-derived proteases are known virulence factors, for example, Zuo & Woo (1997a) found the pathogenic strain of *Cryptobia salmositica* to posses a 200kDa metalloprotease, which is thought to cause anaemia in cryptobiosis by its secretion into host blood (Thomas & Woo, 1989a: Zuo & Woo, 1997b). Therefore, the ability of the host to overcome these virulent proteases may be paramount in eliminating or suppressing the infection. This can be achieved through a variety of host-derived plasma anti-proteases, such as alph-2-macroglobulin (α2M), α1-protease inhibitor and α1-antitrypsin (Ellis, 1987; Salte *et al.,* 1993). For instance, Ramos *et al*. (1997) demonstrated human α2M to inhibit the proteolytic activity of *Trypanosoma cruzi* extracts *in vitro* by inactivation via steric entrapment. The increase in haddock serum anti-trypsin activity with multiple, mature or old infections could result from increased host protease activity at the site of infection due to the increased host tissue damage by multiple parasites or necrosis of the dead parasite. Proteases are involved in the process of necrosis, with the major ones being calpains and cathepsins (Zong & Thompson, 2006). Therefore, as the parasite dies and cell necrosis ensues the proteolytic activity at the local site of infection may increase, as the parasite cephalothorax remains embedded within the host tissue.

Infection of haddock with *L. branchialis* was associated with a significant reduction in SH activity of serum. The depressed SH activity may have resulted from either increased consumption of complement activity as the host tries to overcome the infection, or by suppression of the production of host complement components or their activity by the parasite. For instance, Atlantic cod experimentally infected with the bacterium *Aeromonas salmonicida* ssp. *achromogenes* by intra-peritoneal

injection showed decreased SH activity, and the higher the dose of bacteria the lower the SH activity of the cod (Magnadottir *et al.*, 2002). A reduction in serum haemolysis has also been observed in rainbow trout infected by virulent strains of the bacteria *A. salmonicida* and *Vibrio anguillarum* (Sakai, 1983), and the parasite *C. salmositic*a (Thomas & Woo, 1989b). However, other parasites, such as the digenean blood fluke *S. inermis*, cause an increase in host serum haemolytic activity (Roberts *et al.*, 2005). These authors hypothesised that this resulted either from an increase in macrophages previously observed in the haematopoietic organs of carp, or a reduction in the 'consumption' of host complement by *S. inermis* (Roberts *et al.*, 2005). Many parasites have evolved mechanisms to suppress, inactivate or avoid detection by the complement system of their host. For example, many ticks have been found to produce proteins within their saliva capable of inhibiting their hosts complement activity by preventing the formation of the molecular attack complex (Mejri *et al.*, 2002; Ribeiro, 1987; Astigarraga *et al.*, 1997; Lawrie *et al.*, 1999; Nunn *et al.*, 2005; Tyson *et al.*, 2007). Some parasites are also capable of causing complement 'consumption' by secreting / shedding molecules in their vicinity in order to prevent the formation of molecular attack complexes directly on their surfaces. The first studies to identify this mechanism of complement evasion were by Hammerberg and Williams (1978a, b) in the cestode parasite *Taenia taeniaeformis*, which secretes a polysaccharide, which 'consumes' complement away from its surface. Whether, *L. branchialis* has activated the complement system of haddock serum resulting in its 'consumption', or they possess mechanisms capable of suppressing host complement remain to be determined. However, it is clear that even if *L. branchialis* activates haemolysins, such as complement, leading to 'consumption', the parasite is not eliminated. This

is possibly due to the host not being capable of recovering complement activity post-'consumption' during the infection rendering the host more susceptible to infection. For instance, Zuo & Woo (1997a) found salmonids infected by *C. salmositica* to possess reduced α2M levels, and the ability of a species or strain to recover from infection to be reliant on their capacity to recuperate plasma α2M levels. The reduction in SH activity in the current study seemed to be apparent no matter which infection stage was present. However, due to the smaller sample sizes of infection groups once segregated by parasite maturity and the high variation between fish, no significant differences were observed with that of uninfected haddock. The high variation in SH activity between individuals has previously been reported for numerous species, for example Atlantic cod, halibut (*Hippoglossus hipoglossus*; Linnaeus, 1758) and sea bass (*Dicentrarchus labrax*; Linnaeus, 1758; Magnadottir *et al.*, 1999a, b; Magnadottir, 2000; Magnadottir *et al.*, 2001; Lange & Magnadottir 2003). Factors such as stress, infection and genetic variation have also been found to affect the SH activity of various fish species (Sakai, 1992; Tort *et al*., 1996; Magnadottir *et al*., 1999a; Wiegertjes *et al*., 1993; Magnadottir, 2000; Magnadottir *et al.*, 2001; Magnadottir *et al.*, 2002). Therefore, the fact that wild haddock were sampled by trawling at different locations in this study could account for the high individual variation for this factor, as the sampling regime was highly stressful to the fish, several genetic stocks may have been sampled, and other infections not accounted for may have been present.

Infection of wild haddock with *L. branchialis* was also associated with a significant drop in serum protein content by an average of 5.5mg.ml^{-1}. This may be due to the damage caused by the parasite as it feeds on host blood. Ziskowski *et al*. (2008)

noted that male flounder with fin rot disease possessed lower serum protein levels than normal flounder, and suggested this depletion in protein was possibly to produce new tissue and heal lesions leading to tissue repair of the damaged fins. The blood loss of the haddock as a result of feeding of the parasite on host blood may also have affected the serum protein levels, as the gut of fully mature parasites is capable of holding 100µl of host blood (Sproston & Hartley, 1941). The protein levels of haddock infected by multiple mature parasites also seemed to decrease, but due to the lower sample size for this group no significant differences with uninfected haddock were noted.

The investigation of the infection of wild haddock with *L. branchialis* provided some informative results. However, no definitive conclusions can be drawn from this field study regarding the effect(s) of *L. branchialis*-infection on the systemic immune response of haddock due to the collection of samples from wild fish infected in uncontrolled field conditions. Sampling wild fish by trawling was also not optimal for the investigation of the immune response due to 1) the stress induced by the sampling regime, 2) the fact that the previous infection history of the sampled haddock was not known, 3) the fact that the presence of other infections were not monitored during the sampling, and 4) the fact that the number of Atlantic cod caught during the survey was very low. Gadoids with newly attached female *L. branchialis* were also not obtained during sampling, and therefore, the immune response immediately post-infection could not be monitored. Therefore, the latter part of the study involved the experimental infection of naïve cultured Atlantic cod. The preliminary infection of Machrihanish-cultured cod was intended to run for 56 d.p.i, in order to follow the immune response of the host from the initial attachment

to the full metamorphosis of *L. branchialis*. However, due to a loss of stock fish prior to beginning the study, the infection could only run for 14 d.p.i.. Therefore, another infection experiment was performed which lasted 56 d.p.i., as the parasite had only reached the early penella sub-stage in the preliminary infection study. The cod for the longer infection study were however, obtained from a different source, Viking Fish Farms Ltd. (Ardtoe, Scotland), due to practical reasons.

3.4.2 Systemic immune response of naïve Atlantic cod laboratory-infected with *L. branchialis*

3.4.2.1 Infection status

The prevalence of infection of the laboratory-infected cod was high in both studies. The parasite intensity, on the other hand, was low, reflecting observations from natural infections, and declined over time post-infection, falling approximately one third by 7 d.p.i. in the preliminary infection. This highlights the fact that not all of the attached infective stages will go on to metamorphose and produce egg sacs. This could be as a result of poor attachment, attachment at the 'wrong site' for efficient migration to the ventral aorta / *bulbus arteriosus*, competition for space between parasites as they enter the afferent branchial artery or unfavourable host conditions *e.g.* host immune response. The migration of the parasite up to 14 d.p.i. followed a previous study by Smith *et al*. (2007), even though the fish utilised in the present study were approximately ten times larger by weight. These authors observed penella sub-stages of the parasite infecting Machrihanish-cultured cod 19 days post-exposure (d.p.e.) to infected intermediate hosts, which were principally within the lumen of the ventral aorta. Smith *et al*. (2007) noted parasites to become "readily visible" reaching the late U sub-stage (Chapter 1, Table 1.1) by 33 d.p.e., which were located within the lumen of the *bulbus arteriosus*. In the present longer

infection study of Ardtoe-cultured cod however, the infection was found to subside by 28 and 56 d.p.i., as parasites were not readily visible in the gill cavity and dead parasites were found encapsulated within host tissue. The preliminary infection of Machrihanish-cultured cod only ran for 14 d.p.i. and therefore whether these parasites would have died as in the Ardtoe-cultured cod or metamorphosed into gravid females can not be determined. However, the systemic immune response observed between the two stocks of cod to infection differed in some respects, and although the two fish populations were of similar size, the batch of Machrihanish cod used in this study were found to be in poorer condition with dark skin pigmentation. The lack of success of the infection in Ardtoe cod in the later stages of infection may also have been due to the infection protocol. Free-swimming females were collected for infection of cod post-detachment from the intermediate host. The females usually detach post-copulation on the intermediate host (Schuurmans Stekhoven & Punt, 1937; Sproston & Hartley, 1941; Capart, 1948; Kabata, 1958). However, whether all females used in the bath infection had mated or not could not be determined, and may have affected the infection success. Anstensrud (1990) found both virgin and non-virgin females to be capable of finding a definitive host, and to develop into penella stages five days post-infection. However, it was noted in this study that the majority of virgin females did not develop a cephalothoracic holdfast unlike the copulated females, and none of the virgin females developed further than the post-penella stage compared with 21-59% of copulated females which persisted through the metamorphosis process. They concluded that the presence of seminal fluids in the *seminal receptacles* play a role in inducing large morphological changes in female *L. branchialis*, although the mechanisms behind these effects are not known. Therefore, any detached virgin

females used in the infection would not have developed past the post-penella stage. However, the suggestion that all of the free-swimming females used in the infection protocol were virgins is not probable. The actual infection protocol itself may have also contributed to the lack of success of the infection in the later stages, as ten infective females per fish were added to the bath challenge over a six hour period, rather than a continual exposure to the infective stages by cohabitation with naturally infected intermediate hosts over a 19d period as in Smith *et al.* (2007). The addition of infective stages in a bath challenge was chosen in this study in order to prevent the transmission of other pathogens from wild intermediate hosts, which could occur during cohabitation, and to be able to follow the immune response of cod over a tightly defined time series post-infection. The lower exposure rate to infective stages may have resulted in a decline of successful females infecting cod past the penella sub-stage, as many may initially attach at the 'wrong' site for successful development. However, the observation of early penella sub-stages of *L. branchialis* within the ventral aorta 14 d.p.i. does not support this. In conclusion, the lack of successful infection of Ardtoe cod 28-56 d.p.i. may have resulted from a combination of the innate resistance of the cod population and the infection protocol used.

3.4.2.2 Circulating erythrocyte and leukocyte levels in *L. branchialis*-infected Atlantic cod

The infection of cultured cod with *L. branchialis* did not affect the number of circulating erythrocytes or the blood haematocrit values. This is likely to be due to the immature stages of the parasite, with the infections only lasting up to the early penella stage, and the low parasite intensity observed; as once the parasite matures it possesses an increased nutrient requirement leading to larger blood loss

for the host. For instance, Van Damme *et al*. (1994) found the infection of whiting with single or multiple mature *L. branchialis* to result in reduced haematocrit levels. Kabata (1958) also found the biggest drop in haddock haemoglobin levels when multiple mature parasites were present. However, he suggested that haddock infected with immature females tried to compensate for the blood loss by stimulation of their haematopoietic tissues.

The preliminary infection protocol of Machrihanish-cultured cod was associated with an increasing trend in circulating leukocyte and thrombocyte numbers, and granulocytes up to 7 d.p.i., however this was not significant. The same trend was found in infected cod, however the levels at 7 d.p.i. were found to be significantly higher than pre-infection levels suggesting a slight elevation in circulating leukocytes and / or thrombocytes, and granulocytes 7 d.p.i. with *L. branchialis*. The infection protocol and the infection by *L. branchialis* however, had no significant effect on the number of circulating lymphocytes and thrombocytes, even though they appeared to be higher in infected than sham control cod 1 and 3 d.p.i.. The number of circulating monocytes on the other hand, seemed to increase after the infection protocol 1-7 d.p.i., followed by a recovery back to pre-infection levels 14 d.p.i.. The infection by *L. branchialis* however, seemed to result in a decrease in circulating monocytes, as after an increase 1 d.p.i. the levels fell back down earlier (3 d.p.i.) than that of sham control cod to those observed pre-infection. The high variation between fish for this factor however, meant that no significant differences between sham and infected cod were detected. The lack of significant differences between sham control and infected cod in terms of the counts of leukocytes and thrombocytes circulating in the peripheral blood in the preliminary infection made it

difficult to draw any conclusions as to the effects of infection on them. It would be worth further investigating the blood smears as well as the flow cytometry data as differences in the maturity of the different leukocyte populations may have occurred due to infection which were not observed by flow cytometry. The populations of leukocytes determined by flow cytometry also need to be verified by cell sorting, as although the cell counts gave good correlation with those obtained by microscopy techniques, this was performed on uninfected cod. Infection may result in an increase in immature leukocytes, such as progranulocytes, which may appear in a different population to mature granulocytes on the FSC versus SSC dot plot. For example, Sitja-Bobadilla *et al.* (2006) observed *E. scophthalmi* infection of turbot to result in an increase in circulating progranulocytes. A recent study by Rønneseth *et al.* (2007) has also shown by flow cytometry of cod blood leukocytes using immunofluorescence that the small non-granular population allocated as lymphocytes and thrombocytes in Inoue *et al.* (2002) for other teleost species, also contains some neutrophils.

The longer experimental infection by *L. branchialis* and the infection protocol of Ardtoe-cultured cod did not have any significant effect on circulating leukocyte and thrombocyte numbers in comparison to basal 'resting' levels. However, infection resulted in a reduction in circulating lymphocyte and thrombocyte numbers 3 d.p.i. compared to sham control cod. A thrombus was observed to form around the parasite as it migrated along the afferent branchial artery in accordance with Smith *et al*. (2007). This drop in lymphocytes / thrombocytes corresponds to the migration of *L. branchialis* along the afferent branchial artery towards the ventral aorta. Therefore, the thrombocytes may be involved in the thrombus formation around the

parasite, and clotting at the point of entry to impede haemorrhaging. Smith *et al.* (2007) described *L. branchialis* to be surrounded by a thrombus consisting of red blood cells, degenerative red blood cells and thrombocytes within a fibrin network as it migrated along the afferent branchial artery. In the preliminary infection of Machrihanish-cultured cod no significant change in the number of circulating thrombocytes and lymphocytes was observed. However, due to the fact that the proportion of this cell population composed of lymphocytes or thrombocytes could not be determined due to the flow cytometry protocol used (Inoue *et al.*, 2002), an increase in one cell type followed by a subsequent decrease in the other would not be detected in this study. Therefore, in hindsight it would have been better to determine the differential leukocyte counts manually from the blood smears and microscopic leukocyte counts, in order to be able to differentiate between the lymphocytes and thrombocytes. In the longer infection study, circulating lymphocytes and thrombocytes of infected cod remained below basal 'resting' levels 14 d.p.i. however, this was not significantly different to sham control cod due to the fact that the infection protocol also led to a decreasing but not significant trend in lymphocyte and thrombocyte numbers up to 14 d.p.i.. This was followed by a recovery of these cell types 28 d.p.i. to 'resting' levels, corresponding with the death of the parasite. The recovery of thrombocyte and lymphocyte numbers 28 d.p.i. occurred in both the sham control and infected cod possibly highlighting the expulsion of these cells from the haematopoietic organs to maintain circulating levels after their suppression. The parasites found 28 d.p.i. were dead, and therefore the requirement for thrombocytes at the site of infection reduced as damage caused by the parasite via its increase in size, migration along the blood vessels and/or feeding had ceased. The number of circulating granulocytes was not

significantly affected by the infection, and cycled over the entire study. However, no samples were taken immediately post-infection (1 d.p.i.), and hence whether or not a change occurred at this time-point cannot be determined. The number of circulating monocytes increased over time post-infection with *L. branchialis* peaking 28 d.p.i, increasing an average of 17.7 x 10^6 monocytes.ml^{-1} above basal 'resting' levels. The increase in monocytes observed in Ardtoe cod from 14 d.p.i. to 28 d.p.i. corresponds with the death of the parasite; however this was not significantly different to the sham controls. The circulating monocytes levels of infected fish between 14 to 28 d.p.i., when the parasite died, unfortunately was not measured as an increase may have occurred which was not detected. Richards *et al.* (1994b) observed the infiltration of macrophages and neutrophils around *S. inermis* eggs 1-2 weeks post-shedding (w.p.s.), the macrophage layer surrounding eggs increased 2-3 w.p.s. and 5-6 w.p.s. following which, structural damage and breakdown of the eggs occurred. The cytotoxic effect of phagocytic cell-derived factors on parasites has been highlighted in numerous studies, where parasite tegument damage or death resulted (Whyte *et al*., 1989; Sharp *et al*., 1991; Hamers *et al*., 1992; Nakayasu *et al*., 2005). Cross & Matthews (1993) stated however, that phagocytic responses may be more involved in antigen presentation, mediating a protective response, rather than direct killing of the parasite. However, some parasites employ mechanisms to evade the cytotoxic effects of phagocyte-derived ROS. For instance, the scuticociliate parasite *U. marinum* protects itself from the cytotoxic effects of its hosts' (*Paralichthys olivaceus*; olive flounder) phagocytes by producing excretory / secretory products with superoxide dismutase and catalase activity to scavenge reactive oxygen intermediates, and by causing phagocyte lysis (Kwon *et al*., 2002). Therefore, the effect of *L. branchialis*-infection on cod blood phagocyte

respiratory burst activity was also investigated, as an indication of phagocyte function.

3.4.2.3 Blood phagocyte respiratory burst activity in *L. branchialis*-infected Atlantic cod

The infection protocol of Machrihanish-cultured cod resulted in an increase in the percent of blood phagocytes undergoing respiratory burst 3 d.p.i. and 14 d.p.i. However, infection by *L. branchialis* suppressed the proportion of blood phagocytes undergoing respiratory burst at 14 d.p.i.. This correlated with a decreasing trend in the mean intracellular H_2O_2 production by the phagocytic cells of infected cod from 3 d.p.i., which was also significantly suppressed 14 d.p.i.. The time frame of this suppression of blood phagocyte respiratory burst activity correlates with the metamorphosis of the parasite into the early penella sub-stage in the ventral aorta (14 d.p.i.). Similar phagocyte activity suppression occurs in salmonids after infection with the copepod parasite *L. salmonis* (Mustafa *et al.*, 2000a, b; Fast *et al.*, 2002a). Mustafa *et al.* (2002a) found the suppression of Atlantic salmon phagocyte respiratory burst activity and phagocytosis capacity to correspond with the moulting of the parasite into the more destructive pre-adult stages. They suggested however, that the immunosuppression was due to a stress response in infected fish, as they observed increased plasma cortisol levels post-infection over an extended period. Acute stress of fish has often been associated with an enhancement of some immune parameters whereas, chronic stress has been found to have a generalised suppression on the immune system of fish (Maule *et al.*, 1989; Schreck, 1996; Demers & Bayne, 1997; Ruis & Bayne, 1997; Ortuno *et al.*, 2001; Fast *et al.*, 2008). Fast *et al.* (2002a) however, found that Atlantic salmon and rainbow trout infected by lower numbers of *L. salmonis* than in Mustafa *et al.* (2000a) still possessed

suppressed phagocytic activity as the lice moulted into pre-adult stages in the absence of a stress response. These authors suggested that this suppression is either due to the increased damage caused by *L. salmonis* as it increases in size, or molecules secreted by *L. salmonis* onto the host. The host may also switch to an anti-inflammatory response after chronic exposure to the parasite in order to try to avoid excessive tissue injury induced by ROS production over an extended period (Sitja-bobadilla *et al.*, 2008). Fast *et al.* (2002a) found the host macrophage activity suppression to correlate with the appearance of low molecular weight trypsin-like proteases in the mucus of infected fish. Other arthropod parasites, such as ticks, have also been found to result in host phagocyte activity suppression post-infection. For instance, dogs infected by the tick *Rhipicephalus sanguineus* possess suppressed neutrophil respiratory burst activity (Inokuma *et al.*, 1997). The haematophagous, parasitic nematode *Anguillicola crassus*, which infects the swimbladder of the European eel, has also been found to produce the detoxifying enzyme glutathione-s-transferase thought to quench reactive oxygen species (Nielsen & Buchmann, 1997). Therefore, due to the fact that *L. branchialis* is in direct contact with the phagocytic cells of the blood, the suppression of phagocytic cell respiratory burst activity may be advantageous to the survival of the parasite. However, *L. branchialis* is a very invasive parasite and therefore, the infection, as the parasite migrates, metamorphoses and feeds, is likely to be stressful for the host. The fact that the plasma cortisol levels of cod in this study were not determined therefore means that immunosuppression caused by a chronic stress response cannot be ruled out. Therefore, the plasma cortisol levels of fish over the time course of the infection in comparison to the sham control cod need to be determined, in order to be able to conclude whether these results are due to a

chronic stress response to infection or as a direct response to parasite-derived factors.

The infection protocol of Ardtoe-cultured cod also seemed to result in an increase in the percent of blood phagocytes undergoing respiratory burst 3 d.p.i. however, this was not significant. The mean intracellular production of hydrogen peroxide (H_2O_2) by the phagocytic cells also increased 3 d.p.i. and 28 d.p.i. due to the infection protocol. The infection of Ardtoe cod with *L. branchialis* however, did not affect the proportion of phagocytes undergoing respiratory burst, or the mean intracellular H_2O_2 production of phagocytic cells. The lack of suppression of the phagocyte activity during the infection of Ardtoe cod 14 d.p.i., in contrast to Machrihanish cod, could result from the death of the parasite as they may not have been healthy enough to modulate the immune response of the host at this time and / or the Ardtoe cod may have been more 'resistant' to the suppressive effects of the parasite infection on phagocyte functions. Fast *et al*. (2002a) found that only the macrophage activity of susceptible salmonid hosts was suppressed during infection by *L. salmonis*, whereas the resistant species, coho salmon, rejected *L. salmonis* by 14 d.p.i. without suppression of macrophage activity. Fast *et al.* (2003) also observed coho salmon mucus to not induce *L. salmonis* to secrete products to the same degree as that of the susceptible salmonids, such as Atlantic salmon. They suggested that *L. salmonis*-resistant species were able to block the parasites' secretions and / or digestive enzymes enabling them to elicit an effective innate immune response against *L. salmonis*. This corresponds to a study by Johnson & Albright (1992) who found coho salmon to initiate an acute inflammatory response 1 d.p.i. and epithelial hyperplasia 10 d.p.i.. Therefore, the increase in circulating

monocytes combined with no suppression of phagocyte function in Ardtoe cod may have contributed to the unsuccessful infection by *L. branchialis* in these fish. However, whether the suppression of phagocyte respiratory burst activity in the preliminary infection study would have led to the successful infection of *L. branchialis* to 'full term' in Machrihanish cod cannot be concluded.

3.4.2.4 Serum protein and innate immune parameter levels of *L. branchialis*-infected Atlantic cod

The total protein concentration of Machrihanish-cultured cod serum was slightly elevated in infected cod 1 d.p.i.. However, this was found not to be significantly higher than the protein levels of the sham control cod. The infection of Ardtoe-cultured cod had no effect on the total protein concentration of serum. This is in accordance with Caipang *et al.* (2008) who found Atlantic cod serum protein levels not to change after vaccination with *V. anguillarum*, and Fast *et al.* (2002a) who found that salmonids infected by *L. salmonis* did not display any changes in plasma protein levels.

The infection protocol of Machrihanish-cultured cod led to an increasing trend in serum anti-trypsin activity, which peaked 14 d.p.i.. The infection by *L. branchialis* however, led to an earlier increase in anti-trypsin activity 1 d.p.i., which remained elevated above basal 'resting' levels 3 d.p.i.. However this was 'masked' by the increase in this parameter resulting from the stress of the infection protocol and not significantly different to that of sham control cod. The infection of Ardtoe-cultured cod however, led to a significant increase in serum anti-trypsin activity 3 d.p.i. above that of sham control cod. This increase in Ardtoe cod may have resulted from the host attempting to counteract parasite-derived proteases involved in the

migration and / or development of the parasite in order to eliminate the infection, as it corresponds with the entry of *L. branchialis* into the afferent branchial artery of the fish. For instance, Araujo-Jorge *et al*. (1992) found α2M–treated trypomastigotes to possess an impaired ability to invade macrophages and fibroblasts. However, in order to confirm that an increase in serum anti-trypsin activity of cod occurs post-stimulation with exogenous trypsin, the anti-trypsin activity of cod injected with trypsin is required. A similar increase was observed in Machrihanish cod however, this was not significantly different from the sham controls and therefore, is likely to have resulted from the stress of the infection protocol. The total anti-trypsin activity of the Machrihanish cod serum pre- and post- infection was however, within a similar range to those of Ardtoe cod. Therefore, the significance of the increase in anti-trypsin activity of cod serum in terms of *L. branchialis* resistance can not be concluded, as well as the fact that the preliminary infection only ran for 14 d.p.i.. The components of the anti-trypsin activity of the serum were not determined in the current study. This may be an important aspect to further study, as previous studies have shown the importance of α2M at host-parasite interfaces. Sitja-Bobadilla *et al.* (2006) stated that the α2M serum levels of turbot reflected the effects of *E. scophthalmi* infection better than total antiprotease activity levels. Other studies have also found parasitic infection to result in an increase in host α2M expression, such as infection by the parasitic copepod *Sinergasilus major* in *Ctenopharyngodon idella* (grass carp) and *Trypanoplasma borreli* in carp (Chang *et al*., 2005; Saeij *et al*., 2003).

The serum total IgM concentration of both Machrihanish and Ardtoe -cultured cod did not change due to infection with *L. branchialis*. This is in accordance with

Israelsson *et al*. (1991) who found *L. branchialis* infection in wild cod to not affect serum IgM levels. Other studies of infection in Atlantic cod with bacteria such as *Aeromonas salmonicida* have also not found any correlation between serum total IgM levels and infection (Magnadottir *et al.*, 2002). Magnadottir *et al.* (2001) also found immunisation of cod to not result in any changes in serum total IgM levels.

L. branchialis infection of cod in both of the current studies did not result in any changes in the spontaneous haemolytic activity of cod serum. The level of haemolytic activity in both cod populations however, were very low in comparison to other studies (Magnadottir *et al.*, 2001; Magnadottir *et al.*, 2002), and extremely high variation between individual fish was observed. Some of the cod serum sampled in this study possessed no haemolytic activity, which has been found since 2000 by Magnadottir (2000) in Icelandic-cultured cod. The reasons for this lower activity have not been fully elucidated, however, Magnadottir & Lange (2004) described a strong hydrophobic association between apolipoprotein A-I (ApoL A-I) and C3 in cod serum with no haemolytic activity, and a reduced presence of this association in sera with very high haemolytic activity. ApoL A-I have been shown to have a regulatory role on the complement system, inhibiting the molecular attack complex (MAC; C5b – C9; Jenne *et al*., 1991; Hamilton *et al*., 1993; Choi-Miura *et al*., 1993). ApoL A-I has been found to form a high density lipoprotein complex with clusterin in human plasma which inhibits the terminal lytic complement cascade by interfering with the molecular attack complex formation (Jenne *et al*., 1991). Another reason for the low haemolytic activity observed could be the ‘rearing’ temperature of the cod at 13°C. Magnadottir *et al*. (1999a) found the spontaneous haemolytic activity of cod serum to show a declining trend with increasing temperatures to

around 14°C, although due to the high individual variation this was not significant. The spontaneous haemolytic activity of sera is usually ascribed to the alternative complement pathway (Sakai, 1983; 1992; Sunyer *et al*., 1997; Nakao & Yano, 1998). However, the haemolytic activity of cod serum is unusual in that its optimal activity is at 37°C, it is relatively heat stable, and it is enhanced by EDTA, which normally inhibits complement haemolysis (Magnadottir, 2000), but it does show partial inhibition by zymosan and LPS (Magnadottir, 2000). Magnadottir (2006) later stated that sera from cultured Icelandic cod collected from around 2000 onwards was inhibited by EDTA and was heat sensitive. These unusual features have led many authors to state that the spontaneous haemolytic activity of cod serum cannot be attributed solely to the alternative complement pathway, but that other haemolysins are acting in conjunction with or separately to complement activity (Magnadottir, 2000). This has also made it hard to elucidate the true nature of the lytic pathway in cod (Magnadottir, 2006). Therefore, it is difficult to conclude anything about the effects of *L. branchialis* infection on the haemolytic activity of cod serum as the activities observed in these studies were very low or absent prior to infection. This made it difficult to detect any ‘consumption’ or suppression of host haemolysin activity by the parasite. The complement system, a known haemolysin, however, has proven an important factor in the host immune response to parasitic infections, capable of killing parasites *in vitro* (Harris *et al.*, 1998; Buchmann, 1998; Rubio-Godoy *et al.*, 2004), and thought to contribute to family resistance to certain parasites in some host species (Forward & Woo, 1996).

The two different stocks of cultured-cod used in this study showed differences in their immune response to infection by *L. branchialis,* and the stress induced by the

infection protocol. The infection of Machrihanish-cultured cod ran for only 14 d.p.i. and the parasite reached the early penella sub-stage within the ventral aorta. The infection of Ardtoe-cultured cod resolved by 28 d.p.i., with the death of the parasite. However, whether the parasites would have died or completed metamorphosis in the preliminary infection of Machrihanish-cultured cod cannot be determined. The latter cod however, appeared in poorer condition with dark skin pigmentation in comparison to the other stock of cod used, and the basal 'resting' levels of some of the immune factors measured appeared lower. This study does not however, allow the direct comparison of these results as the infections were carried out at different times and over different time periods, so no conclusion as to the susceptibility of the two batches of cod used can be drawn. The batch of Machrihanish-cultured cod used in this study were also in poor condition and not 'representative' of Machrihanish-cultured cod as some deaths occurred in these stock cod prior to the experiment; however, after consulting with a veterinarian no reason for these deaths could be found.

3.4.2.5 Conclusion

In conclusion, the systemic immune response observed in both wild haddock and cultured-cod depended on the maturation stage of the *L. branchialis* infection, and in the former the infection intensity. Infection in wild haddock was associated with a slight elevation of circulating granulocytes, which appeared to be more pronounced when immature post-penella parasites were present. Infection by fully metamorphosed *L. branchialis* was associated with an increase in haddock circulating thrombocytes and a decrease in serum protein levels. However, the infection of haddock with multiple mature *L. branchialis* was associated with a reduction in circulating monocytes, and an increase in circulating thrombocytes and

serum anti-trypsin activity. These responses may be due to the increased damage caused by the massive increase in size and the feeding of the mature parasite. Infection by multiple parasites was associated with a more pronounced effect on some of the immune parameters measured, which could be due to the increased damage caused by multiple parasites and / or an increase in the quantity of parasite derived secretions at the site of infection resulting in a systemic effect. Infection by *L. branchialis* was also associated with a suppressive effect on some immune parameters, such as the spontaneous haemolytic activity of wild haddock serum. However, the field nature of the sampling and the use of observations from wild haddock made it difficult to draw definitive conclusions from these results, due to the likely presence of infections by other pathogens which were not accounted for in this wild, preliminary study. Therefore, it cannot be conclusively stated that the systemic immune responses observed in the *L. branchialis*-infected haddock were resultant solely from the effect(s) of *L. branchialis*-infection. This led to the study of the immune response of naïve cultured-cod experimentally infected by *L. branchialis* in a controlled environment in comparison to sham control cod, in order to elucidate the immune response of cod to *L. branchialis*. However, the immune response of cod as *L. branchialis* metamorphosed fully could not be examined due to the fact that the infection of Ardtoe-cultured cod subsided with the death of the parasites by 28 d.p.i.

The preliminary infection of cultured-cod was also associated with a suppression of the proportion of circulating phagocytes undergoing respiratory burst and the mean H_2O_2 production of those phagocytic cells when the parasite developed into an early penella sub-stage in the ventral aorta. The unsuccessful infection of Ardtoe-cultured

cod, however, did not lead to any significant changes in circulating phagocyte respiratory burst activity. This could highlight the importance of the circulating phagocytic leukocytes and their ROS production in the host's immune response to infection. The serum anti-trypsin activity of cod in the longer unsuccessful infection study was also elevated as the parasite entered the afferent branchial artery, and not in the preliminary infection. The importance of this parameter in the immune response to *L. branchialis* is debatable though, as the activity in the Machrihanish-cultured cod was still within the levels of the Ardtoe-cultured cod. The individual components of the total anti-trypsin activity need to be assessed during the infection, such as α2M levels. However, the immune response to infection needs to be determined over the entire metamorphosis of *L. branchialis* to determine whether the infection was successful or not, and preferably in populations with varying susceptibility to *L. branchialis*. This will not be possible without further studies into the resistance of different stocks of cultured-cod, if any differences exist. Lysne & Skorping (2002) however, found the existence of small groups of inherently susceptible cod which were infected and re-infected if the parasite was lost, and groups of resistant hosts with a lower chance of infection. In hindsight, a positive control of cultured-cod immunised with a known immuno-stimulant, such as Freund's complete adjuvant or zymosan, would have been useful due to the low response of many parameters to the infection, and the low infection intensity observed. The immune response at the 'local level' *i.e.* the site of infection, also needs to be further studied to better characterise the host-parasite interface, and to determine whether *L. branchialis* modulates the local immune response to increase the success of the infection. Whether this suppression in the immune response of infected gadoids is due to the host attempting to eliminate the infection and unable

to recuperate pre-infection levels, immuno-suppressive parasite secretions and / or a stress response to the infection also needs to be further investigated.

Chapter 4 Local immune response of *Gadus morhua* to infection by *Lernaeocera branchialis*

4.1 Introduction

During infection and / or following injury, inflammation occurs as part of the primary innate immune response to eliminate infection. Cytokines and the cells that produce them are key mediators of this process. Cytokines are small proteins or glycoproteins which act as signals mediating the immune response (Thomson, 1994; Callard & Gearing, 1994, cited in Secombes *et al.*, 1996). The production of these inflammatory mediators at the site of injury / infection occurs early, and is key to the initiation of the inflammatory response, which occurs following binding of cytokines to specific receptors on cell membranes. This specific binding instigates a cascade that results in an increase or decrease in the production of cytokine-regulated proteins. Even though studies of the immune response elicited during parasitic infections in teleosts are hindered by a lack of antibody markers, an expanding number of gene sequences for immune-relevant proteins, such as cytokines, have been identified in teleosts to date. These include interleukin-1beta (IL1β), tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), transforming growth factor beta (TGFβ), IL10, IL8, IL22 and IL26 (Fujiki *et al.*, 2000; Scapigliati *et al*., 2001; Laing *et al.*, 2001; Zou *et al.*, 2003; Garcia-Castillo *et al.*, 2002; Milev-Milanovic *et al.*, 2006; Furnes *et al*., 2009; Seppola *et al*., 2008; Corripio-Miyar *et al.,* 2009; Igawa *et al*., 2006). This has enabled an increasing number of studies examining the teleost host's immune response to parasitic infections to be carried out (Lindenstrom *et al*., 2003, 2004; Sigh *et al*., 2004a & b; Saeij *et al*., 2003; Bridle *et al*., 2006a & b). These studies however, measure the immune response at the 'molecular transcript level' and not at the 'post-translation level' through measurement of functional proteins. However, with the tools available these studies

provide valuable information concerning the processes involved in the local and systemic immune response to parasitic infections.

The importance of the local immune response at the site of parasitic infection has been highlighted in numerous studies (Lindenstrom *et al*., 2003; Sigh *et al*., 2004a & b; Bridle *et al*, 2006a & b; Covello *et al*., 2009). The successful elimination of the parasite by the host is thought to be largely mediated by the local cytokine response elicited at the site of infection. The pro-inflammatory cytokine IL1β is part of the IL1 family and was the first cytokine characterised in teleosts. It is also the most extensively studied cytokine in parasitic infections of teleost hosts to date (Buchmann & Bresciani, 1998; Lindenstrom *et al*., 2003; Sigh *et al*., 2004a; Engelsma *et al*., 2003; Bridle *et al*., 2006a and b; Fast *et al*., 2006; Morrison *et al*., 2007; Gonzalez *et al*., 2007a). It is thought to play a pivotal role in the instigation of an immune response and inflammation post-injury and / or infection (Dinarello, 1997). For instance, it primes leukocytes for respiratory burst activity and phagocytosis after exposure to bacteria (Secombes *et al.*, 2001; Peddie *et al.*, 2002). This function is supported by observations of up-regulation of its gene expression in teleost hosts during bacterial, viral and parasitic infections (Grayson *et al*., 2002; Engelsma *et al*., 2003; Lindenstrom *et al*., 2003; Pressley *et al*., 2005; Purcell *et al*., 2004; Saeij *et al*., 2003; Sigh *et al*., 2004a; Tafalla *et al*., 2005; Zou *et al*., 1999; Bridle *et al*., 2006a & b). This cytokine has also been reported to have a prominent role in the initiation of the inflammatory response to ectoparasites in fish (Gonzalez *et al*., 2007a), and Buchmann (1999) and Lindenstrom *et al*. (2003) highlighted its importance in the initiation of host anti-gyrodactylid immune responses at the local infection site. IL1β mediates the majority of its effects by up-

or down- regulating the expression of other cytokines and chemokines (Dinarello, 1997). Chemokines are a subfamily of cytokines whose function is to recruit leukocytes to the site of injury / infection (Fernandez & Lolis, 2002). A pro-inflammatory chemokine, sequenced in teleosts including Atlantic cod, and thought to be up-regulated by IL1β, is IL8, also known as CXCL8 (Seppola *et al*., 2008). IL8 is a member of the CXC chemokine family and is the most widely studied chemokine in teleosts. It has been reported to be pro-inflammatory, inducing neutrophil migration, activation and degranulation in mammals (Peveri *et al*., 1988; Schroder *et al.*, 1987; Thelen *et al*., 1988; Scapini *et al*., 2000). The function of IL8 in teleosts has not been extensively studied yet, and the IL8 gene of most fish species studied to date lacks the ELR-motif found in mammals (Lee *et al.*, 2001; Laing *et al.*, 2002; Chen *et al*., 2005). This motif is vital for the angiogenesis function of IL8 in mammals (Rosenkilde & Schwartz, 2004; Strieter *et al*., 1995; Belperio *et al.*, 2000), and this has therefore led authors to hypothesise that fish IL8 posses fewer functions than their mammalian counterparts. However, the gene sequence for IL8 of the gadoids *Melanogrammus aeglefinus* (haddock) and *Gadus morhua* (Atlantic cod) do posses the ELR-motif as in mammals (Corripio-Miyar *et al*., 2007; Seppola *et al*., 2008). IL8 has been found to be up-regulated in teleosts post-infection with parasites (Sigh *et al.,* 2004a; Jones *et al.*, 2007) and bacteria (Mulder *et al*., 2007), and after vaccination with bacterial antigens (Fast *et al*., 2007; Seppola *et al*., 2008; Caipang *et al.*, 2008). In response to its pro-inflammatory function, some haematophagous parasites, such as ticks, have established mechanisms to prevent its activity, hypothesised to suppress neutrophil migration and activation at the site of feeding (Kocakova *et al*., 2003).

Anti-inflammatory cytokines with an immunosuppressive effect, such as IL10, also exist in order to regulate the inflammatory response (Dinarello, 1997). In mammals, IL10 is vital for the regulation of the inflammatory response, which it achieves by down-regulating other cytokines such as IL1β and IL8 (de Waal Malefyt *et al*., 1991). The cytokine IL10 also down-regulates neutrophil reactive oxygen species (ROS) production by inhibiting $p47^{PHOX}$ phosphorylation (Dang *et al*., 2006). Even though an anti-inflammatory function of IL10 has not been confirmed yet in teleosts, recent studies have suggested IL10 to posses a suppressive effect on pro-inflammatory cytokines in teleosts, such as Atlantic cod, due to the fact that it displays the opposite gene expression profile to IL1β following polyinosinic: polycytidylic acid (poly IC) injection of Atlantic cod (Seppola *et al*., 2008).

Parasite establishment, however, may be enabled by parasite-modulation of the local host response. This may be achieved by manipulating the mediators of the immune response, such as cytokines, by 1) inhibiting or initiating the production of particular immune proteins, 2) preventing their functionality by occupying their corresponding cell receptors or binding to them directly, and / or 3) degradation post-production by mechanisms such as proteolysis. For instance, the salivary gland extracts (SGE) of ixodid ticks have been found to suppress the expression of host pro-inflammatory cytokines IL1, TNFα and IFNγ mRNA (Fuchsberger *et al*., 1995), up-regulate the production of the anti-inflammatory cytokine IL10 (Kopecky *et al*., 1999; Ferreira & Silva, 1998), and bind to host cytokines preventing them from binding to their specific receptors (Gillespie *et al*., 2001; Kocakova *et al*., 2003). Protozoan parasites and bacteria have also been observed to produce proteases, which degrade complement proteins (Reed *et al*., 1995; Oda *et al*.,

1990). However, it will only be possible to study the latter two methods of parasite manipulation of host immune mediator function once species-specific recombinant proteins and antibody markers are available for them. Nevertheless, the capacity of a parasite to suppress or initiate the production of immune factors by the host can be partially determined by molecular techniques allowing the quantification of their gene expression post-infection and / or by *in situ* hybridisation to detect localised modulation of their gene expression. The utility of such techniques depends on the satisfaction of the assumption that the mRNA gene expression for a protein correlates to its post-translation processing into a functional protein.

The infective stage of *L. branchialis* initially attaches to the gill filament of Atlantic cod, this being followed by its entry into the afferent branchial artery (Chapter 3; Smith *et al*., 2007). The innate immune response of teleost gills is thought to be of great importance due to their broad exposure to the external environment and to the fact that numerous pathogens, including bacteria, parasites and viruses, are thought to enter their hosts via the gills (Morris *et al*., 2000; Rimstad *et al*., 1999; Lee *et al*., 1999; Ramsay *et al*., 2002; Smith *et al*., 2007). The gills along with the other mucosa-associated lymphoid organs, such as skin and gut, provide the initial barrier to pathogen entry and subsequent establishment in the host, and the ability of the host to initiate a local immune response is crucial in preventing pathogen infection. This is achieved both through the physical barrier provided by the mucus layer and by an assortment of innate immune response elements, such as IgM which is the primary immunoglobulin of teleosts. IgM is a major component of the humoral immune response to bacteria, parasites and viruses (Ellis, 1986). An increase in IgM gene expression has been reported at the local sites of parasitic

infection, suggesting antibody production, such as in the skin of rainbow trout after infection with *Ichthyophthirius multifiliis* (a protozoan parasite of skin and gills; Sigh *et al.*, 2004b). Parasitic infections in teleosts have also been associated with increasing numbers of antibody secreting cells (ASC) at the site of infection (Bermudez *et al.*, 2006; Zhao *et al*., 2008). For instance, channel catfish skin showed a 20-fold increase in ASC post-immunisation against *I. multifiliis* (Zhao *et al*., 2008). The authors suggested that the number of ASC in the target organ is "dynamic and increases in response to parasitic infection" (p500, Zhao *et al*., 2008). B cells are therefore thought to play a role in surveillance at sites vulnerable to pathogen entry, such as the gills and skin (Zhao *et al.*, 2008). Recent studies on rainbow trout have also found B cells to have a phagocytic function (Li *et al.*, 2006). Li *et al.* (2007) found phagocytosis by B cells to be enhanced when particles were opsonised by complement, and LPS-stimulated B cells to increase their expression of anaphylatoxin (C3a and C5a) receptors. They went on to suggest that the B cells of teleosts play a role in the development of an inflammatory response. However, whether B cells of Atlantic cod are phagocytic remains to be determined.

The complement system is also a key part of the innate and adaptive immune response, and has been found to play a crucial role in responses to parasitic infections in teleosts (Wehnert & Woo, 1980; Forward & Woo, 1996; Buchmann, 1998; Rubio-Godoy *et al.*, 2004). The functions of complement include the production of inflammatory anaphylatoxins, opsonisation of 'non-self' surfaces leading to enhanced phagocytosis and antigen processing, solubilisation of immune complexes, and cell lysis by formation of the membrane attack complex (Gasque, 2004; Carroll, 2004; Dempsey *et al.*, 1996; Boshra *et al*., 2006). It is composed of

numerous soluble proteins and receptors produced mainly by the liver (Dalmo *et al*., 1997). Three pathways of activation have been described in fish (the alternative, classical and mannose lectin-binding) which all converge with the central C3 protein (Boshra *et al*., 2006; Sunyer *et al*., 2003). Both IgM and C3 have been stated to be opsonic factors binding to foreign molecules resulting in the adherence of host leukocytes to parasites (Hoole & Arme, 1986; 1988). This adherence of leukocytes, such as granulocytes and macrophages/monocytes to parasites can result in parasite death. For instance, Nakayasa *et al.* (2005) observed the infection site of the haematophagous monogenean parasite *Neoheterobothrium hirame* in the Japanese flounder (*Parcalichthys olivaceus*) to be infiltrated by granulocytes and monocytes, which adhered to the parasite tegument resulting in its collapse, phagocytosis, and ultimately parasite death. Host leukocytes are thought to adhere to parasites via Fc and C3b receptors, as occurs on the schistosomula of the trematode *Schistosoma mansoni* (Butterworth, 1984). To date the majority of studies relating to parasitic infections in teleosts, and the role of host complement involve the measurement of serum complement activity (Cuesta *et al.*, 2006b; Roberts *et al*., 2005), C3 gene expression post-infection (Sigh *et al*., 2004b; Chang *et al.*, 2005; Gonzalez *et al*., 2007b), or the complement-mediated killing of parasites by fish serum/plasma *in vitro* (Wehnert & Woo, 1980; Buchmann, 1998; Rubio-Godoy *et al*., 2004; Leiro *et al.*, 2008). There are however, few studies determining the binding of C3 to the parasite *in vivo* at the site of infection. Buchmann (1998) incubated *Gyrodactylus derjavini* (a monogenean parasite of salmonids) *in vitro* with rainbow trout plasma and observed the binding of host C3 to carbohydrate-rich epitopes of the parasite resulting in death by host complement activity. *L. branchialis* is intimately exposed to immuno-reactive molecules in the

host's blood during the infection process as it migrates through the blood vessels, and its haematophagous lifestyle also means that its gut is exposed to a wide array of host-derived immuno-reactive molecules taken in with the blood meal. These include factors involved in non-host recognition, such as C3 and immunoglobulin binding, which will lead to the activation of the host's immune response, reduced feeding, damage to the parasite's gut and possibly parasite death without further intervention by the parasite.

The aims of the following study were to investigate the local immune response of naïve hatchery-reared *Gadus morhua*, in terms of immune factors involved in pathogen detection and mediation of the immune response, to laboratory infection with *L. branchialis*. Due to the localised histo-pathological responses reported in the literature and the limited systemic immune response to the initial stages of the infection (see Chapter 3), it was thought important to look at the immune response at the local host-parasite interface. This was achieved by immunohistochemistry (IHC) to detect host IgM and C3 proteins, and *in situ* hybridisation (ISH) to detect the gene expression of the host cytokines IL1β, IL8 and IL10, and the localisation of host plasma and B cells at the host-parasite interface over a time series infection. The possible modulation of the local host immune response by products actively produced by *L. branchialis* to allow the initial infection was also investigated by examining the gene expression of cytokines, binding of IgM and C3 proteins, and the presence of plasma and B cells at the host-parasite interface with recently dead and live parasites. The work was carried out using a working hypothesis that live parasites may produce products at the host-parasite interface to suppress a strong immune response and hence parasite elimination; whereas, recently dead

parasites, producing no immunomodulatory factors, are exposed to the host immune response without the production of these products.

4.2 Materials and methods

4.2.1 Localised immune response to *Lernaeocera branchialis* over time

4.2.1.1 Laboratory infection of naïve *Gadus morhua*

Laboratory infections of naïve Atlantic cod with *L. branchialis* were performed as stated in Chapter 2 (Section 2.3), and paraffin wax embedded samples of *L. branchialis*-infected Atlantic cod arising from the two experiments outlined in Chapter 3 were employed to follow the localised immune response of the host over time. Briefly, an infection involving 81 naïve Atlantic cod from Machrihanish Marine farm Ltd. hatchery (Machrihanish, Scotland) was undertaken. Uninfected control fish were sampled at 0 days post infection (d.p.i.; n=9), half the remaining fish were infected and samples taken at 1, 3, 7 and 14 d.p.i. (n=9 infected & 9 control fish in duplicate tanks at each time point). A second infection was carried out in order to follow the infection as the female *L. branchialis* metamorphosed. This involved the infection of naïve Atlantic cod from Viking Fish Farms Ltd. (Ardtoe, Scotland). However, the infection resolved with the death of the parasite by the end of the experiment. Samples were processed from all the time points of the former infection to follow the local immune response over a time-series post-infection. However, due to time and material constraints only the samples taken at 56 d.p.i. (*i.e*. post-parasite death) were processed from the latter infection for the investigation of the local immune response as the parasite died.

4.2.1.2 Fixation and wax processing of *L. branchialis in situ*

The gills and *bulbus arteriosus* of Atlantic cod were fixed in 10% (v/v) neutral buffered formalin (as in Chapter 2, Section 2.11.1.1) for examination with a stereomicroscope back at the laboratory in order to count parasites and identify the parasite stage and the site of infection. Sites of infection and uninfected controls were routinely processed for wax embedding. Five µm thick paraffin wax sections from 5 fish per time point and treatment in duplicate tanks were cut and dried onto poly-L-lysine (Sigma) coated slides or RNAse-free 3-aminopropyltriethoxysilane (APES) coated slides at 60°C for 1 h, for IHC and ISH, respectively.

4.2.1.3 Detection of IgM and C3 by immunohistochemistry

Sections on poly-L-lysine coated slides were de-waxed with two rinses of 100% xylene for 5 min each, and then rehydrated through an ethanol series into distilled water. The sections were pre-treated with 3% (v/v) hydrogen peroxide in methanol for 10 min to quench endogenous peroxidise activity. The sections were washed 3 times in Tris buffered saline (TBS; 50mM Tris, 150mM NaCl, pH 7.6) prior to blocking for 30 min with 10% (v/v) goat serum in TBS. The sections were then incubated with 1:250 mouse anti-cod C3 (a monospecific polyclonal antibody which was a gift from Dr. Bergljot Magnadóttir) in TBS overnight at 4°C or 20µg.ml^{-1} mouse anti-cod IgM light chain (supernatant 29, ADL, Stirling, U.K.) for 1 h, washed 3 times in TBS and incubated with 1:100 anti-mouse biotin (Sigma) in TBS for 30 min at room temperature. The sections were washed as previously described with a last rinse in phosphate buffered saline (PBS) and incubated with 1:200 streptavidin-HRP (Vector Labs, Peterborough, England) in PBS for 30 min. Following 3 washes in PBS, sections were incubated with VIP substrate kit (Vector Labs) or the AEC substrate (Sigma), respectively according to the manufacturers' instructions. The

sections were then counterstained with methyl green (Vector Labs) for 5 min at 60°C, rinsed in distilled water and permanently mounted with VectaMount (Vector Labs), or counterstained with haematoxylin for 3 min, rinsed in water and mounted with Citifluor (Agar Scientific Ltd, Essex, England), respectively.

4.2.1.4 Detection of IL1β, IL8, secretory IgM and transmembrane IgM mRNA by *in situ* hybridisation

RNAse treatment of glassware, plasticware and buffers

All glassware and glass slides were oven baked in foil for 3 h at 180°C, and non-sterile plasticware was submerged in 0.1M sodium hydroxide in nanopure water overnight and rinsed in nanopure water. All buffers were treated with diethyl pyrocarbonate (Sigma) following manufacturers' instructions, except those with amines, such as Tris, and other compounds which cannot be autoclaved, such as detergents or proteins. Buffers containing the latter were made up using RNAse-free reagents, DEPC-treated water and in baked bottles and lids.

cRNA probe production

The cRNA probes to mRNA transcripts of *Gadus morhua* secretory IgM heavy chain, transmembrane IgM heavy chain, interleukin 1β, and interleukin 8 were produced using a non-cloning synthesis method using a DIG RNA labelling kit (Roche). This method involved the application of an RT-PCR product with a T7 RNA promoter sequence (Table 4.1) added to the 5' end of the antisense strand as a template to produce an antisense cRNA probe by *in vitro* transcription. The RNA promoter sequence is added onto the 5' end of the antisense primer so that during the PCR it is incorporated into the PCR product. During *in vitro* transcription digoxigenin (DIG) labelled-UTP is integrated into the cRNA probe to allow detection

with anti-DIG antibodies post-hybridisation with the target tissue. Sense cRNA probes labelled with DIG were also made by incorporating an SP6 RNA promoter sequence (Table 4.1) on the 5' end of the sense primer, in order to act as negative controls for non-specific binding.

RNA extraction

Head kidney, spleen and gill tissues for RNA extraction were collected from six hatchery-reared Atlantic cod weighing approximately 50g (Viking Fish Farm, Ardtoe) 2d post-laboratory-infection with *L. branchialis* arising from the experiment described in Chapter 6. Tissues were collected into 1ml RNA Later (Sigma) and stored at 4°C for 48 h in order to allow tissue penetration prior to RNA Later removal and long term storage at -70°C.

Total RNA was extracted from Atlantic cod head kidney, spleen and gill tissue using Tri-reagent (Sigma) following manufacturers' instructions. Briefly, 1ml Tri-reagent was added to 50-100mg of tissue and homogenised using a manual tissue homogeniser and sterile plastic pestles. The sample was incubated for 5 min at room temperature, and 200µl chloroform (Sigma) added for 15 min prior to centrifugation at 12,000 xg for 15 min at 4°C. The aqueous layer containing RNA was transferred to a fresh RNase-free microfuge tube (Eppendorf, Hamburg, Germany) and 1:10 (v/v) isopropanol (molecular grade; Sigma) added for 5 min at room temperature prior to centrifugation at 12,000 xg for 10 min at 4°C. The supernatant was transferred to a fresh microfuge tube and 500µl isopropanol added prior to incubation for 10 min at room temperature to precipitate RNA prior to centrifugation at 12000 xg for 10 min at 4°C. The RNA pellet was washed by

vortexing in 1ml ice-cold 75% (v/v) ethanol (molecular grade; Sigma) followed by centrifugation at 7500 xg for 5 min at 4°C. The pellet was air-dried for 10 min and re-suspended in a minimal volume of DEPC-treated water. RNA concentration and quality were analysed on a ND-100 nanodrop spectrophotometer (Labtech Inc., Arizona, USA), prior to storage at -70°C for later use.

RT-PCR production of PCR template for cRNA probe production

Reverse-transcription (RT) of total RNA samples to cDNA was performed using Reverse-iT^{TM} MAX 1st Strand Synthesis Kit (Abgene) following manufacturers' instructions. Briefly, 1µg total RNA from head kidney, spleen and gill were each combined with 4µl 5x 1st strand synthesis buffer, 2µl dNTP mix (5mM each), 1µl 500ng/µl anchored oligo-dT, 1µl QRTase enhancer, 1µl ABsoluteTM MAX QRTase Blend (50U/µl), and made up to 20µl with DEPC-treated water in PCR tubes (Thermo tubes, Thermo Scientific). Prior to the RT reaction the RNA template was heated at 70°C for 5 min in order to remove any secondary structures, and then placed immediately on ice. The RT reaction was performed on a Biometra^{R} thermocycler by incubating at 42°C for 1 h, followed by 10 min at 75°C in order to inactivate the DNA polymerase. The cDNA samples were placed immediately on ice for PCR or frozen at -20°C for later analysis.

PCR was performed using a 2x PCR master mix (ABgene) following manufacturers' instructions. Oligonucleotide primers (Eurofins MWG Operon) for *Gadus morhua* secretory IgM constant heavy chain domain 3 and 4, transmembrane IgM *i.e.* constant heavy chain domain 3 to transmembrane region, interleukin 1β, interleukin 8 and interleukin 10 (see Table 4.1) were designed using Primer Select Lasergene version 6.0 (DNAStar Inc) and Primer3 version 0.4.0. Software. The specificity of

the oligonucleotide primers was verified with the BLASTN program (National Center for Biotechnology Information). Briefly, 75 -125ng cDNA template from spleen, head kidney and gill was mixed with 0.5μM sense and 0.5μM antisense primers (Table 1), 1x PCR mix and made up to 25μl total volume with DEPC-treated water. The PCR was performed on a Biometra^{R} thermocycler with an initial denaturation of 94°C for 5 min, followed by 36 cycles of 94°C for 20 seconds, annealing temperature (see Table 4.1) for 30 seconds and 72°C for 1 min, followed by a single final elongation cycle step of 72°C for 5 min.

PCR products were resolved on a 1.2% agarose gel with 83ng.ml^{-1} ethidium bromide (Sigma) run at 250V for 15 min in 1x sodium hydroxide-boric acid buffer. Seven μl PCR product was mixed with 1μl 10x reddyrun loading dye (Abgene) and loaded onto the agarose gel. DNA molecular weights were calibrated using DNA Molecular Weight Marker IX (72-1353bp; Roche, Basel, Switzerland). Gel imaging was performed under ultraviolet illumination.

Sequencing of RT-PCR products prior to cRNA probe production

The PCR cDNA products were sequenced in order to confirm that the proper target had been amplified. Briefly, cDNA PCR products were purified into nanopure water using QIAquick PCR purification kit (Qiagen Ltd, West Sussex, UK) following manufacturers' instructions and sequenced on a CEQ sequencer (Beckman Coulter, High Wycombe, UK). Products were sequenced using CEQ dye terminator cycle sequencing with a GenomeLab™ Quick Start kit (Beckman Coulter) following manufacturers' instructions. Post CEQ analysis, the sequences were analysed with Lasergene version 8.0 Seqman software (DNA Star, WI, USA), and the sequences queried using BLASTN (NCBI).

In vitro transcription of cRNA probe

cRNA probes were produced using the DIG RNA labelling kit (SP6/T7) (Roche), following manufacturers' instructions. Briefly, PCR products were purified using QIAquick PCR purification kit (Qiagen) following manufacturers' instructions. Purified PCR products were quantified on an ND-100 nanodrop spectrophotometer (Labtech) and 200ng product made up to 13µl with DEPC-treated water. This was placed on ice and 2µl 10x NTP labelling mix, 2µl 10x transcription buffer, 1µl RNAse inhibitor and 2µl T7 RNA polymerase (for antisense cRNA probe) or 2µl SP6 RNA polymerase (for sense cRNA probe) added to the DNA template. This was mixed and incubated at 37°C for 2 h in a Biometra^{R} thermocycler. Two µl DNAse (RNAse-free) was added and incubated at 37°C for 15 min to remove the DNA template. The reaction was stopped by the addition of 2µl 0.2M EDTA, pH8.0. The cRNA probes were then precipitated to remove unincorporated nucleotides from the reaction, following manufacturers' instructions. Briefly, 2.5µl 4M Lithium chloride and 75µl ice-cold molecular grade ethanol (Sigma) were added to the reaction mix and left to precipitate overnight at -20°C. The precipitate was pelleted at 13,000 xg at 4°C for 15 min, washed in 50µl ice-cold 75% ethanol, centrifuged at 13,000 xg for 10 min at 4°C, and allowed to dry. The labelled cRNA probes were then dissolved in 75µl DEPC-treated water at 37°C for up to 20 min, quantified on a ND-100 nanodrop spectrophotometer (Labtech), and aliquoted into RNAse-free tubes and stored at -70°C. Probes complementary to interleukin 10 mRNA could not be produced, presumably due to the lack of interleukin 10 expression in the tissues used to prepare the RT-PCR products.

cRNA probe DIG labelling efficiency

DIG- labelled cRNA probes and DIG- labelled actin control RNA (Roche) were sequentially diluted in RNA dilution buffer and 1µl spotted onto a positively charged Hybond N+ nylon membrane (Amersham Biosciences) 1cm apart. The control RNA was diluted in the range of 0.01pg.µl^{-1}-10ng.µl^{-1} to serve as a standard for probe labelling efficiency. The membrane was air dried and baked at 120°C for 30 min to fix the nucleic acid to the membrane. The membrane was blocked for 30 min in blocking buffer, incubated with 1:5000 anti-DIG-alkaline phosphatase (Roche) in blocking buffer for 30 min. This was followed by two steps of washing for 15 min in wash buffer, equilibration in detection buffer for 5 min and finally antibody binding detection using an Alkaline phosphatase detection kit (Vector) following manufacturers' instructions. The diluted probe was compared to the control actin RNA to determine whether sufficient labelled probe was produced during *in vitro* transcription following manufacturers' instructions.

Table 4.1 Oligonucleotide primers with T7 (antisense) and SP6 (sense) RNA promoter sequences added to the 5' end used for reverse transcription–polymerase chain reaction DNA template production for *in vitro* transcription.

Primer Target	Accession no.		Nucleotide sequence 5'-3'	Length (bp)	Annealing temperature (°C)
Secretory IgM heavy chain	X58870	sense	**ATTTAGGTGACACTATAGAA**TCCTCCATCCGTTTATCTGC	423	72.5
Secretory IgM heavy chain	-	antisense	**TAATACGACTCACTATAGGG**ACATCTACTGGGGCAAGCAC	-	-
Transmembrane IgM	X58871	sense	**ATTTAGGTGACACTATAGAA**GGTGAGGTGTTATCCGTGCT	342	71.0
Transmembrane IgM	-	antisense	**TAATACGACTCACTATAGGG**ATCTTGATGGCAGTGGTTCC	-	-
Interleukin 1β	AJ535730	sense	**ATTTAGGTGACACTATAGAA**AAGGGCAGCCAGTCCGAGAAGG	360	74.2
Interleukin 1β	-	antisense	**TAATACGACTCACTATAGGG**AGGGCAACTGGCTGGGCTGAA	-	-
Interleukin 8	AJ535731	sense	**ATTTAGGTGACACTATAGAA**AAAATCCCCATCGGCTCCCTACT	377	71.7
Interleukin 8	-	antisense	**TAATACGACTCACTATAGGG**TCGTCCACAAACAGCCCATCATT	-	-
Interleukin 10	EU004087	sense	**ATTTAGGTGACACTATAGAA**CGATCAGAGCATTGCACACT	384	69.6
Interleukin 10	-	antisense	**TAATACGACTCACTATAGGG**GTCGTCGTTTTGAACCAAGG	-	-
T7 promoter sequence	-	-	**TAATACGACTCACTATAGGG**	-	-
SP6 promoter sequence	-	-	**ATTTAGGTGACACTATAGAA**	-	-

In situ hybridisation

Pre-treatment of tissue sections

Sections on APES-coated slides were de-waxed twice in 100% xylene for 20 min each, and then rehydrated through an ethanol series into nanopure water. Sections were treated with 1% (v/v) hydrogen peroxide (Sigma) in PBS for 20 min in order to quench endogenous peroxidise activity. To retrieve antigens after formalin fixation, sections were submerged in 10mM sodium citrate buffer, pH 6.0 (25 slides in 500ml) and microwaved for 4 min at 850W. The slides and buffer were cooled on ice for 10 min and then rinsed in Proteinase K buffer pre-warmed to 37°C. The tissue sections were digested with 10µg.ml^{-1} proteinase K (Promega) at 37°C for 12 min. Digestion was stopped by a brief rinse in PBS followed by 0.2% (w/v) glycine (Sigma) in PBS for 10 min. Glycine was washed from the sections with PBS and sections post-fixed in 4% (w/v) paraformaldehyde (Sigma) in PBS for 5 min on ice. Sections were rinsed three times in PBS prior to hybridisation.

Hybridisation

Sections were encircled using the Gene Frame® (Abgene) product and pre-equilibrated with 100µl hybridisation buffer with a plastic coverslip (Gene Frame, Abgene) for 30 min at 37-42°C on a humidified hotplate (Hybaid Ltd, Hampshire, UK). RNA secondary structures were eliminated by diluting the probes in hybridisation buffer, heating them to 80°C for 5 min and placing them immediately on ice for 10 min. The probes were further diluted to 1-3ng.µl^{-1} (depending on the optimum dilution for the probe, see Table 4.2) in hybridisation buffer and pre-heated to 37-42°C. Hybridisation buffer was removed from sections and the probe added at 10µl.cm$^{2-1}$ tissue. Hybridisation took place overnight at 37-42°C on a humidified

hotplate (Hybaid). Negative controls consisted of sections hybridised with sense probes and no probe, respectively.

Post-hybridisation washes

All glassware, plasticware and buffers used post-hybridisation were not treated for RNAses, due to the fact that annealed cRNA probes:target mRNA hybrids are no longer vulnerable to RNAse degradation.

Table 4.2 cRNA probe hybridisation temperatures and concentrations.

cRNA probe	*Hybridisation temperature (°C)*	*Probe concentration for hybridisation (ng.µl^{-1})*
Antisense secretory IgM	42	1
Sense secretory IgM	42	1
Antisense transmembrane IgM	37	3
Sense transmembrane IgM	37	3
Antisense Interleukin 1β	37	3
Sense Interleukin 1β	37	3
Antisense interleukin 8	37	3
Sense interleukin 8	37	3

Coverslips were removed and the sections washed in 2x SSC buffer at room temperature for 20 min, and 2x SSC buffer for 20 min at 37-42°C. The sections were then rinsed three times in RNAse buffer prior to incubation with 150µg.ml^{-1} RNAse A (Sigma) at 37°C for 1 h. The sections were then rinsed in 2x SSC at 37°C for 45 min prior to immunostaining. The wash and RNAse steps above were performed in order to eliminate cRNA probes not properly hybridised to the target mRNA.

Detection of DIG-labelled cRNA

Alkaline phosphatase detection of secretory IgM

Immunostaining was performed on sections in order to visualise DIG-labelled cRNA probe binding to secretory IgM mRNA. Sections were rinsed three times in TBS for

5 min and blocked with blocking buffer for 30 min. Sections were incubated with sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche) at 1/500 in blocking buffer for 2 h at room temperature. Unbound antibodies were washed off with three washes in TBS, and Vector® alkaline phosphatase red substrate kit (Vector labs) used for the detection of bound antibodies following manufacturers' instructions. Sections were counterstained with methyl green (Vector labs) for 2 min at room temperature, rinsed in distilled water, 1 min 95% ethanol, 1 min 100% ethanol and cleared in xylene prior to permanent mounting with VectaMount (Vector).

Horseradish peroxidase detection with tyramide signal amplification (TSA) kit

Immunostaining was performed on sections in order to visualise DIG-labelled cRNA probe binding to transmembrane IgM, IL8 and IL1β mRNA. Due to the low abundance of the mRNA transcripts of these genes within the tissues an amplification kit utilising a relatively novel technology 'TSA' was employed, following manufacturers' instructions (Perkin Elmer®, U.S.A.). Briefly, sections were rinsed three times in TNT wash buffer (Perkin Elmer®, U.S.A.) for 5 min and blocked with 100µl TNB block (Perkin Elmer®, U.S.A.) for 30 min. Sections were incubated with mouse anti-DIG (Roche) at 1/50 in TNB block for 30 min at room temperature. Unbound antibodies were washed off with three 5 min washes in TNT wash buffer, and incubated with 1:100 goat anti-mouse-HRP (Sigma) in TNB block for 30 min. The sections were then washed again in TNT wash buffer as before and incubated with 1:50 biotinyl tyramide in amplification diluent (TSA kit Perkin Elmer) for 30 min. The sections were washed as described previously and incubated with 100µl 1:100 streptavidin-HRP in TNB block (TSA kit, Perkin Elmer) for 30 min at 37°C. Finally, the sections were washed again and the AEC kit (Sigma) used for the detection of

bound antibodies following manufacturers' instructions. Sections were counterstained with haematoxylin for 3 min, rinsed in tap water and mounted with Citifluor (Agar Scientific Ltd, Essex, UK). Negative controls consisted of sections immunostained without primary antibodies.

4.2.1.5 Light Microscopy

Light microscope images were taken following the protocols described in Chapter 2.11.5.

4.2.2 The localised immune response to live versus dead *L. branchialis*

4.2.2.1 Staining of infective free-swimming *L. branchialis* for visualisation of the settled infective stages on the gills

Free-swimming *L. branchialis* females were stained in order to visualise the newly attached *L. branchialis* females on the gills of laboratory-infected Atlantic cod. Initially, 0.01g.l^{-1}neutral red in seawater for 1 h was used to stain the parasites following Anstensrud (1989). However, preliminary infection experiments showed this to be too similar to the colour of cod gills to distinguish neutral red-stained *L. branchialis* from host gill by the naked eye. Therefore, alternative dyes were investigated, these comprising methylene blue, brilliant cresyl blue, nile blue and azure A. Five free-swimming *L. branchialis* females were stained in 0.001%, 0.01% and 1% (w/v) of each of the stains in seawater for 1 h, 1 h and 45 min, respectively at 10°C. The intensity of the stain and survival rates were evaluated and compared to parasites kept in seawater without stain over a period of 72 h. Methylene blue was the only stain found not to kill the parasite, and a staining concentration of 1% (w/v) methylene blue for 45 min was chosen due to its staining intensity.

4.2.2.2 Killing *L. branchialis in situ*

Different techniques to kill free-swimming *L. branchialis* were investigated, including:

1. abdominal damage by sterile 25G needle frozen in liquid nitrogen

2. abdominal damage by acupuncture needle frozen in liquid nitrogen

3. abdominal damage by sterile 25G needle heated in Bunsen burner

4. abdominal damage with flat edged forceps

Three parasites per 'killing' technique were incubated at 10°C in seawater and their survival rate monitored over 24 h compared to control parasites.

4.2.2.3 Effect of anaesthetic on *L. branchialis* survival

In order to make sure that the anaesthesia of infected fish did not kill newly attached *L. branchialis*, the effect of 2-phenoxyethanol (Sigma) at a concentration of 1:10,000 in seawater on the survival of the parasite was investigated. Five free-swimming female *L. branchialis* were incubated with 2-phenoxyethanol (1:10,000) for 5 min, washed twice in seawater and their survival rate observed at 1, 12, 24 and 48 h post-anaesthesia at 10°C.

4.2.2.4 Preliminary infection of naïve *G. morhua* with stained *L. branchialis*

A preliminary infection of Atlantic cod with methylene blue stained *L. branchialis* was undertaken in order to determine if the staining affected the parasites capacity

to infect the host, and if the parasite 'killing' technique was successful *in situ*. Four naïve Atlantic cod (238-281g; 25.2-31.3cm) from Machrihanish Marine farm Ltd. hatchery were infected by a bath challenge with 40 methylene blue stained and 40 unstained free-swimming *L. branchialis* females, as described in Chapter 2. One week prior to infection two cod were panjet-marked under anaesthetic in order to identify them. One day post infection (d.p.i.) one panjet-marked cod was euthinased by concussion rendering the animals instantly unconscious whilst simultaneously destroying the brain, and the gill cavity checked by eye for stained parasites. The stained parasites were then dissected *in situ* and observed under a stereomicroscope in seawater to ensure that the parasites were live *i.e*. moving/gut movement. Another panjet marked cod 1 d.p.i. was anaesthetised and 5 stained parasites located within the gill cavity. Two of these stained parasites were killed by damage to the abdomen, and the fish allowed to recover before being placed back in the experimental tank. One hour post-parasite 'killing' the cod was euthinased by concussion rendering the animals instantly unconscious whilst simultaneously destroying the brain, and the gills examined under a stereomicroscope. This was to check that the two parasites with abdominal damage had died within 1 h and that the other 'untouched' stained and unstained parasites were still alive. The remaining two cod were euthinased at 5 d.p.i. by concussion rendering the animals instantly unconscious whilst simultaneously destroying the brain, and the gills and *bulbus arteriosus* examined under a stereomicroscope. This was to check that the stained parasites had maintained their infection of the host for an extended period of time.

4.2.2.5 Infection of naïve *G. morhua* with stained *L. branchialis*

Thirty naïve Atlantic cod from Viking Fish Farms Ltd. (Ardtoe, Scotland) were acclimatised for 2 weeks in 4 500L tanks (2 x infection tanks n=10; 2 x sham control tanks n=5) at 13°C. Eleven days prior to infection all fish were anaesthetised and panjet-marked (5 on the left of the underbelly and 5 on the right of the underbelly in each infection tank). Twenty fish (n=10 per infection tank) were infected by a bath challenge with methylene blue stained *L. branchialis* at a ratio of 10 parasites per fish. The sham control tanks underwent the same procedure except no parasites were added to the tanks. One day post infection (d.p.i.) 10 fish marked on the left hand side of the underbelly (n=5 per infection tank) were anaesthetised, the branchial cavity checked for stained parasites which were killed by damage to the abdomen, and the fish allowed to recover before being placed back in the experimental tanks. The other 10 infected fish were also anaesthetised and handled in the same manner as the previous fish, except that the parasites were not killed. The 10 sham control fish were also anaesthetised and handled in the same manner in order to rule out any differences measured from these fish to be due to the stress of anaesthesia or handling. All fish were sacrificed by destruction of the brain 36 h post-infection *i.e* 12 h post-parasite killing and the gills, *bulbus arteriosus* and head kidney placed immediately into 4% (w/v) paraformaldehyde in PBS, pH 7.4.

4.2.2.6 Fixation and wax processing of *L. branchialis in situ*

Gills, *bulbus arteriosus* and head kidney were fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.4 for 24 h at 4°C. The tissues were then transferred into 70% ethanol for 10 h, in order to prevent ‘masking’ of mRNA or epitopes by excessive cross-linking by the fixative, prior to processing for paraffin wax embedding. Five µm thick paraffin wax sections from 5 fish per treatment were cut and dried onto poly-L-lysine

(Sigma) coated slides or RNAse-free APES-coated slides at 60°C for 1 h, for immunohistochemistry and *in situ* hybridisation, respectively.

4.2.2.7 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining of 5µm thick paraffin wax sections was performed using the protocol described in Chapter 2.11.4.

4.2.2.8 Immunohistochemistry detection of IgM and C3 at attachment site of live versus dead *L. branchialis*

Immunohistochemistry was carried out as described in Section 4.3.1.3.

4.2.2.9 *In situ* hybridisation detection of IL8, secretory IgM and transmembrane IgM mRNA at attachment site of live versus dead *L. branchialis*

In situ hybridisation was carried out as described in Section 4.3.1.4.

4.2.2.10 Light microscopy

Microscopy was performed as described in Chapter 2.11.5.

4.3 Results

4.3.1 IL1β, IL8, secretory IgM and transmembrane IgM RT-PCR products

The primers outlined in Table 4.1 were successful in producing amplified RT-PCR products for IL1β, IL8, sIgM and tIgM mRNA from gill, head kidney and spleen tissue dissected from Ardtoe-cultured cod 2 d.p.i. with *L. branchialis*. However, no RT-PCR product for IL10 could be produced, probably due to the lack of mRNA expression of this gene 2 d.p.i. of cod with *L. branchialis*.

Expression of IL1β, sIgM and tIgM was detected in the gill, head kidney and spleen of infected cod (Figure 4.1 and 4.2, respectively). IL8 was also found to be expressed in the gill, head kidney and spleen (Figure 4.3a). Even though semi-quantitative or quantitative RT-PCR was not performed, the band for the RT-PCR product of IL8 was found to be very faint in all organs tested, especially the gills, in comparison to the other genes examined with the same amount of total template RNA and number of PCR cycles. Therefore, 40 cycles of PCR post-reverse transcription were performed for IL8, in order to collect enough cDNA product to produce a probe (Figure 4.3b). Negative controls performed with only the forward or the reverse primer or no cDNA template for each pair of primers were all negative.

Sequencing revealed that the purified RT-PCR products were the intended cDNA for Atlantic cod IL1β, IL8, sIgM and tIgM mRNA, and they were therefore used to produce the cRNA probes.

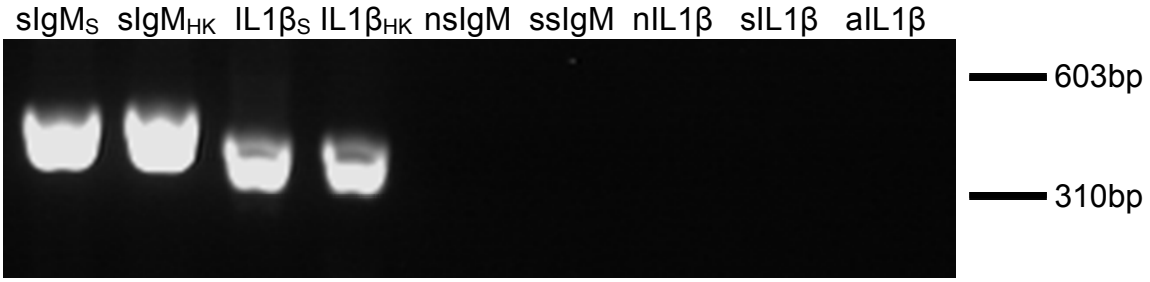

Figure 4.1 Interleukin 1β (IL1β) and secretory IgM (sIgM) mRNA reverse transcription-polymerase chain reaction (RT-PCR) products from spleen ($_S$) and head kidney ($_{HK}$) of Atlantic cod 2d post-infection with *L. branchialis*, after 36 PCR cycles, resolved on a 1.2% agarose gel, and stained with ethidium bromide. Negative controls: n, no cDNA template; s, only sense primer on spleen cDNA template; a, only antisense primer on spleen cDNA. Bars at side illustrate molecular weight markers in base pairs.

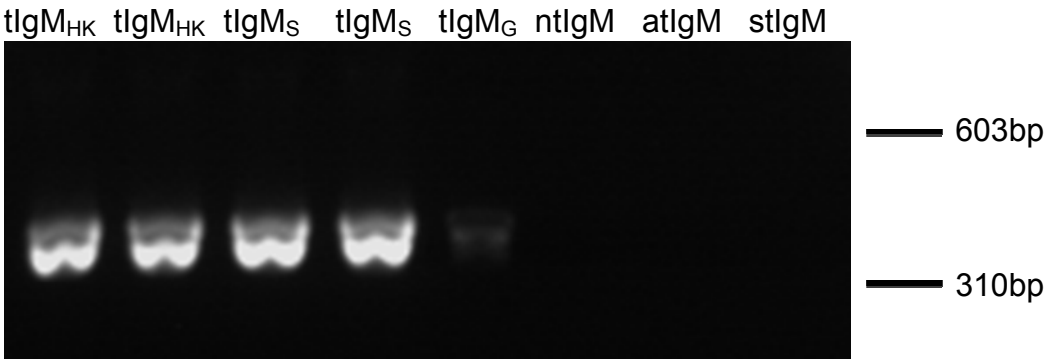

Figure 4.2 Transmembrane IgM (tIgM) mRNA reverse transcription-polymerase chain reaction (RT-PCR) products from head kidney ($_{HK}$), spleen ($_S$) and gills ($_G$) of Atlantic cod 2d post-infection with *L. branchialis*, after 36 PCR cycles, resolved on a 1.2% agarose gel, and stained with ethidium bromide. Negative controls: n, no cDNA template; a, only antisense primer on spleen cDNA; s, only sense primer on spleen cDNA template. Bars at side illustrate molecular weight markers in base pairs.

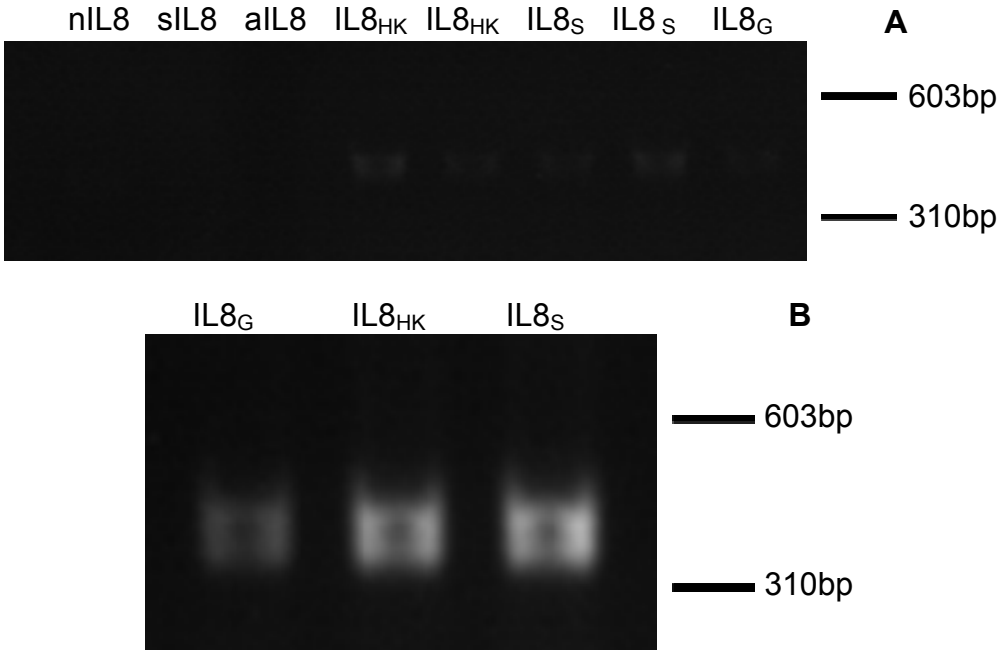

Figure 4.3 Interleukin 8 (IL8) mRNA reverse transcription-polymerase chain reaction (RT-PCR) products from head kidney ($_{HK}$), spleen ($_{S}$) and gills ($_{G}$) of Atlantic cod 2d post-infection with *L. branchialis*, after (A) 36 PCR cycles and (B) 40 PCR cycles, resolved on a 1.2% agarose gel, and stained with ethidium bromide. Negative controls: n, no cDNA template; s, only sense primer on spleen cDNA template; a, only antisense primer on spleen cDNA. Bars at side illustrate molecular weight markers in base pairs.

4.3.2 The localised immune response to *L. branchialis* infection over time

4.3.2.1 Localisation of IgM by immunohistochemistry (IHC) at the site of infection

The monoclonal anti-cod IgM antibody did not stain cells within the gills and heart of the cod samples processed in this experiment, but seemed to stain extracellular IgM within the vascular system. The negative controls without the primary antibody showed no positive staining. At 0 d.p.i. no positive staining was found in the gill tissue (Figure 4.4a), however, positive staining was found in the blood plasma of gill

arteries. One d.p.i. the parasites were attached to the base or the tip of the gill filaments and intense IgM positive staining was observed in the affected gill filament blood vessels (Figure 4.4b). However, no positive IgM staining was found at the immediate attachment site of the parasites (Figure 4.4b). At 3 d.p.i. the majority of parasites had migrated into the gill arch of the fish entering the afferent branchial artery. Positive IgM staining was observed encircling the host reaction around the parasites which had migrated into the gill arch (Figure 4.4c), and within the haemorrhage that occurred at the parasite's entry point into the blood vessels. However, a minority of parasites at 3 d.p.i. were still attached to the gill filament by their antennae. The gill filament underwent epithelial hyperplasia with fusion of the secondary lamellae and positive IgM staining was observed to accumulate in the affected filament associated with the vascular system (Figure 4.4d & e). By 7 d.p.i. the parasites had migrated into the ventral aorta and no positive IgM staining was found associated with the parasites within the ventral aorta (Figure 4.4f). Fourteen d.p.i. the cephalothorax of the parasites remained in the host's ventral aorta and they had developed into an early penella stage. Positive IgM staining was again observed at the periphery of the host reaction within the ventral aorta (Figure 4.4g). Dead, necrotic parasites were observed 56 d.p.i. of Ardtoe-cultured cod. Positive IgM staining was observed on the degrading parasite and within the local vicinity (Figure 4.4h).

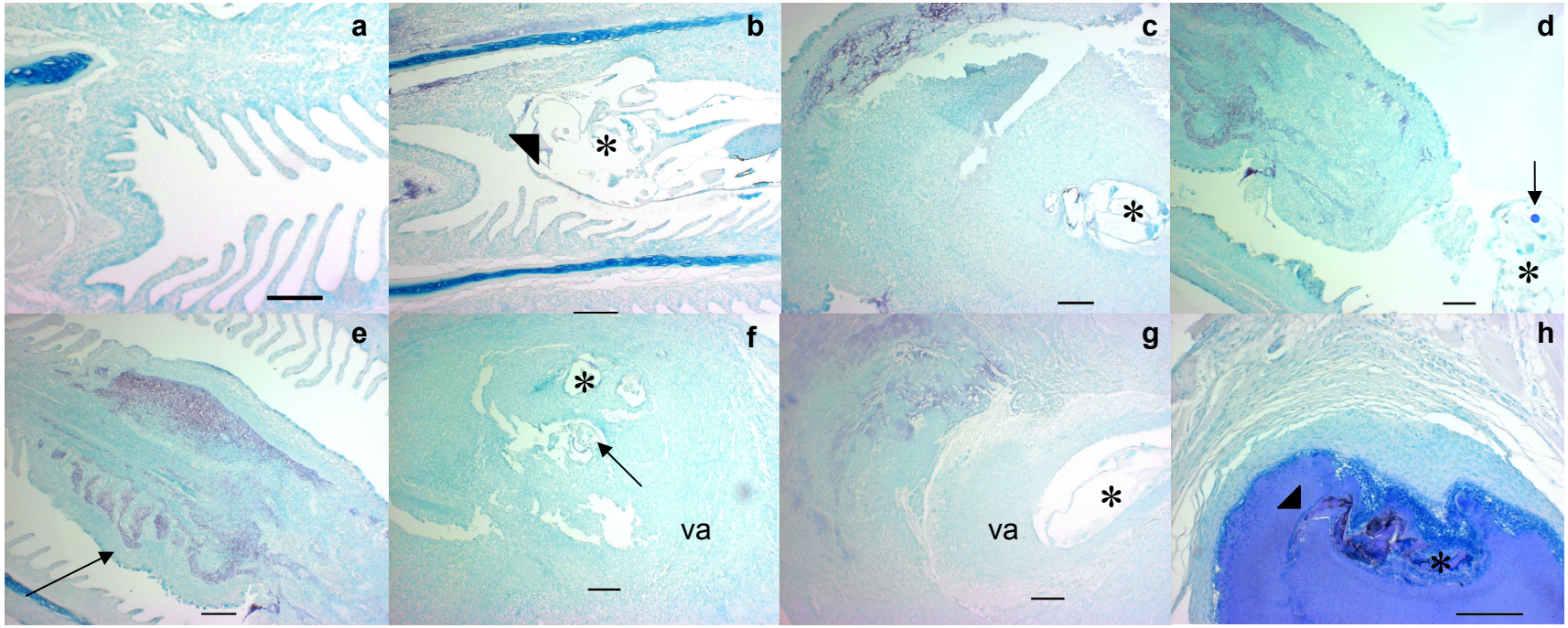

Figure 4.4 Localisation of host IgM (with positive reaction indicated by purple colour) at the infection site in *Lernaeocera branchialis* – infected cultured cod by immunohistochemistry at 0, 1, 3, 7, 14 and 56 days post infection (d.p.i.). (a) Uninfected gills 0 d.p.i.; (b) initial attachment of *L. branchialis* to gill filament 1 d.p.i.; (c) *L. branchialis* migrated into the gill arch 3 d.p.i.; (d) a few *L. branchialis* were still attached to the gill filament 3 d.p.i., arrow = parasite lens; (e) infected gill filament anterior to the parasite's attachment site, note fusion of the secondary lamellae (arrow); (f) *L. branchialis* migrated into the host's ventral aorta 7 d.p.i., arrow = parasite thoracic segment; (g) metamorphosing *L. branchialis* remains within the ventral aorta at the tip of the *bulbus arteriosus*14 d.p.i.; and (h) degrading dead *L. branchialis* within the host's blood vessels 56 d.p.i.. * = parasite; black arrow head = parasite antennae; va = ventral aorta lumen. Bar = 100µm.

4.3.2.2 Localisation of C3 β chain by IHC at the site of infection

The gills of uninfected fish at 0 d.p.i. showed no staining within the tissue (Figure 4.5a), while some faint staining for C3 was seen within the plasma of the vascular system of the gill arch and filaments. However, 1 d.p.i. the staining within the blood vessels of the infected gill filament was more intense (Figure 4.5b) suggesting the accumulation of C3 within the infected filament. This stronger positive staining was also observed in adjacent gill filaments where abrasion by, or contact with, the parasite had occurred. Once the parasite entered the gill arch, at 3 d.p.i., faint C3 positive staining was observed at the periphery of the host reaction (Figure 4.5c). By 7 d.p.i. the parasites had migrated into the host's ventral aorta where no C3 staining was observed within the host reaction (Figure 4.5d), however, a faint staining remained around the edges of the cellular aggregate encircling the parasite. At 14 d.p.i. intense C3 binding occurred within the periphery of the host reaction to the parasite within the ventral aorta (Figure 4.5e). The gut of the parasite stained positive for C3 by IHC (Figure 4.5d & e), together with occasional staining of the cuticle epithelium. However, whether this was specific for host C3 bound to the gut post-feeding or a parasite-derived protein which the antibody cross-reacts with remains to be determined. No C3 was observed to be associated with the cuticle of the parasite up to 14 d.p.i.. However, C3 binding was observed on the exterior of the degrading, dead parasites within the vascular system of the Ardtoe-cultured cod 56d.p.i. (Figure 4.5f).

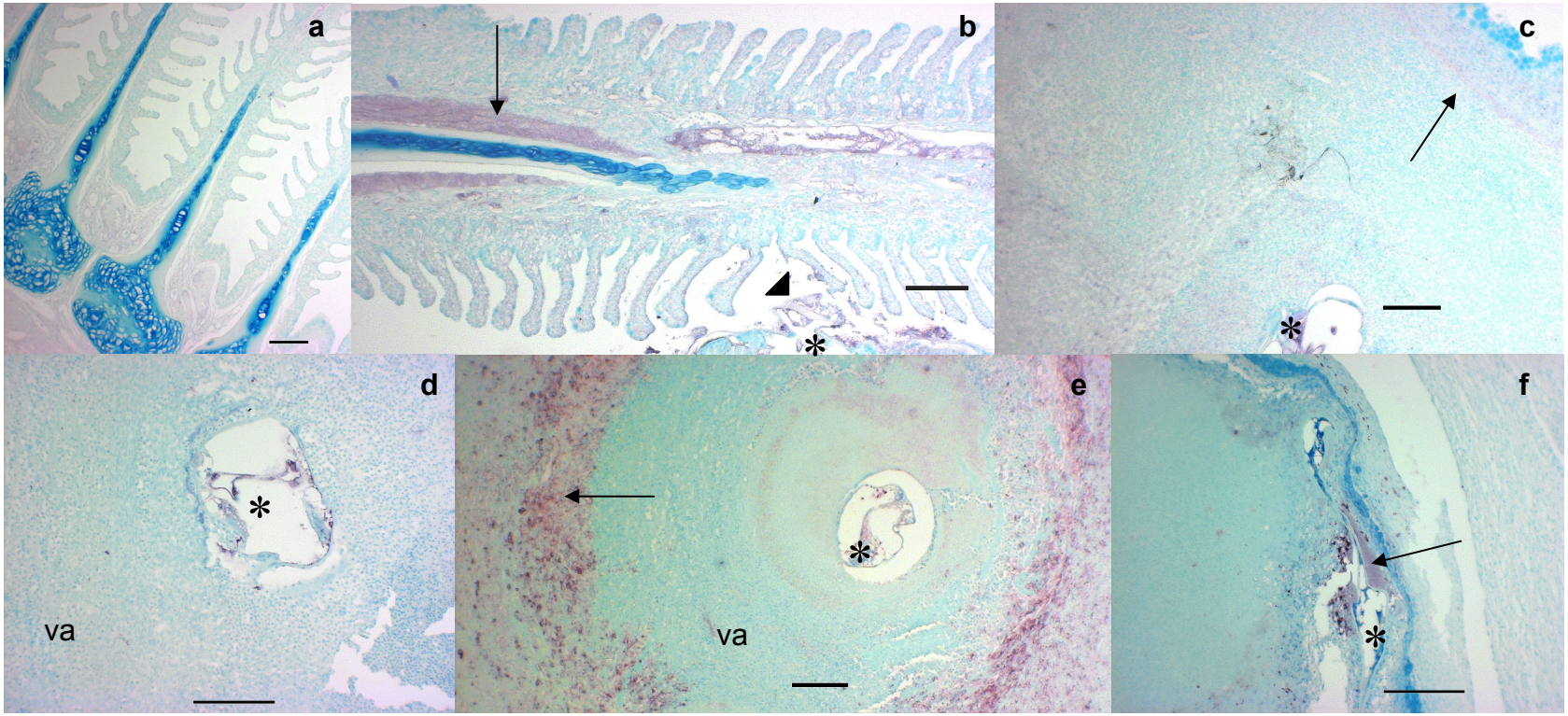

Figure 4.5 Localisation of host C3 (positive reaction indicated by purple colour) at the infection site in *Lernaeocera branchialis* – infected cultured cod by immunohistochemistry at 0, 1, 3, 7, 14 and 56 days post infection (d.p.i.). (a) Uninfected gills 0 d.p.i.; (b) initial attachment of *L. branchialis* to gill filament 1 d.p.i., arrow head = parasite 2nd maxialla; (c) *L. branchialis* migrated into the gill arch 3 d.p.i. (arrow = faint C3 positive reaction); (d) *L. branchialis* migrated into the host's ventral aorta 7 d.p.i.; (e) metamorphosing *L. branchialis* remains within the ventral aorta at the tip of the *bulbus arteriosus* 14 d.p.i.; and (f) degrading dead *L. branchialis* within the host's blood vessels 56 d.p.i.. * = parasite; black arrow = C3 binding; va = ventral aorta. Bar = 100µm.

4.3.2.3 Localisation of secretory IgM gene expression (mRNA) by *in situ* hybridisation (ISH) at the site of infection

The specificity of the antisense secretory IgM (sIgM) transcript cRNA probe was tested on Atlantic cod head kidney tissue. The staining was clearly cell-associated with no non-specific background staining detected. The sIgM transcript positive cells were found grouped in defined clusters often associated with the tissue vascular system lining the blood vessel walls (Figure 4.6a). Positive cells were also scattered throughout the haematopoietic tissue and revealed a large cytoplasm:nucleus ratio, suggesting that the probe was specific for plasma cells (Figure 4.6b). All tissue sections hybridised with sense sIgM cRNA probes or in the absence of a probe were negative (Figure 4.6c & d).

Sections of uninfected gills at 0 d.p.i. revealed sIgM positive cells in the connective tissue surrounding the branchial arteries in the gill arch (Figure 4.7a) and the blood vessels in the gill filaments. The initial attachment of *L. branchialis* 1 d.p.i. did not appear to affect the distribution of sIgM positive cells, and positive cells were still observed in the connective tissue surrounding the blood vessels of the affected and adjacent gill filaments (Figure 4.7b and c). Epithelial hyperplasia was evident at the parasite attachment site and no sIgM positive cells were in the direct vicinity of this area (Figure 4.7c). *L. branchialis* migrated through the gill arch entering the afferent branchial artery 3 d.p.i., proceeding into the ventral aorta 7 and 14 d.p.i.. Sub-epithelial haemorrhaging occurred at the point of entry and a host reaction, including the formation of a thrombus, encircled the parasite within the blood vessels but no sIgM positive cells were found to be present at these stages (Figure 4.7d-f). Fifty-six d.p.i. all of the parasites were dead and encapsulated by host tissue and undergoing degradation. No sIgM positive cells were found within the

host tissue immediately surrounding the parasite (Figure 4.7g), however, numerous positive cells were observed at the periphery of the encapsulated parasite (Figure 4.7h).

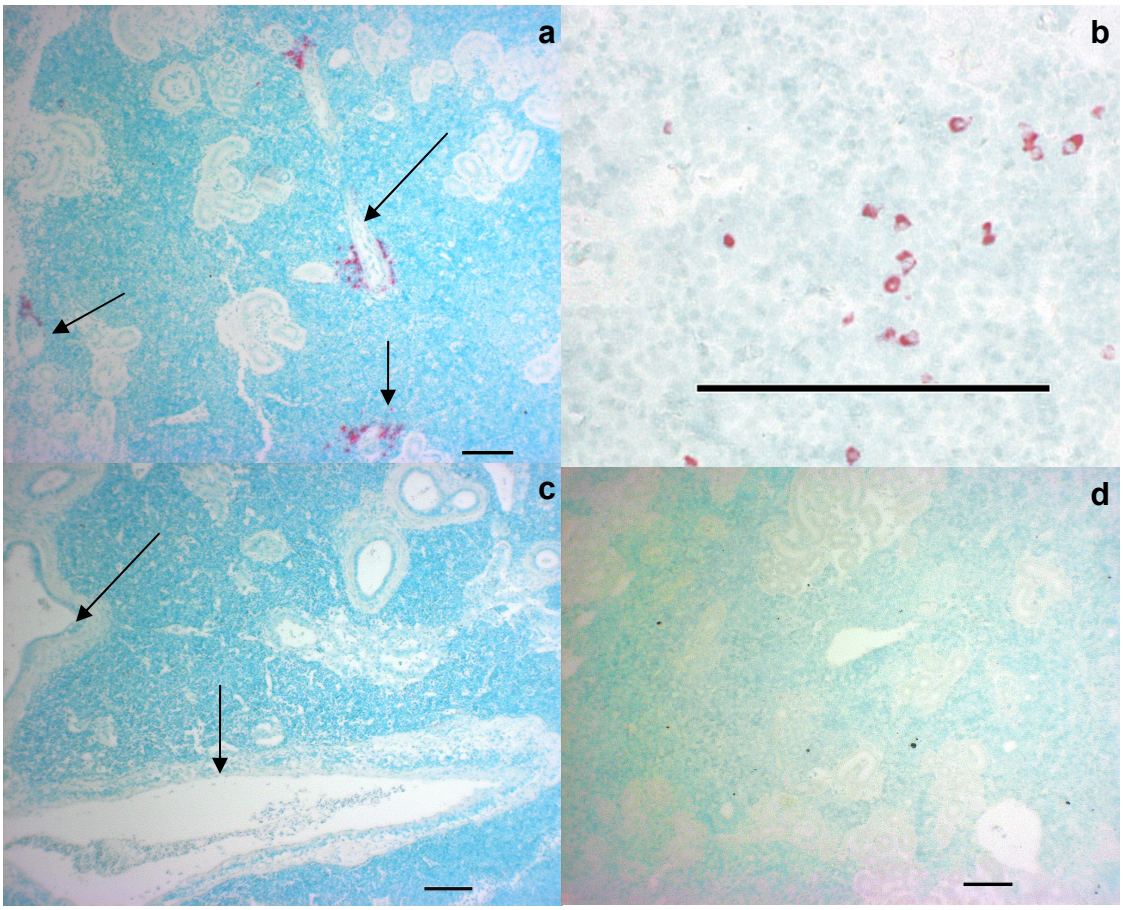

Figure 4.6 Localisation of secretory IgM (sIgM) transcripts in Atlantic cod head kidney to test specificity of cRNA probe (positive reaction indicated by red colour). (a) Clusters of sIgM positive cells associated with the vascular system and (b) scattered throughout the haematopoietic tissue of uninfected cultured-cod head kidney probed with antisense sIgM cRNA probe. No positive staining in head kidney probed (c) without any cRNA probe or (d) with the sense sIgM cRNA probe showing the specificity of antisense sIgM probe. Black arrow = blood vessesls. Bar = 100µm.

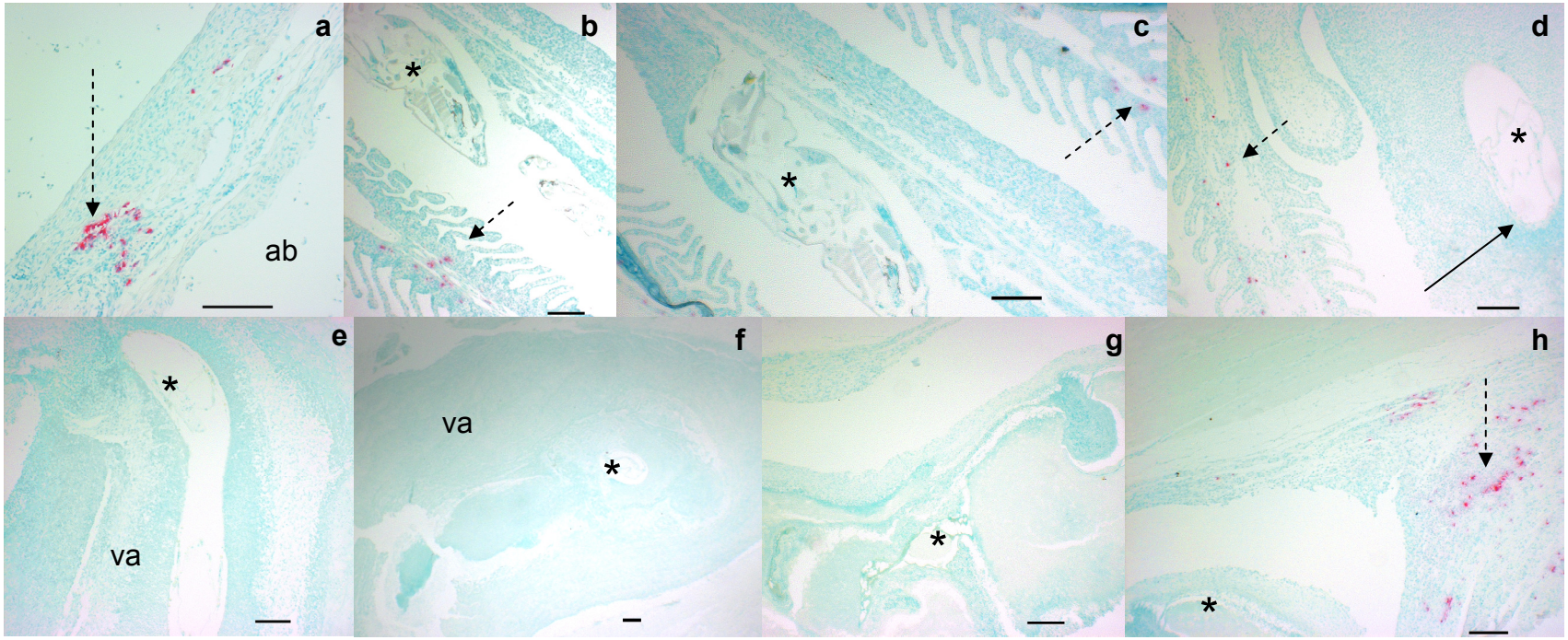

Figure 4.7 Localisation of host secretory IgM transcripts, *i.e.* gene expression, by *in situ* hybridisation (positive reaction indicated by red colour) at the infection site in *Lernaeocera branchialis*-infected cultured-cod 0, 1, 3, 7, 14 and 56 days post infection (d.p.i.). (a) Gill arch 0 d.p.i. illustrating sIgM positive cells in the connective tissue surrounding the branchial arteries' ab = afferent branchial artery; (b & c) initial attachment of *L. branchialis* to gill filament 1 d.p.i.; (d) *L. branchialis* migrated into the gill arch 3 d.p.i. with the genitor-abdominal region surrounded by a subepithelial haemorrhage (arrow = haemorrhage); (e) *L. branchialis* migrated into the lumen of the host's ventral aorta 7 d.p.i.; (f) metamorphosing *L. branchialis* remains within the lumen of the ventral aorta at the tip of the *bulbus arteriosus*14 d.p.i.; (g) degrading dead *L. branchialis* within the host's blood vessels 56 d.p.i.; (h) and the periphery of an encapsulated, dead and degrading *L. branchialis* 56 d.p.i.. * = parasite; dashed black arrow = sIgM mRNA positive cells; va = ventral aorta. Bar = 100μm.

4.3.2.4 Localisation of transmembrane IgM gene expression (mRNA) by ISH at the site of infection

The specificity of the antisense transmembrane IgM (tIgM) transcript cRNA probe was tested on Atlantic cod head kidney tissue. The staining was clearly cell-associated with no non-specific background detected. The tIgM transcript positive cells were scattered evenly throughout the head kidney, and in contrast to sIgM positive cells they were not found in clusters or to have a preferential association with the tissue vascular system (Figure 4.8a). The ISH protocol was not sensitive enough to detect cells expressing tIgM transcripts; therefore Tyramide Signal Amplification was performed in order to locate positive cells. This, corroborated by the fact that positive cells were observed to possess a smaller cytoplasm:nucleus ratio than sIgM positive cells, suggests that the probe was specific for B cells (Figure 4.8a). All tissue sections hybridised with sense tIgM cRNA probes (Figure 4.8b and c) or in the absence of a probe with the TSA step were negative (Figure 4.8d).

Sections of uninfected gills at 0 d.p.i. revealed tIgM positive cells within and lining the blood vessels of the gill filaments (Figure 4.9a). The initial attachment of *L. branchialis* 1 d.p.i. did not appear to affect the distribution of tIgM positive cells, and positive cells were still observed within the blood vessels of the affected and adjacent gill filaments as well as the arterioles of the secondary lamellae (Figure 4.9b and c). It was also noted that the dorsal cuticle surface of the parasite's cephalothorax stained positive (Figure 4.9b – d), however this was thought to be a non-specific reaction. No tIgM positive cells however, were observed in the direct vicinity of parasite attachment (Figure 4.9c). Once *L. branchialis* migrated through the gill arch entering the afferent branchial artery, at 3 d.p.i., a thrombus had formed around the parasite (Figure 4.9d). This thrombus remained around the parasite as it

migrated into the ventral aorta by 7 d.p.i. (Figure 4.9e) and metamorphosed into an early penella sub-stage at 14 d.p.i. (Figure 4.9f). However, no tIgM positive cells were found to be present in the local host reaction at these stages (Figure 4.9d, e and f). No tIgM positive cells were observed in the direct vicinity of encapsulated, dead, degrading parasites found 56 d.p.i. of Ardtoe-cultured cod (Figure 4.9g). However, numerous positive cells were observed to the periphery of the encapsulated parasite (Figure 4.9h).

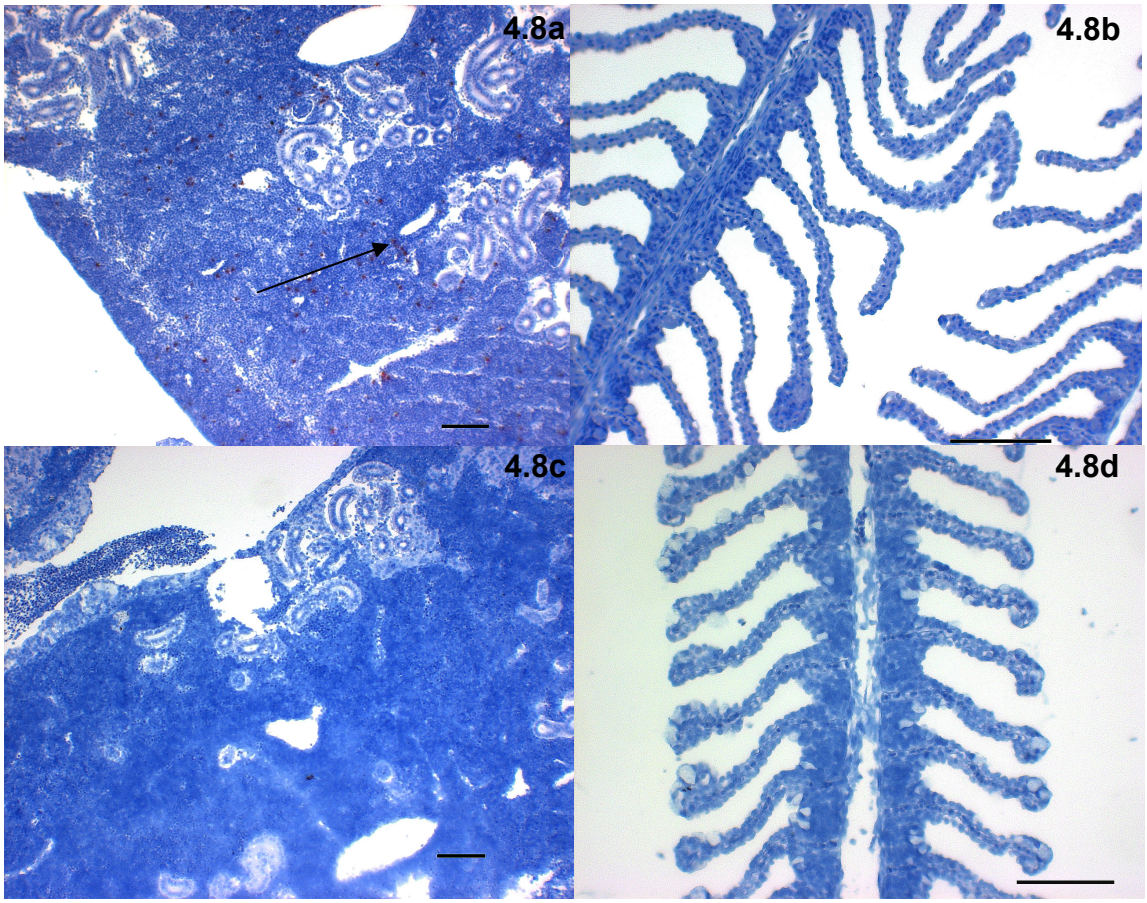

Figure 4.8 Localisation of transmembrane IgM (tIgM) transcripts (positive reaction indicated by red colour) in Atlantic cod head kidney to test specificity of cRNA probe. (a) Evenly scattered tIgM positive cells within the haematopoietic tissue of uninfected cultured-cod head kidney probed with antisense tIgM cRNA probe, black arrow = tIgM mRNA positive cells. No positive staining in the (b) gills probed with sense tIgM cRNA probe, in the (c) head kidney probed with sense tIgM cRNA probe or in (d) the gills without any cRNA probe, showing the specificity of the antisense sIgM probe. Bar = 100µm.

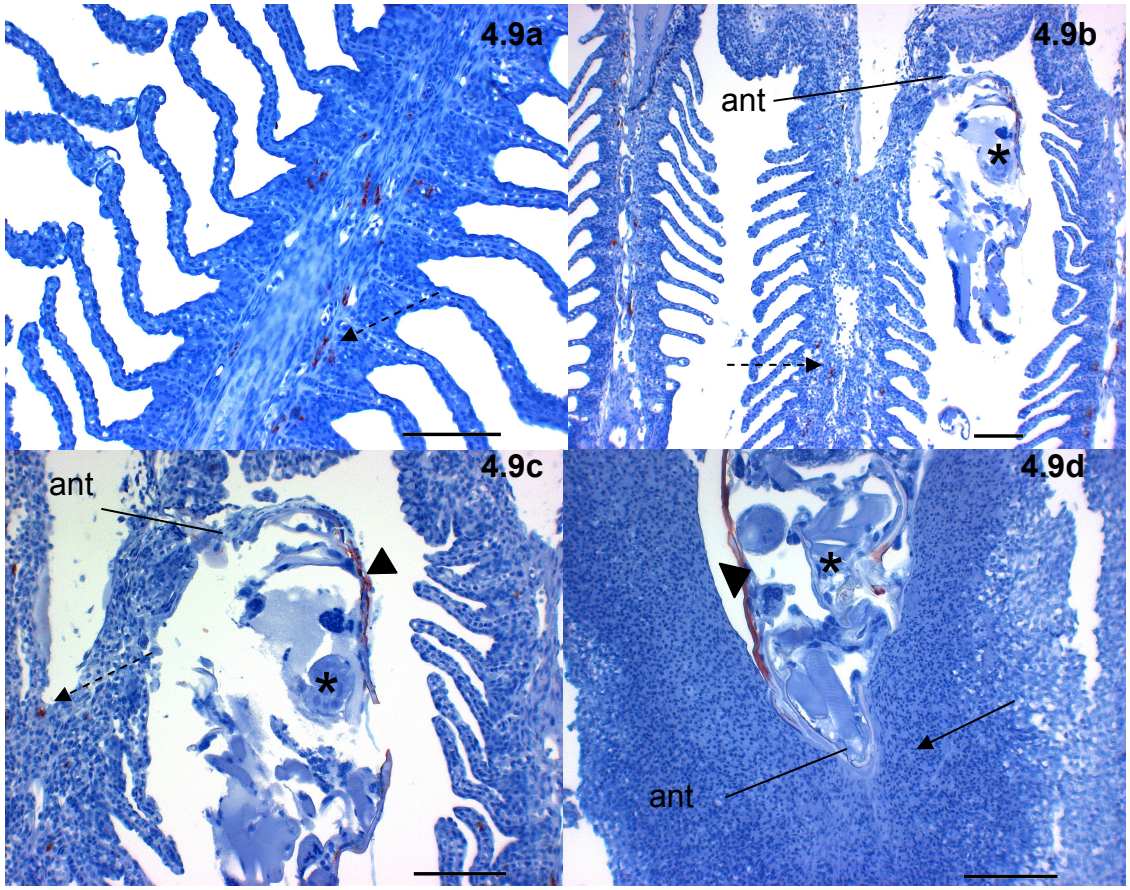

Figure 4.9 Localisation of host transmembrane IgM (tIgM) transcripts, *i.e.* gene expression, by *in situ* hybridisation (positive reaction indicated by red colour) at the infection site in *Lernaeocera branchialis*-infected cultured-cod 0, 1, and 3 days post infection (d.p.i.). (a) Gill arch 0 d.p.i. illustrating tIgM positive cells within and lining the blood vessels of the gill filament; (b) initial attachment of *L. branchialis* to host gill filament 1 d.p.i.; (c) close-up of initial attachment of *L. branchialis* to host gill filament 1 d.p.i., note positive staining of the dorsal cuticle surface of the parasite (arrow head = positive cuticle); (d) *L. branchialis* migrated into the gill arch 3 d.p.i. residing within the afferent branchial artery surrounded by a thrombus (arrow) * = parasite; dashed arrow = tIgM mRNA positive cells; ant = antennae. Bar = 100µm.

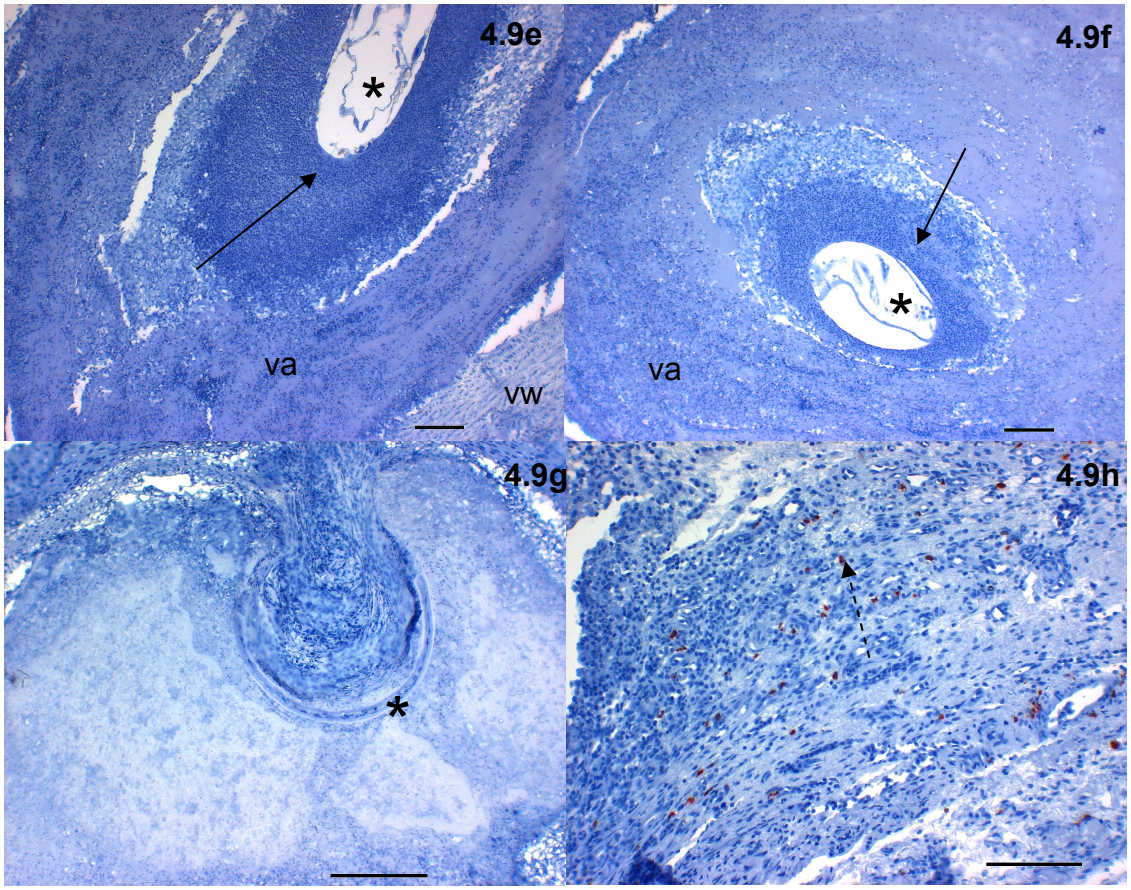

Figure 4.9 continued Localisation of host transmembrane IgM (tIgM) transcripts, *i.e.* gene expression, by *in situ* hybridisation (positive reaction indicated by red colour) at the infection site in *Lernaeocera branchialis*-infected cultured-cod 7, 14 and 56 days post infection (d.p.i.). (e) *L. branchialis* migrated into the lumen of the host's ventral aorta 7 d.p.i. surrounded by a thrombus (thrombus = arrow); (f) *L. branchialis* remains within the lumen of the ventral aorta metamorphosing into the early penella sub-stage at the tip of the *bulbus arteriosus* 14 d.p.i.; (g) degrading dead *L. branchialis* within the host's blood vessels 56 d.p.i.; and (h) tIgM positive cells at the periphery of an encapsulated, dead and degrading *L. branchialis* 56 d.p.i.. * = parasite; va = ventral aorta; vw = ventral aorta wall. Bar = 100µm.

4.3.2.5 Localisation of interleukin-8 gene expression (mRNA) by ISH at the site of infection

The specificity of the antisense interleukin 8 (IL8) transcript cRNA probe was tested on Atlantic cod head kidney and gill tissue. The staining was clearly cell-associated with no or little non-specific background detected. The IL8 transcript positive cells were very rare within the head kidney (Figure 4.10a), which meant that no pattern of localised expression could be detected, in comparison to tIgM and sIgM positive cells. This was corroborated by the faint RT-PCR product band produced from total RNA of head kidney (Figure 4.3a). The ISH protocol was not sensitive enough to detect cells expressing IL8 transcripts; therefore a double step Tyramide Signal Amplification (TSA) was performed in order to locate positive cells. All tissue sections hybridised with sense IL8 cRNA probes, or in the absence of a probe with the double TSA step were negative (Figure 4.10b). Sections probed with the antisense IL8 cRNA probe and the double TSA step in the absence of the primary anti-DIG antibody were also negative (Figure 4.10c).

Sections of uninfected gills, at 0 d.p.i., revealed rare, scattered IL8 transcript (faintly) positive cells within the afferent branchial artery of the gill arch. The initial attachment of *L. branchialis* 1 d.p.i. sometimes led to epithelial hyperplasia of the gill filament over the anterior cephalothorax of the parasite (Figure 4.11a). Once the parasite entered the blood vessels, a few faintly IL8 positive cells were observed in the gill filament blood vessels near to the attachment site and oral cone of the parasite (Figure 4.11a and b). It was also noted that the dorsal cuticle surface of the parasite's cephalothorax stained positive, as observed for tIgM probe staining (Figure 4.11a). At 3 d.p.i., *L. branchialis* had usually migrated into the gill arch entering the afferent branchial artery. The entry point was marked by sub-epithelial

haemorrhaging at the base of the gill filament (Figure 4.11c). However, no IL8 positive cells were observed within the vicinity of the entry point of the parasite and subsequent haemorrhage (Figure 4.11c). A thrombus had also formed around the parasite in the afferent branchial artery. This thrombus remained around the parasite as it migrated into the ventral aorta by 7 d.p.i. (Figure 4.11d) and metamorphosed into an early penella sub-stage at 14 d.p.i. (Figure 4.11e). However, no IL8 positive cells were found to be present in the local host reaction within the ventral aorta 7 or 14 d.p.i. (Figure 4.11d and e). Numerous leukocytes within the ventral aorta to the periphery of the host reaction to the parasite at 14 d.p.i. were, however, observed to be intensely positive for IL8 transcripts (Figure 4.11f). These leukocytes were identified in the free-flowing blood within the host's ventral aorta. They were large leukocytes with copious cytoplasm (Figure 4.11g). However, no IL8 positive cells were observed in the local vicinity of the degenerating encapsulated parasite in Ardtoe-cultured cod 56 d.p.i.

4.3.2.6 Localisation of interleukin-1β gene expression (mRNA) by ISH at the site of infection

The *in situ* hybridisation protocol with the antisense interleukin-1β probe did not work, and due to time and material constraints could not be repeated and optimised. This merits further work due to the central role of this cytokine in the inflammatory response of teleosts.

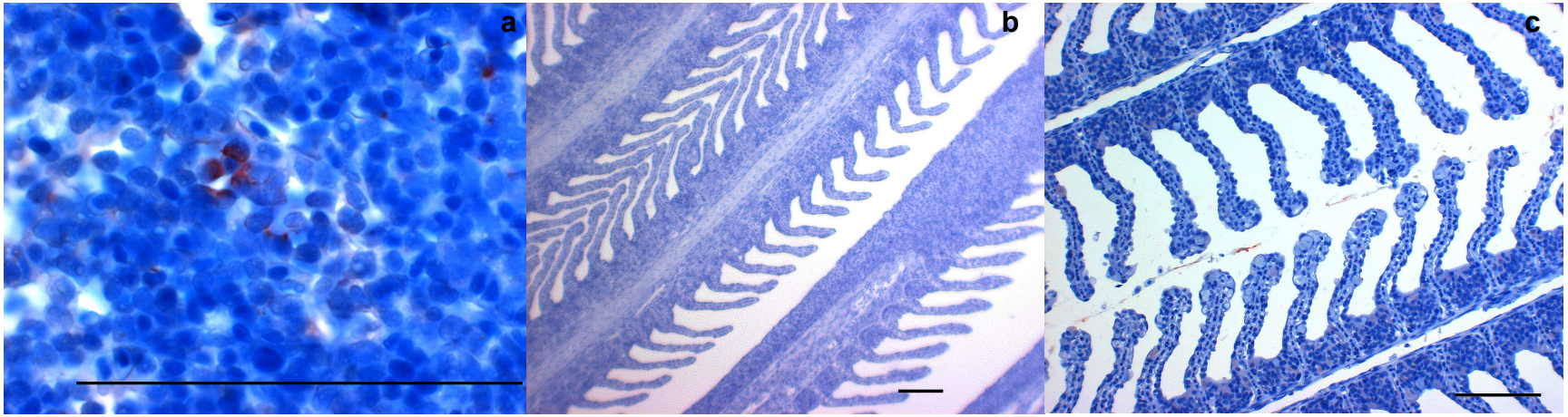

Figure 4.10 Localisation of interleukin 8 (IL8) transcripts in Atlantic cod head kidney and gills to test the specificity of the antisense IL8 cRNA probe (positive reaction indicated by red colour). (a) Extremely rare IL8 transcript positive cells observed within the haematopoietic tissue of uninfected cultured-cod head kidney probed with antisense IL8 cRNA probe. No positive staining in (b) the gills probed without any cRNA probe, showing the specificity of the antisense IL8 probe; or in (c) the gills probed with the antisense IL8 probe and processed without the primary anti-DIG antibody, showing no background after double tyramide signal amplification step. Bar = 100µm.

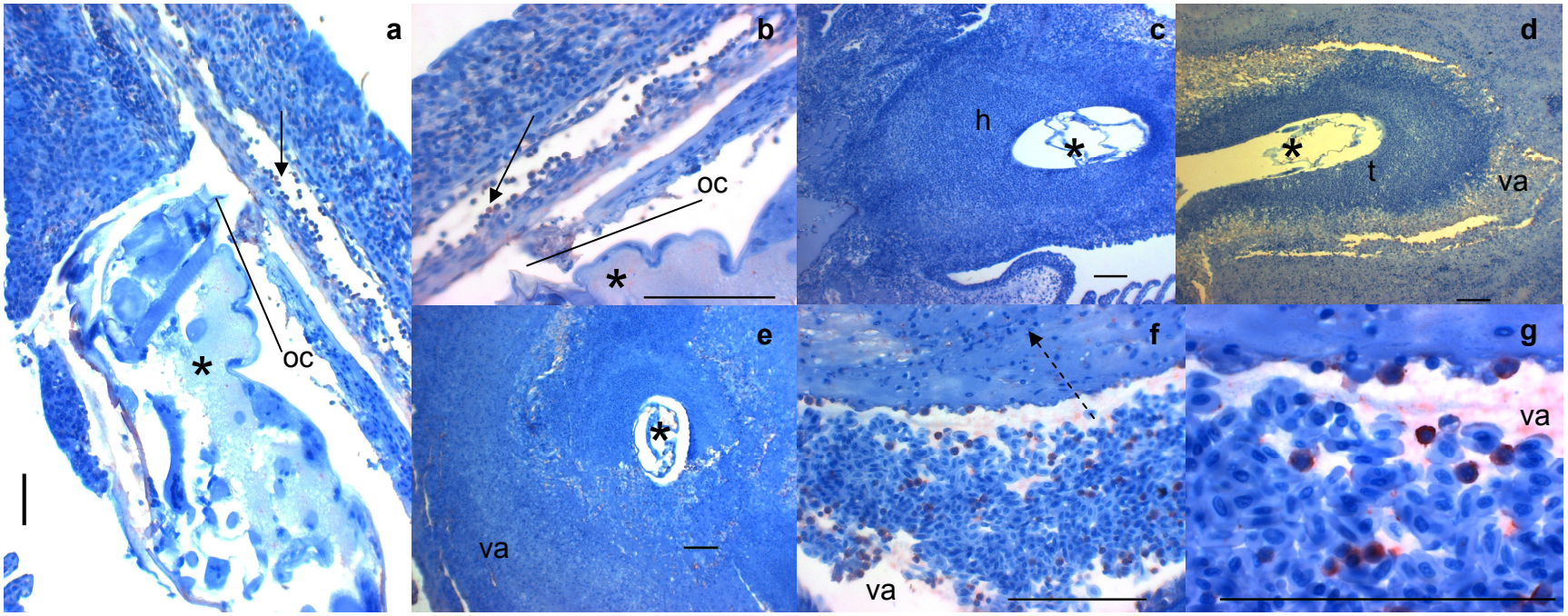

Figure 4.11 Localisation of host interleukin 8 (IL8) transcripts, *i.e.* gene expression, by *in situ* hybridisation at the infection site (positive reaction indicated by red colour) in *Lernaeocera branchialis*-infected cultured-cod 1, 3, 7, and 14 days post infection (d.p.i.). (a & b) The initial attachment of *L. branchialis* to host gill filament 1 d.p.i.; (b) close up of attachment site of *L. branchialis* 1 d.p.i., note faint positive staining of some leukocytes in the infected gill filament blood vessel (arrow;); (c) *L. branchialis* migrated into the gill arch 3 d.p.i. with subepithelial haemorrhage (h) at the point of entry around the genitor-abdominal region; (d) *L. branchialis* migrated into the lumen of the host's ventral aorta 7 d.p.i. surrounded by a thrombus (t; thrombus); (e) *L. branchialis* remains within the lumen of the ventral aorta metamorphosing into the early penella sub-stage at the tip of the *bulbus arteriosus* 14 d.p.i.; (f) IL8 transcript positive leukocytes in the ventral aorta to the periphery of the host reaction to the parasite, dashed arrow; (e) close-up of f showing cell-associated staining of large leukocytes. * = parasite; oc, oral cone; va = ventral aorta. Bar = 100µm.

4.3.3 Localised immune response to live versus dead *L. branchialis*

4.3.3.1 Killing method of infective *L. branchialis* stage

Damage to the abdomen of free-swimming female *L. branchialis* with flat-edged forceps was found to be very effective at killing the parasites with 100% mortality 4 h post-damage (h.p.d.; Figure 4.12). The other 'killing' methods using needles were found to be less effective, and less practical than the method using forceps, with less than 100% mortality of parasites and / or their longer survival post-damage (Figure 4.12).

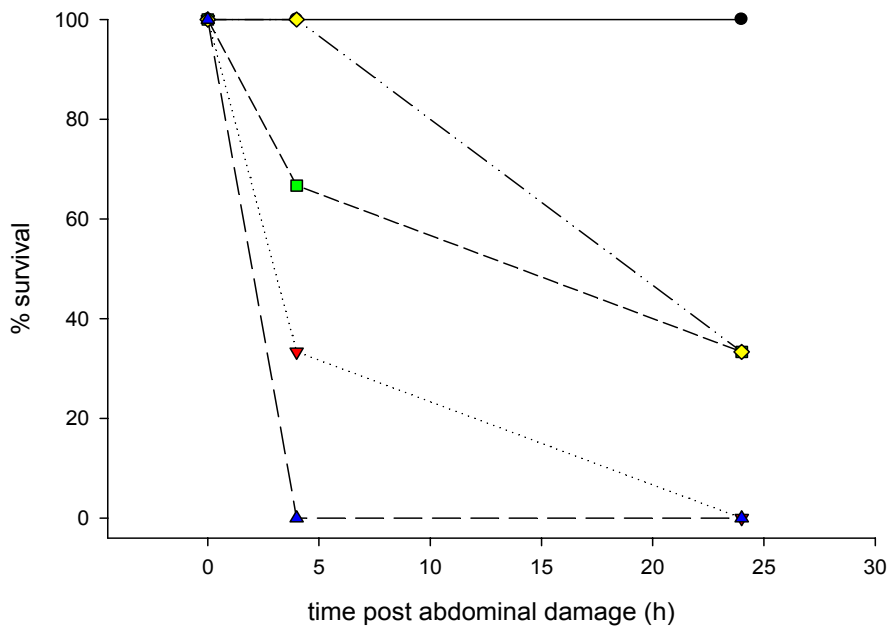

Figure 4.12 The survival rate of free-swimming, infective stages of *L. branchialis* over a 24 h period post-abdominal damage with a hot needle (red upside-down triangle), a frozen needle (green square), a frozen acupuncture needle (yellow diamond) and flat-edged forceps (blue triangle), in comparison to untouched parasites (controls = black circle; n = 3 per treatment).

4.3.3.2 Effect of anaesthetic on *L. branchialis* survival

All five parasites submerged in anaesthetic for 5 min survived for up to 48 h post-anaesthesia. Therefore, anaesthetising the fish in order to kill the parasite by

abdominal damage for the comparison of the local immune response to live versus dead *L. branchialis* was not thought to affect parasite survival *i.e.* live parasites on cod anaesthetised in the same way as cod with dead parasites would not affect their survival. The survival of the parasites, however, was not monitored after 48 h.

4.3.3.3 Preliminary infection of Atlantic cod with methylene blue stained *L. branchialis*

One d.p.i. with stained and unstained infective parasite stages, the stained parasites were found to infect the cod and be alive on the gill filaments (due to parasite and gut movements). The parasites damaged by forceps at 1 d.p.i. were found to be dead (no appendage movement or gut movement) 1 h later, and still attached on the gill filament. However, the undamaged parasites were still alive and attached to the gill filament. The stained parasites on cod 5 d.p.i. were found to have migrated into the gill arch, and the cephalothorax to reside within the ventral aorta in some cases. There was no obvious effect of parasite staining on their survival or their ability to infect cod in comparison to unstained parasites. Therefore, methylene blue stained infective stages of *L. branchialis* were used to infect cod in the live versus dead parasite experiment in order to be able to locate the parasites at the early stages of infection to kill them quickly and easily.

4.3.3.4 Histopathology of the site of infection of live versus dead *L. branchialis*

The histology at the local infection site was examined in cod infected with live or dead parasites at 36 h.p.i.. No qualitative differences were observed between the 'histological picture' at the infection site of live versus dead parasites at these stages in the infection. Live and dead *L. branchialis* were found to be in a variety of locations on the hosts' gills dependent on their stage of migration. Some were

attached to the gill filament by their antennae, others were embedded in the filament blood vessel (Figure 4.13a), and some were found to have migrated into the gill arch within the afferent branchial artery (Figure 4.13g). This made the comparison of the local immune response to live versus dead parasites difficult at this infection stage, as the histology depended largely on whether or not the parasite had entered the host's vascular system. Few histopathological changes were associated with the attachment of the parasite to the gills by the second antennae. When the parasite was attached to the gill filament, epithelial hyperplasia with the fusion of the secondary lamellae often occurred on the affected gill filament with numerous mucous cells present. However, once the parasite entered the filament blood vessel, numerous thrombocytes were observed at the point of entry (Figure 4.13b-c), and some neutrophils were also observed in the infected filament blood vessel. The majority of these thrombocytes were found to possess a spindle-shaped morphology (Figure 4.13c). Host tissue was also found inside the oral cone of some parasites post-penetration into the blood vessels, for example thrombocytes and blood plasma (Figure 4.13c). Haemorrhage occurred at the entry point as the parasite migrated into the blood vessels (Figure 4.13 d-f). To the periphery of these haemorrhages leukocytes were observed including thrombocytes and neutrophils (Figure 4.13d-e). Once the parasites had migrated into the afferent branchial artery of the gill arch the host proceeded to form a thrombus around them (Figure 4.13g). This consisted of red blood cells of various stages of degeneration, numerous spindle-shaped thrombocytes and neutrophils within a mesh, which resembled fibrin (Figure 4.13h).

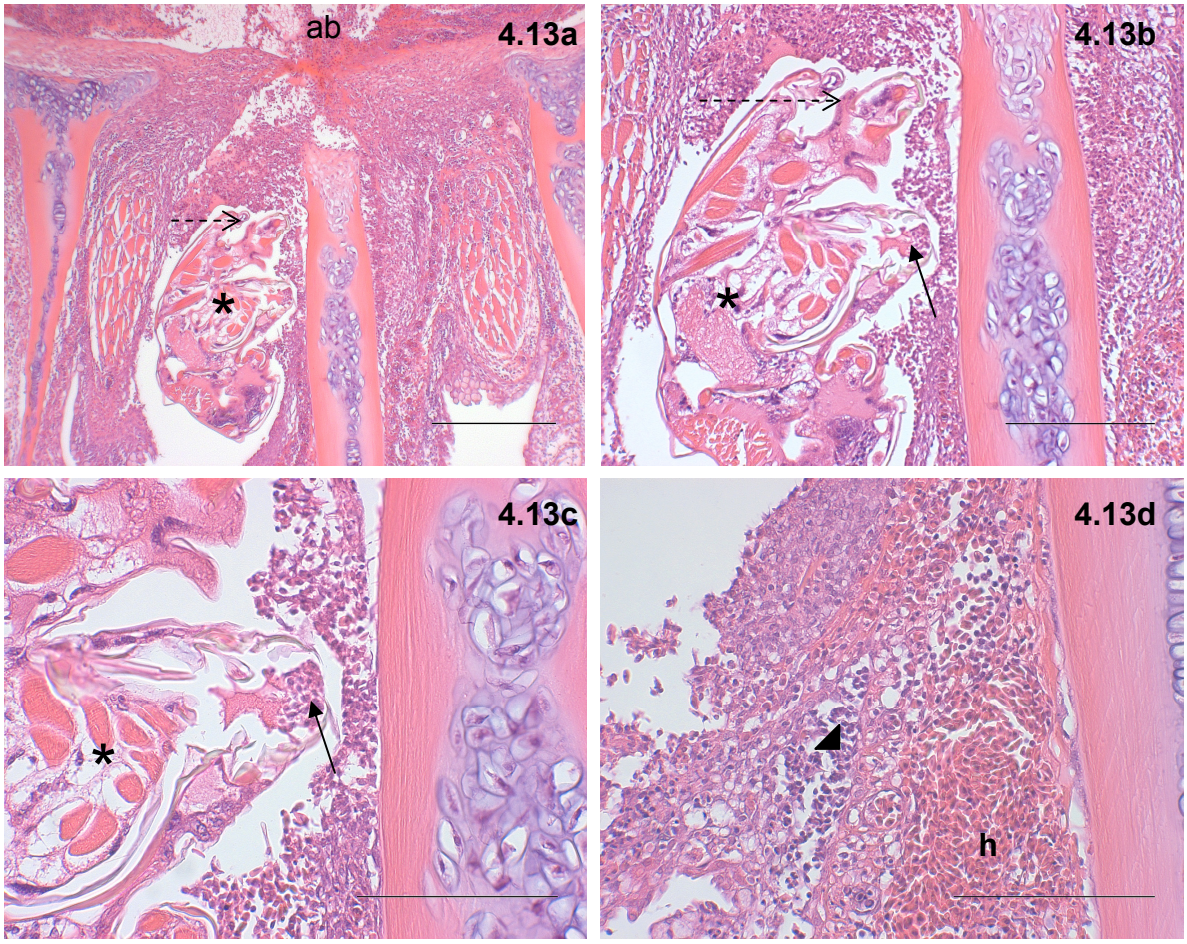

Figure 4.13 Local infection sites of dead *L. branchialis* 12 h post-killing by abdominal damage 36 h post-infection of Ardtoe-cultured cod, stained with H&E. (a) Dead *L. branchialis* cephalothorax burrowed into the base of the gill filament into the blood vessel to migrate into the afferent branchial artery (ab). (b) Close-up of the parasite cephalothorax embedded within the gill blood vessel, illustrating the oral cone (arrow). (c) Close-up of dead parasite oral cone filled with host tissue (arrow). (d) Haemorrhage (h) due to parasite attachment on gill filament infected by dead parasite, with leukocytes to the periphery (arrowhead). * = parasite; dashed arrow = parasite antennae. Bar = 100µm.

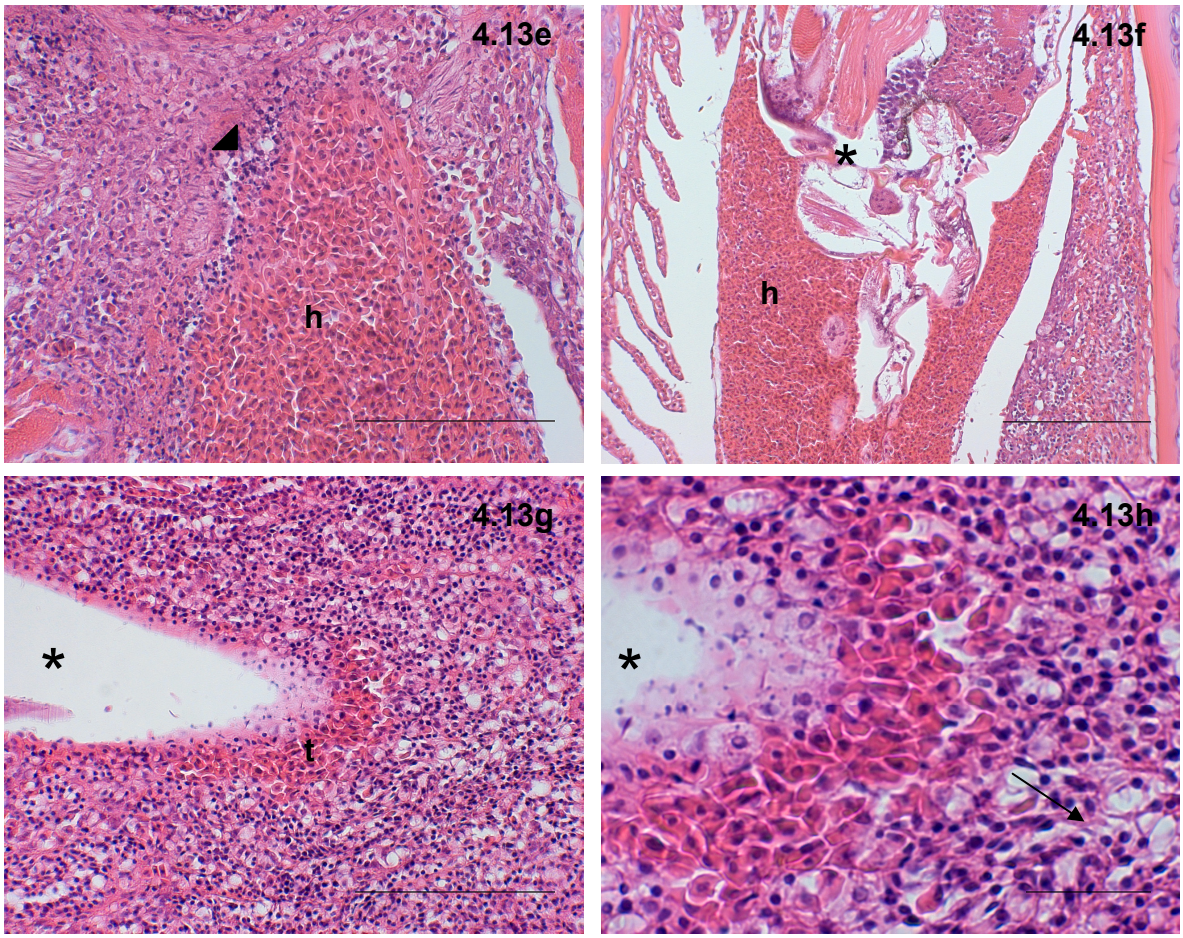

Figure 4.13 continued Local infection sites of live *L. branchialis* 36 h post-infection of Ardtoe-cultured cod, stained with H&E. (e) Haemorrhage (h) at entry point of live *L. branchialis* into the gill arch at the base of the gill filament as the cephalothorax migrates into the afferent branchial artery with leukocytes to the periphery (arrowhead). (f) Haemorrhage (h) surrounding a live parasite cephalothorax as it burrows into the gill arch at the base of the gill filament. (g) A live parasite that migrated into the afferent branchial artery with the beginning of the formation of a thrombus (t) around it. (h) Close-up of g illustrating the host cells surrounding the parasite in the afferent branchial artery including thrombocytes and neutrophils (arrow). * = parasite. Bar = 100µm.

4.3.3.5 Localisation of IgM by IHC at the site of infection of live versus dead *L. branchialis*

Positive IgM staining was observed in the plasma of the gill blood vessels of uninfected Ardtoe-cultured cod (Figure 4.14d). However, the blood vessels of gill filaments infected by dead or live parasites possessed a more intense positive IgM stain (Figure 4.14a and c). No positive IgM stain, however, was found on the cuticle or the gut of the live or dead parasites. Once parasites had entered the blood vessels of the gill filaments or the afferent branchial artery, haemorrhage ensued which contained positive IgM staining (Figure 4.14a and c). The oral cones of parasites that had entered the blood vessels occasionally contained host tissue which stained positive for IgM (Figure 4.14b). However, parasites attached to the exterior of the gill filaments by their antennae did not possess IgM in their oral cones. In conclusion, no qualitative differences were observed in the IgM staining patterns of the gills infected by dead or live parasites.

4.3.3.6 Localisation of C3 β chain by IHC at the site of infection of live versus dead *L. branchialis*

The gills of uninfected Ardtoe-cultured cod showed some slight positive staining for C3 in the vascular system (Figure 4.15c and d). However, gills infected by live or dead parasites possessed positive C3 staining in the gill filament blood vessels of infected and adjacent gill filaments (Figure 4.15a). This positive staining remained in the filament blood vessel when the parasite had migrated into the gill arch with a slight positive stain in the afferent branchial artery (Figure 4.15b). The haemorrhage that occurred upon the parasite burrowing into the filament blood vessel or the afferent branchial artery did not however stain positive for C3 (Figure 4.15b).

It was also noted that the parasite gut and parts of the epithelium under the cuticle stained positive after IHC staining (Figure 4.15a). However, no qualitative differences in the positive C3 staining of the cod gills 36 h.p.i. with live or dead parasites were observed.

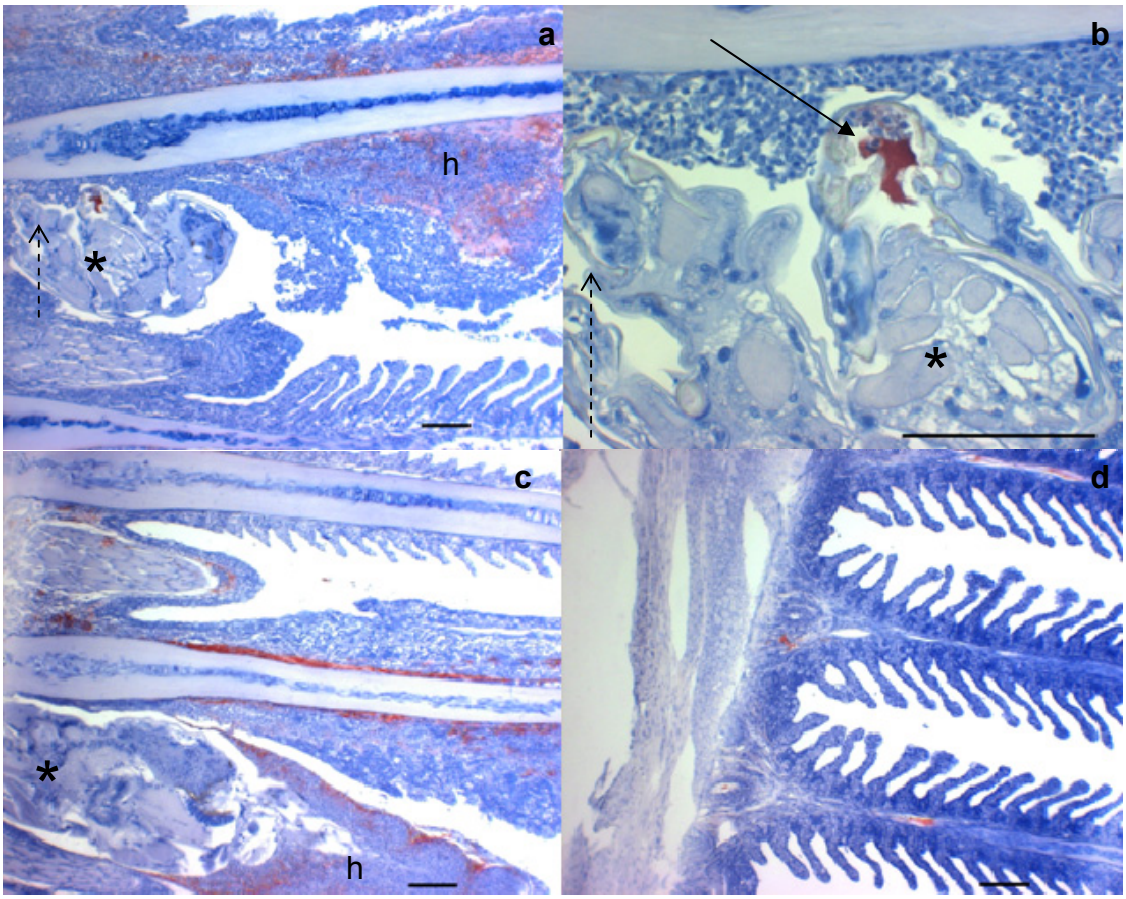

Figure 4.14 Localisation of host IgM (positive reaction indicated by red colour) at the infection sites of (a-b) dead *L. branchialis* 12 h post-killing by abdominal damage and (c) live *L. branchialis* 36 h post-infection of Ardtoe-cultured cod by immunohistochemistry. (a) Dead *L. branchialis* cephalothorax burrowed into the gill filament blood vessel to migrate into the afferent branchial artery. (b) Close-up of the cephalothorax in (a), illustrating the host tissue and positive IgM staining in the oral cone of the parasite (arrow). (c) A live parasite as it burrows into the gill arch at the base of the gill filament, surrounded by a haemorrhage (h) at the point of entry. (d) Location of host IgM in an uninfected cod gill. * = parasite; dashed arrow = parasite antennae. Bar = 100µm.

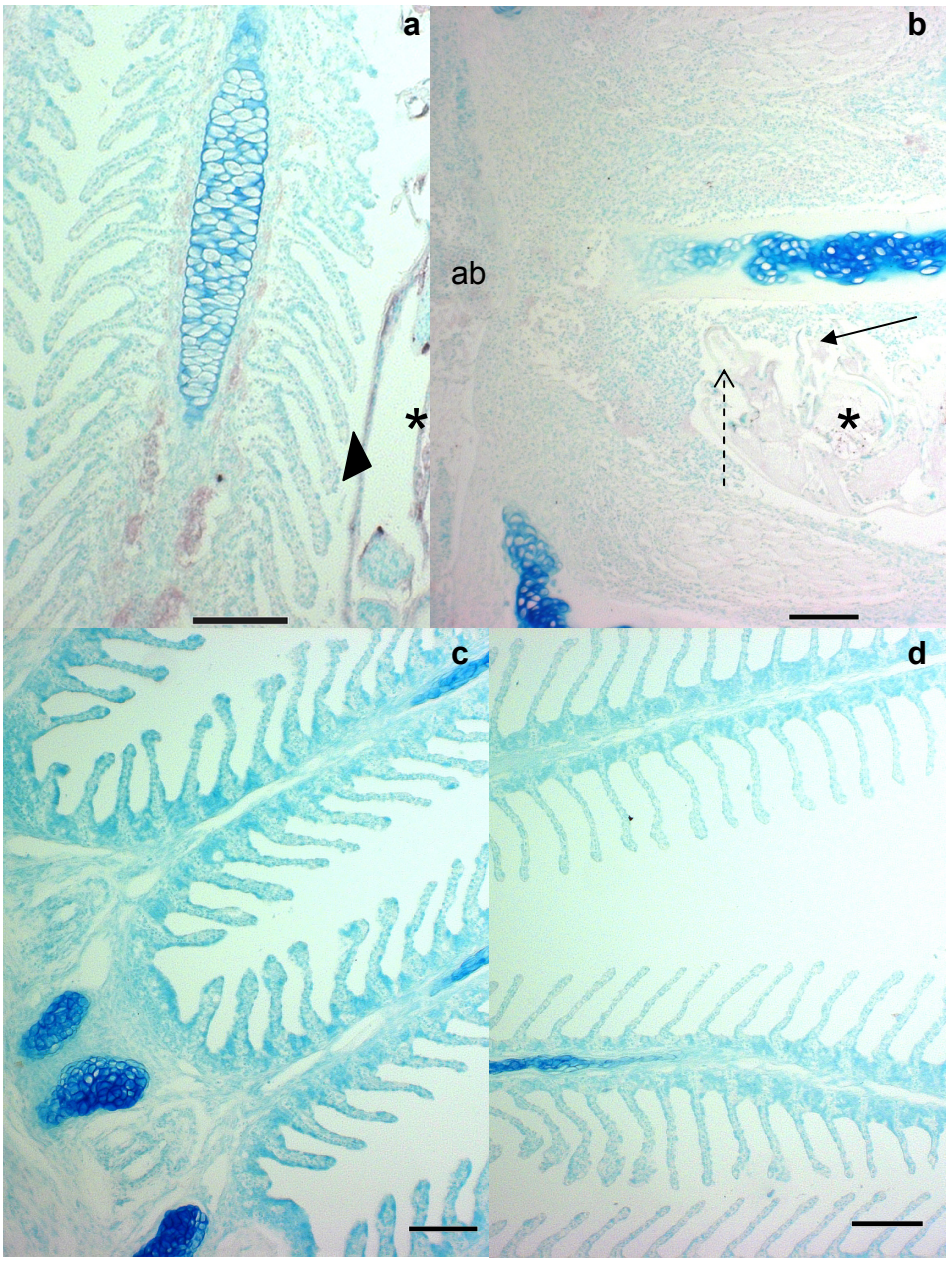

Figure 4.15 Localisation of host C3 (positive reaction indicated by purple colour) at the infection sites of (a) live *L. branchialis* and (b) dead *L. branchialis* 12 h post-killing by abdominal damage 36 h post-infection of Ardtoe-cultured cod by immunohistochemistry. (a) Live parasite attached to gill filament by antennae with C3 within the filament blood vessel. (b) Dead parasite embedded in gill filament with faint C3 staining in the afferent branchial artery (ab). Location of host C3 in the (c) base and (d) tips of an uninfected cod gill. * = parasite; arrow = parasite oral cone; dashed arrow = parasite antennae; arrowhead = parasite genito-abdominal region. Bar = 100µm.

4.3.3.7 Localisation of secretory IgM gene expression (mRNA) by *in situ* hybridisation (ISH) at the site of infection of live versus dead *L. branchialis*

Cells expressing the sIgM gene were observed lining the branchial arteries of the gill arches and the blood vessels of the gill filaments within the connective tissue of the uninfected gills of Ardtoe-cultured cod (Figure 4.16c). This pattern of distribution of sIgM positive cells was also observed in the gills of cod infected by live and dead parasites. No sIgM positive cells were found at the attachment site of parasites to the gill filaments (Figure 4.16a) or within the blood vessels, such as the afferent branchial artery, as the parasite migrated within it (Figure 4.16b).The haemorrhage that followed the entry of the parasite into the host blood vessels also did not show any positive sIgM cells. Therefore, no qualitative differences were observed in the distribution of sIgM positive cells of gills 36 h.p.i. with dead or live parasites.

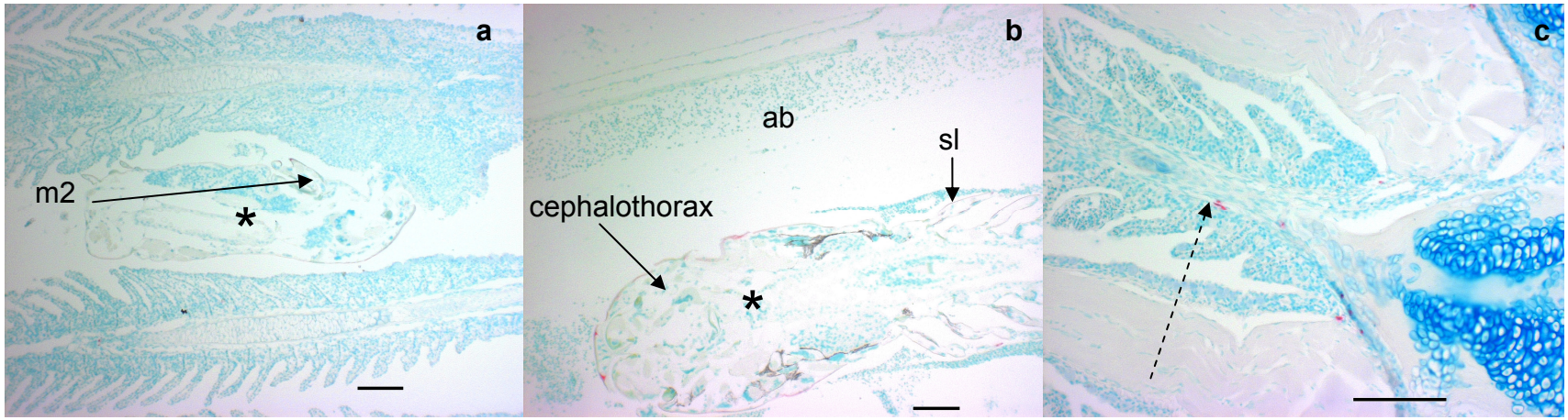

Figure 4.16 Localisation of secretory IgM (sIgM) gene expression (positive reaction indicated by red colour) at the infection sites of (a) dead *L. branchialis* 12 h post-killing by abdominal damage and (b) live *L. branchialis* 36 h post-infection of Ardtoe-cultured cod by *in situ* hybridisation. (a) Dead parasite attached to gill filament by antennae. (b) Live parasite migrated into the afferent branchial artery of the gill arch. (c) sIgM gene expression in an uninfected cod gill. * = parasite; dashed arrow = sIgM mRNA positive cell; ab = afferent branchial artery; m2 = 2nd maxilla; sl = swimming leg. Bar = 100μm.

4.3.3.8 Localisation of transmembrane IgM gene expression (mRNA) by ISH at the site of infection of live versus dead *L. branchialis*

The gills of uninfected Ardtoe-cultured cod possessed tIgM positive cells within and lining the blood vessels of the gill filaments from the base (Figure 4.17b) to the tips (Figure 4.17c). This distribution of tIgM positive cells was maintained in the gills of cod infected by dead or live parasites. No tIgM positive cells were observed at the attachment site of parasites on the gill filaments (Figure 4.17a), within the haemorrhage ensuing parasite entry into the blood vessels, or within the thrombus forming around the parasite as it migrates along the afferent branchial artery. Therefore, no qualitative differences were observed in the distribution of tIgM positive cells in the gills of cod 36 h.p.i. with live or dead parasites.

4.3.3.9 Localisation of interleukin-8 gene expression (mRNA) by ISH at the site of infection of live versus dead *L. branchialis*

The gills of uninfected Ardtoe-cultured cod possessed rare, scattered and faintly IL8 transcript positive cells within the blood vessels of the gill arch. This distribution of IL8 positive cells was maintained in the gills of cod infected by dead or live parasites. However, no IL8 positive cells were observed at the attachment site of dead or live parasites on the gill filaments (Figure 4.18a and b), within the haemorrhage ensuing parasite entry into the blood vessels, or within the thrombus forming around the parasite as it migrates along the afferent branchial artery. Therefore, no qualitative differences were observed in the distribution of IL8 positive cells in the gills of cod 36 h.p.i. with live or dead parasites.

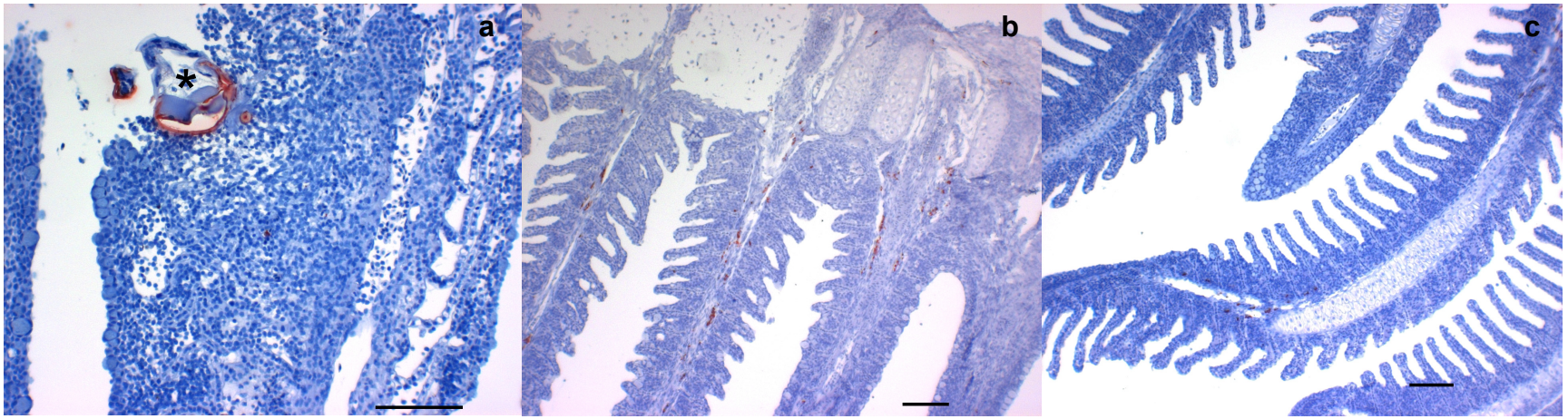

Figure 4.17 Localisation of transmembrane IgM (tIgM) gene expression (positive reaction indicated by red colour) at the infection sites of (a) dead *L. branchialis* 12 h post-killing by abdominal damage 36 h post-infection of Ardtoe-cultured cod by *in situ* hybridisation. Note artefact of positive staining of parasite cuticle. (a) Dead parasite attached to gill filament. sIgM gene expression at the (b) base and (c) tips of an uninfected cod gill. * = parasite. Bar = 100µm.

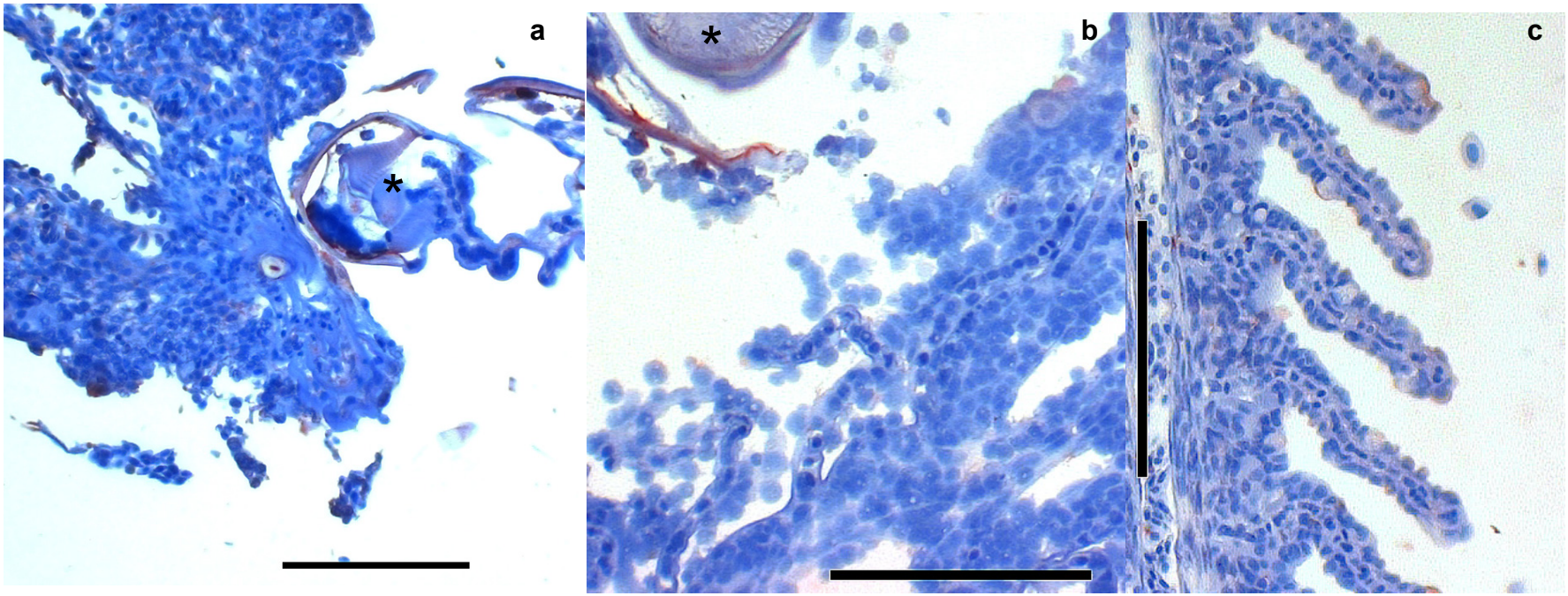

Figure 4.18 Localisation of interleukin 8 (IL8) gene expression (positive reaction indicated by red colour) at the infection sites of (a) dead *L. branchialis* 12 h post-killing by abdominal damage and (b) live *L. branchialis* attached to the gill filament 36 h post-infection of Ardtoe-cultured cod by *in situ* hybridisation. (c) IL8 gene expression in an uninfected cod gill. Note artefact positive staining of parasite cuticle. * = parasite. Bar = 100µm.

4.4 Discussion

4.4.1 The localised immune response to *L. branchialis* infection over time

The local immune response of Atlantic cod to infection by *L. branchialis* was studied in terms of the localisation of the host proteins IgM and C3, host cells positive for transcripts of the proinflammatory cytokine IL8, and host cells positive for tIgM and sIgM transcripts, *i.e*. B and plasma cells, at the site of infection over a time series. The histopathology at the local site of infection is not described in great detail due to the presentation of similar findings in a previous publication by Smith *et al.*, (2007). *L. branchialis* initially attached to the gill filaments by their antennae 1 d.p.i., which led to histopathological changes which were limited to the affected gill filament. By 3 d.p.i., the majority of parasites had migrated into the gill arch residing within the afferent branchial artery, with haemorrhaging at the point of entry and thrombus formation around the parasite, as described by Smith *et al.* (2007). The parasite's migration continued with its presence in the ventral aorta at the tip of the *bulbus arteriosus* 7 d.p.i., where it remained 14 d.p.i., continuing its metamorphosis to resemble an early penella sub-stage. The thrombus remained around the parasite as it migrated into the ventral aorta becoming more fibrin-rich and organised. Smith *et al.,* (2007) described the thrombus formation to be a dynamic process following the migration and growth of the parasite (p10), which became more organised with the in-growth of granulation tissue from the arterial wall into the fibrin-rich thrombus. They suggested that in time, the thrombus would become an endothelial swelling of the arterial wall, embedding the parasite within the wall of the artery or *bulbus arteriosus*. A similar scenario occurs in green turtles (*Chelonia mydas*) infected by cardiovascular spirorchid flukes. The flukes and their egg shells are found within

resolving arterial thrombi with re-canalisation of the blood vessel and the transfer of the thrombus to the exterior of the artery lumen (Gordon *et al.*, 1998). These authors described the fluke egg shells within thrombi to be resistant to destruction by phagocytosis, resulting in chronic granulomatous inflammation with the formation of a granuloma around the parasite, which resulted in the parasite residing to the exterior of the artery lumen.

A longer *L. branchialis* infection study was carried out which followed the infection up to 56 d.p.i., at which point the parasites were dead and undergoing degradation, and were seen to be encapsulated within host tissue. These parasites had failed to undergo metamorphosis. The rejection of other copepod parasites, such as *Lernaea cyprinacea* and *L. polymorpha,* reported in naïve and previously infected fish is suggested to be partially as a result of the host's cellular response (Shields & Goode, 1978; Woo & Sharriff, 1990). The degradation of parasites has often been associated with their encapsulation within host tissue and the adherence of host leukocytes to their tegument (Kirk & Lewis, 1998; Richards *et al*., 1996; Nakayasu *et al*., 2005). For instance, the eggs and emigrating miracidia of *Sanguinicola inermis* blood flukes are encapsulated within host granulomatous inflammatory tissue of the gills 42 d.p.i. of carp with their subsequent degradation 90 d.p.i. (Kirk & Lewis, 1998).

4.4.1.1 Localisation of host IgM and C3 β chain by IHC at the site of infection

The monoclonal anti-cod IgM and polyclonal anti-cod C3 antibodies stained extracellular host IgM and C3, respectively, within the plasma of the gills of Atlantic cod. The C3 staining is in accordance with Abelseth *et al*. (2003) who observed C3

protein within the veins and arteries of *Anarhichas minor* (wolfish) liver, head kidney, gills and spleen, but not associated with any of the blood cells. This was found to be more intense in the blood vessels of filaments infected by the parasite 1 d.p.i. This suggested an accumulation of IgM and C3 in the affected filaments, but could have also been due to congestion and widening of the blood vessels resulting in increased host plasma containing IgM and C3 at the site, as a consequence of the attachment of the parasite. However, adjacent gill filaments in contact with the parasite also showed intense C3 staining of the blood plasma. This suggests that the parasite, possibly due to its large size, irritated the adjacent filament by abrasion resulting in an increase in C3. C3 has been found to be an acute phase protein in teleosts and its transcription is induced by pro-inflammatory cytokines (Bayne & Gerwick, 2001; Bayne *et al.*, 2001). A minority of parasites remained attached to the gill filament by their antennae 3 d.p.i., compared to the majority which migrated into the gill arch. Local histopathological changes of these filaments were largely comprised of epithelial hyperplasia and fusion of the secondary lamellae, with numerous mucous cells present. An intense accumulation of host IgM was also observed in the infected filament blood vessel. It would be interesting to determine whether these parasites are rejected from the host due to their slower migration, however, due to the terminal sampling of fish this was not examined. As the majority of parasites migrated into the afferent branchial artery of the gill arch at 3 d.p.i., haemorrhage occurred at the point of entry, which stained positive for IgM, and a cellular aggregate and thrombus formed around the parasite which followed the migration of the parasite into the ventral aorta at 7 and 14 d.p.i.. Host C3 and IgM were both identified around the periphery of this thrombus and cellular aggregation during the migration of the parasite at all subsequent sampling times.

This probably represented positive staining of C3 and IgM proteins within the plasma of the free-flowing blood within the afferent branchial artery and ventral aorta around the cellular aggregate within the vessel lumen. The more intense staining of IgM than C3 within the plasma of the afferent branchial artery and ventral aorta may be due to the fact that Atlantic cod plasma IgM concentrations are known to be relatively high in comparison to that of salmonids (Israelsson *et al*., 1991; Pilstrom & Petersson, 1991). A lack of increase in the intensity of the positive staining of the plasma in infected fish compared to uninfected fish also correlates with the fact that cod possess a low or absent specific antibody response to immunisation or infection (Espelid *et al*., 1991; Pilstrőm & Petersson, 1991; Magnadóttir *et al*., 2001; Magnadóttir *et al*., 2002), and the total IgM concentrations of these cod did not change post-infection compared to uninfected fish (see Chapter 3, Section 3.3.2.10). However, by 14 d.p.i. a clear binding of C3 occurred within the outer portion of the cellular aggregate around the parasite. The binding of complement has also been observed to occur on the periphery of sarcoid granulomas in human lymph nodes (Ghose *et al*, 1974). C3 is the central component of the complement system and its up-regulation has previously been associated with parasitic infections in teleosts (Chang *et al*., 2005; Sigh *et al*., 2004b; Gonzalez *et al*., 2007b). This has sometimes been observed to be an up-regulation in the gene expression of extra-hepatic C3 and other complement molecules in the parasite's target host tissue, for example the skin of rainbow trout and carp infected by *I. multifiliis* (Sigh *et al*., 2004b, Gonzalez *et al*., 2007b). However, no C3 or IgM was found on the cuticle of the parasite at any of these sampling times. Numerous parasites have developed mechanisms of preventing C3 and IgM binding to their exterior surface, such as the adsorption of host molecules

or capturing complement inhibitors on their surface. For instance, adult male *Schistosoma mansoni* are thought to adsorb host proteins to their surface masking alternative complement activation sites (Rasmussen & Kemp, 1987), *Onchocerca volvulus* microfilariae bind the soluble host alternative pathway inhibitor factor H to their surfaces (Meri *et al*., 2002), and *Sanguinicola inermis* (a blood fluke of carp) possess 'host-like' molecules on their surface thought to aid their evasion of their host's immune response, including IgM binding (Roberts *et al*., 2005). Positive C3 staining did occur on the gut epithelium of the parasites at all stages of the infection, and on parts of the parasite's cuticular epithelium. However, whether the positive reaction observed was host C3 or a parasite-derived protein with which the anti-cod C3 antibody cross-reacted requires further investigation. This is especially, the case as the parasite is not thought to start feeding, *i.e*. intake host material, until it reaches the ventral aorta or *bulbus arteriosus*, and as the stained parasite epithelial cells have no direct contact with the host. However, at 56 d.p.i., C3 was observed on the exterior of the cuticle of dead and degrading parasites encapsulated within host tissue. Host IgM was also identified on the degrading parasite, as well as within the necrotic centre of the host reaction. The co-localisation of C3 and extracellular IgM on the dead parasite could suggest the involvement of the classical complement system. However, confirmation of this would require further in-depth investigation. IgM and C3 are also known opsonins involved in the adherence of leukocytes to parasites (Hoole & Arme, 1986; 1988). For instance, macrophages and granulocytes adhere via Fc and C3b receptors directly to the tegument of schistosomula of the trematode *Schistosoma mansoni,* inducing structural damage and eventually death of the parasite (Butterworth, 1984). The activation of complement could also initiate the formation of the membrane attack

complex and the release of anaphylatoxins, which recruit leukocytes to the site of infection (Gasque, 2004; Sunyer & Lambris, 1998). However, to further elucidate the scenario leading to the death of the parasite in these fish, the local immune response of samples taken from Ardtoe-cultured cod prior to parasite death should be examined. The lack of C3 and IgM staining on the cuticle or in the direct vicinity of live *L. branchialis* in Machrihanish-cultured cod does, however, suggest that the parasite could be capable of evading C3 and IgM binding, at least up to 14 d.p.i.. This may be due to the cuticle of the parasite being less vulnerable to IgM and C3 binding and their downstream effects, whereas the gut of the parasite is more susceptible to these immune factors. This is highlighted by the fact that the midgut, *i.e.* the non-cuticularised gut portion, stained positive for cod C3. However, many haematophagous parasites have developed evasion mechanisms to prevent the deleterious effects of the immune response occurring subsequent to or prior to the binding of host molecules to their gut. Bricknell *et al*. (2003) stated that the function of host immuno-reactive molecules, such as C3 and antibodies, are likely to be severely impeded by the environmental conditions within the gut of salmon lice, such as the presence of proteolytic enzymes and a difference in the pH and osmolarity of the gut compared to host plasma. Bricknell *et al*. (2002) found Atlantic salmon IgM to have a reduced affinity for their epitope when the environmental pH increased or decreased by more than 2 units of salmon plasma. Therefore, the conditions within the gut lumen of *L. branchialis* deserve further study, as they may impair the function of the host immune factors taken in with the blood meal. Whether or not *L. branchialis* possesses a peritrophic matrix is also a key question which requires addressing. Other haematophagous insects and marine crustaceans, such as calanoids and caligids (Brunet *et al.*, 1994; pers comm. Dr. J.

Bron), possess a peritrophic matrix which forms a layer between the midgut epithelium and the food, protecting the gut from mechanical damage, toxins and pathogen insult (Lehane, 1997). It forms a size-selective barrier to macromolecules larger than 90kDa within the gut lumen, and therefore it has been hypothesised that it could prevent host antibody binding to the gut epithelial cells of haematophagous parasites (Bricknell *et al*., 2003).

The later stages of infection post-fourteen d.p.i. still need to be studied, which was the purpose of the longer infection study of Ardtoe-cultured cod. However, this was not possible in this study due to the death of the parasite prior to the achievement of full metamorphosis.

4.4.1.2 Localisation of sIgM and tIgM gene expression (mRNA) by ISH at the site of infection

Atlantic cod gill, head kidney and spleen tissues 2 d.p.i. with *L. branchialis* were all found to possess expression of the genes encoding IL1β, IL8, tIgM and sIgM. This is in accordance with studies by Seppola *et al*. (2008), Caipang *et al*. (2009), and Schroder *et al.* 1998, who found constitutive expression of these genes in uninfected cod, and upregulation of the proinflammatory cytokines (IL1β and IL8) post-injection with inactivated *V. anguillarum* or Poly I:C. Unfortunately, these tissues were not found to express IL10 transcripts in the current study and therefore, a probe to locate IL10 transcript positive cells could not be produced.

Host cells positive for sIgM transcripts *i.e.* plasma cells were found to be grouped in clusters associated with the vascular system and scattered throughout the haematopoietic tissue of the head kidney, whereas the cells positive for tIgM *i.e*. B

cells were observed to be scattered evenly throughout the haematopoietic tissue of the head kidney. This is in accordance with previous studies in Atlantic cod by Stenvik *et al.* (2001) and Schroder *et al*. (1998). The clusters of plasma cells have previously been found to be specific for a particular VH-family, and it is not known how they are formed (Stenvik *et al*., 2001). They have been found to occur in non-immunised cod and are thought to be induced spontaneously or by 'natural antigens' resulting in the high serum IgM concentrations observed in cod (Stenvik *et al.*, 2001). The gills of uninfected cod constitutively expressed sIgM within cells of the connective tissue lining the branchial arteries in the gill arch and the blood vessels of the filaments. The same was observed for tIgM expression, which was observed in cells lining the blood vessels of the gills but also circulating within the blood. These results were also in corroboration with Schroder *et al*. (1998). The association of cells positive for IgM transcripts with the vascular system has led researchers to highlight the likely importance of these areas in antigen trapping and lymphocyte stimulation (Schroder *et al.*, 1998; Stenvik *et al.*, 2001).

Post-infection with *L. branchialis* the distribution of tIgM and sIgM positive cells did not change in the gills, and no positive cells were observed in the local vicinity of the parasite *i.e.* within the initial attachment site, the haemorrhage at the point of entry into the gill arch, the thrombus and cellular aggregate around the parasite as it migrated along the afferent branchial artery into the ventral aorta, or within the host tissue encapsulating dead parasites. This lack of IgM transcript positive cells at the site of infection 1-14 d.p.i. was expected due to the fact that IgM positive cells usually appear later post-infection or post-vaccination in teleosts, and that the parasite is migrating constantly to new locations until it resides within the ventral

aorta between 7 and 14 d.p.i.. For instance, Bermudez *et al.* (2006) observed IgM positive cells to increase progressively in the intestine (the target organ) of turbot post-infection with *Enteromyxum scophthalmi* (a myxozoan parasite) and to peak 78d post-exposure to the parasite. Sitja-Bobadilla *et al*. (2006) hypothesised that T lymphocytes are recruited to the site of infection initially, whereas B cells appear later. IgM positive cells are also rarely associated with certain parasitic infections, such as in the lesions of amoebic gill disease (AGD; K.A. Grove unpublished results cited in Morrison *et al.*, 2006). In the present study, at 56 d.p.i. numerous tIgM and sIgM transcript positive cells were observed to the periphery of the encapsulated dead *L. branchialis* found in Ardtoe-cultured cod. Many studies have reported the localisation of IgM positive cells at the site of parasitic infections and vaccination in teleosts (Bermudez *et al*., 2006; Steinhagen & Rombout, 1994; Zhao *et al*., 2008; Feng *et al*., 2009). The location of B and plasma cells at the periphery of the host cellular reaction corresponds to other reports where IgM positive cells were identified at the periphery of granulomas and in granulomatous tissue (de Brito & Franco, 1994; Hurst *et al*., 2006; Pirarat *et al*., 2007). Piraret *et al*. (2007) observed IgM positive cells in the marginal zones of granulomas and within the granulomatous tissue post-infection of tilapia (*Oreochromis niloticus*) with the bacteria, *Edwardsiella tarda*. These authors suggested that the IgM positive cells influenced and enhanced the phagocytic activity of cells involved in the granuloma enhancing bacteria clearance. The identification of plasma cells in the current study suggests localised antibody production, which correlates with the identification of extracellular IgM on the dead and degrading parasites.

4.4.1.3 Localisation of the gene expression of the proinflammatory cytokine IL8 (mRNA) by ISH at the site of infection

The gills of uninfected cod were found to possess rare leukocytes faintly positive for IL8 transcripts within the afferent branchial artery of the gill arches. This constitutive expression of IL8 correlates with Seppola *et al*. (2008) and Caipang *et al.* (2008) who also observed constitutive expression of IL8 in the gills and blood of cod, respectively. Post-infection with *L. branchialis* no IL8 transcript positive cells were observed in the direct vicinity of the parasite *i.e.* within the initial attachment site, the haemorrhage at the point of entry into the gill arch, the thrombus and cellular aggregate around the parasite as it migrated along the afferent branchial artery into the ventral aorta, or within the host tissue encapsulating dead parasites. Faintly positive leukocytes were however, identified within the affected gill filament blood vessels 1 d.p.i., but only when the parasite had burrowed into the filament blood vessel. This is likely to be due to the increased damage caused by the parasite, as it burrows into the filament blood vessel, activating the host leukocytes initiating the recruitment of more leukocytes to the site to help prevent the establishment of the infection. However, the staining of these leukocytes was still faint, suggesting a low level of IL8 transcript production at this time point. On the other hand, by 14 d.p.i., leukocytes to the periphery of the host reaction around the parasites within the lumen of the ventral aorta were found to stain intensely for IL8 transcripts, suggesting higher IL8 production by these leukocytes at this stage of the infection. Increased host IL8 gene expression has been associated with other parasitic infections in teleosts (Jones *et al*., 2007; Bridle *et al*., 2006b; Sigh *et al*., 2004a; Covello *et al*., 2009), sometimes being observed in the target tissue, such as in the skin of rainbow trout during *I. multifiliis* infection (Sigh *et al*., 2004a) and the gills of *Latris lineata* (three striped trumpeter) post-infection by the ectoparasite,

Chondracanthus goldsmidi (Covello *et al*., 2009). In *Oncorhynchus gorbuscha* (pink salmon), the increased expression of pro-inflammatory cytokine genes including IL8, has been suggested to be involved in the innate immune response involved in the rejection of *Lepeophtheirus salmonis* in this relatively resistant salmonid host (Jones *et al*., 2007). In the present study, these leukocytes were identified within the free-flowing blood of the ventral aorta, and resembled large phagocytic cells. However, the exact identification of the type of leukocyte expressing IL8 transcripts requires further study with specific cell markers. The mammalian cell types associated with IL8 production comprise primary endothelial cells, macrophages / monocytes, fibroblasts, epithelial cells and neutrophils (Peveri *et al.*, 1988; Scapini *et al.*, 2000; Murphy *et al*., 2008; Jimenez *et al*., 2006). Caipang *et al*. (2008) suggested that infiltrating neutrophils releasing IL8 resulted in the sustained IL8 expression in the blood of cod post-vaccination with *Vibrio anguillarum*. The main functions of IL8 observed in mammals include the induction of neutrophil activation, migration and degranulation (Peveri *et al*., 1988; Schroder *et al*., 1987; Scapini *et al*., 2000). Therefore, the expression of IL8 transcripts in leukocytes within the ventral aorta of *L. branchialis*-infected cod may be involved in recruiting and activating neutrophils at the site of infection. IL8 has also been found in mammals to induce a more rapid maturation of granulation tissue during wound healing (Moyer *et al*., 2002) and therefore, may be involved in enhancing the granulation tissue described by Smith *et al*. (2007), which is present as the thrombus around the parasite becomes more organised. Atlantic cod IL8 also possesses an ELR motif (Seppola *et al*., 2008), which is vital for the angiogenesis function of IL-8 in mammals (Rosenkilde & Schwartz, 2004; Strieter *et al*., 1995; Belperio *et al.*, 2000). Therefore, IL8 expression could be involved in neovascularisation at the infection

site, as Smith *et al*. (2007) described local neovascularisation of the granulation tissue resulting in the re-canalisation of the vessel. However, until species-specific recombinant proteins and antibodies are available for IL8 its exact functions in Atlantic cod cannot be concluded. The dead and degrading parasites at 56 d.p.i. however, were not associated with IL8 transcript positive cells.

The *in situ* probe produced to detect IL1β did not work in this study and, due to time and material constraints, could not be optimised. This merits further study, as high IL1β expression was observed by RT-PCR in the gills, head kidney and spleen of cod 2 d.p.i. with *L. branchialis*. This is especially the case as IL1β is a key pro-inflammatory cytokine involved in the host response to numerous bacterial, viral and parasitic infections in teleosts (Grayson *et al*., 2002; Engelsma *et al*., 2003; Lindenstrom *et al*., 2003; Pressley *et al*., 2005; Purcell *et al*., 2004; Saeij *et al*., 2003; Sigh *et al*., 2004a; Tafalla *et al*., 2005; Zou *et al*., 1999; Bridle *et al*., 2006a & b).

4.4.2 Localised immune response to live versus dead *L. branchialis*

The local immune responses of Atlantic cod to live and recently dead *L. branchialis* at the initial stages of the infection, *i.e.* 36 h.p.i., were qualitatively compared. It was originally hoped that a quantitative comparison of the number of cells positive for the transcripts of the immune genes per area of host tissue examined could be achieved. However, this was not possible due to the differences in the location of parasites 36 h.p.i., *i.e.* some still attached to the filament and others proceeded to burrow into the gill vascular system, as well as the scarcity of positive host cells at the infection site.

The ‘histological picture’ at the site of infection of live and dead parasites did not differ, and was influenced by the phase of the migration that the parasite had achieved, and was similar to reports from previous publications (Kabata, 1958; Smith *et al*., 2007). The pathology caused by the parasites that were still attached to the gill filaments by their antennae tended to be restricted to the affected gill filament, with epithelial hyperplasia and fusion of the secondary lamellae with numerous mucous cells present. This is in accordance with Kabata (1958), who observed the juvenile stages of the parasite to result in thickening of the terminal points of the filament and lamellar fusion as a result of host tissue proliferation. The proliferation of mucous cells deserves further study to quantitatively determine if there is a significant increase in their number post-infection. For instance, other ectoparasitic copepods, such as *Ergasilus sieboldi* infecting the gills of *Abramis brama,* have been found to result in mucous cell proliferation within the respiratory epithelium (Dezfuli *et al.,* 2003). Fish mucus contains numerous innate immune factors, such as complement, lectins, lysozyme, C-reactive protein, anti-bacterial peptides, IgM, and haemolysins (Alexander & Ingram 1992; Rombout *et al* 1993; Aranishi & Nakane 1997). Therefore, the effect of host mucus on the establishment of *L. branchialis* should not be overlooked at these early stages of the infection. For instance, Buchmann & Bresciani (1998) observed a significant negative correlation between the intensity of *Gyrodactylus derjavini* (monogean) infection and mucous cell density on the skin surface of rainbow trout. The ectoparasitic stage of the initial infection by *L. branchialis* however, is short, only lasting a few days, but the genito-abdominal region of the parasite remains exterior to the host within the gill cavity exposed to fish mucus.

The parasites further advanced in their migration through the host, had entered the gill vascular system, either the blood vessels of the filament or they had already migrated into the afferent branchial artery of the gill arch. Numerous spindle-shaped thrombocytes were found at the point of entry into the blood vessels, and neutrophils were also observed in the filament blood vessel. Thrombocytes are an important part of the blood clotting system in teleosts and are thought to be functionally analogous to mammalian platelets (Rowley *et al.*, 1997; Hill & Rowley, 1996). Teleost thrombocytes have also recently been suggested to be involved in antigen presentation and immune regulation. This was highlighted by a study of rainbow trout thrombocytes where they were observed to express immune genes including those encoding the pro-inflammatory cytokines IL1β and TNFα (Kollner *et al.*, 2004). The point of parasite entry in the current study was often marked by haemorrhaging, which clotted with thrombocytes and leukocytes at the periphery, and seemed capable of preventing further blood loss. Smith *et al.* (2007) did not observe newly attached females, however, they did hypothesise that the haemorrhagic foci they described at the base of the gill filaments were the entry points for the parasites. This was confirmed in the present study, as parasites were observed to migrate either along the filament blood vessel or into the gill arch at the base of the filament entering the afferent branchial artery resulting in local haemorrhaging. The aggregations of leukocytes, such as neutrophils, and thrombocytes at the periphery of the haemorrhage were probably recruited due to the tissue damage, to prevent further infection and to impede blood loss. Infection of *L. lineata* by the gill ectoparasite *C. goldsmidi* also resulted in the infiltration of neutrophils at the base of the filament near to the parasite's attachment site, as well as epithelial hyperplasia and mucous cell proliferation (Covello *et al*., 2009). In the

present study the host also formed a thrombus around the parasite as it migrated within the afferent branchial artery, which consisted of numerous thrombocytes and erythrocytes within a mesh likely to be fibrin. Smith *et al.* (2007) previously described this thrombus but stated that few leukocytes were present in the lesion. However, in the present study an aggregation of leukocytes had also occurred around the parasite, including numerous neutrophils. Many studies have also reported the appearance of neutrophils at the site of injured tissue in the primary stages of inflammation, where they are suggested to destroy pathogens by phagocytosis and / or cytotoxic-like activity (Afonso *et al*., 1998; Matsuyama *et al*., 1999; Reite & Evensen, 2006). This could be due to the fact that these thrombi were at an earlier stage of formation at 36 h.p.i., whereas those described in Smith *et al*. (2007) were sampled later at 5 d post-exposure to parasites. In mammals, haemorrhages and inflammatory sites with platelet-rich thrombi accumulate neutrophils around the platelets (Henry, 1961; Wester *et al*., 1979). This is thought to be due to a number of mechanisms including activated platelets releasing chemotactic proteins for leukocytes (Deuel *et al*., 1981; Deuel *et al.*, 1982), and a membrane glycoprotein of platelets and endothelial cells which binds neutrophils (Hamburger & McEver, 1990). The exact mechanisms involved in teleosts however, remain to be elucidated. Recent studies by Ronneseth *et al.* (2007) have, however, suggested that neutrophils possess a central role in the immune response of cod due to the fact that they make up a high proportion of the peripheral blood leukocytes in comparison to other teleosts.

The distribution of host IgM and C3 proteins, and tIgM, sIgM and IL8 transcript positive cells also did not show any qualitative differences between the gills of fish

infected with live or dead parasites. The distribution of the above factors observed in the gills of Ardtoe-cultured cod followed the same pattern as that described for the infected Machrihanish-cultured cod 1-3 d.p.i.. However, some of the parasites that had burrowed into the blood vessels showed host cells and blood plasma within their oral cones, which was positive for IgM but not C3. Whether this was host blood flowing into the oral cone or blood actively taken into the oral cone by the parasite during feeding remains to be determined, however the parasite is thought not to feed until it reaches the final infection location. For instance, Smith *et al*. (2007) found host red blood cells in the gut of *L. branchialis* once it had reached the late U form at 33 d post-exposure to infected intermediate hosts. This highlights the intimate exposure of the parasite to host immune molecules due to their meso-parasitic and haematophagous lifestyle, and therefore, the likelihood that this parasite possesses immune evasion techniques. The lack of differences between the immune response to live versus dead parasites suggests that this early stage of the parasite does not actively produce products involved in the modulation / evasion of the immune parameters measured in this study. This is based on the assumption that once the parasite is dead the release of parasite-derived molecules ceases, and no ‘leakage’ occurs into the host tissue. However, from this part of the study it cannot be concluded whether this stage of the parasite produces molecules involved in the evasion or modulation of other host immune responses, or that later stages of the parasite produce molecules involved in immune evasion of the studied host factors. This is especially the case due to the fact that, IL8 transcript positive cells and intense C3 binding were not associated with the infection site of live parasites until much later, at 14 d.p.i. This therefore, requires further study involving the killing of parasites over time to look at the differences between dead versus live

parasites at different stages of the infection. This study also did not take into account immune evasion techniques other than the active secretion of immuno-modulatory molecules, such as the properties of the parasites cuticle. For instance, some parasites such as *S. inermis* possess host-like proteins on their surface thought to aid evasion of host recognition as non-self (Roberts *et al*., 1995). Therefore, the cuticle of *L. branchialis* also deserves further study.

4.4.3 Conclusions

In summary, this study has highlighted the possible importance of host C3 and IgM in the host's elimination of *L. branchialis*. This is due to the fact that live parasites up to 14 d.p.i. did not show IgM or C3 binding to their cuticle, whereas dead and degrading parasites 56 d.p.i. showed positive staining for both parameters. This may also imply the involvement of complement-fixing antibodies in the process. However, this is not conclusive as the local immune response leading up to the death of the parasite at earlier time points needs to be investigated and compared to that of live successful parasites. The immune response of hosts infected by successful parasites undertaking full metamorphosis should also be investigated, as, due to the termination of the study at 14 d.p.i. (due to an unforeseen loss of stock cod), whether the parasites examined would have been successful to the end of their infection or rejected by the host at a later stage can not be concluded. This could have considerable relevance to the question of whether the C3 binding and IL8 transcript positive leukocytes, observed at 14 d.p.i., and the positive C3 staining of the parasites gut are or are not involved in the elimination of the parasite. However, the positive staining of the gut requires further investigation as the gut of the newly attached parasites prior to entering the hosts' blood vessels also stained positive for host C3. The observation of IL8 transcript positive leukocytes 14 d.p.i. at

the periphery of the cellular aggregate within the ventral aorta lumen may reflect their involvement in the recruitment to and activation of leukocytes at the site, and the maturation and neovascularisation of granulation tissue. However, this suggestion remains speculative until the exact functions of IL8 in Atlantic cod are elucidated. In hindsight, a positive control for the parameters measured, such as the use of tissues sampled from cod post-immunisation with a known immuno-stimulant, such as Freund's complete adjuvant or bacteria, would have been useful in order to confirm that the techniques were working, and to ascertain what a strong response to infection would look like in cod. This is especially true for the IHC staining with the anti-cod IgM and C3 antibodies which was relatively weak. The ISH technique was chosen in this study due to the large size of the parasite and the localised host pathology. However, this was time-consuming and only a small number of immune genes could be studied. Therefore, further work should include the quantification of host immune gene transcripts by QRT-PCR or microarray / deep sequencing of tissue from the local infection sites *i.e.* gill, blood and ventral aorta, in order to study a wider array of immune genes post-infection, which would also narrow down the list of candidate genes for further study by ISH. The large intravascular thrombi observed around parasites as they migrate and metamorphose in the vascular system have been observed previously by Smith *et al*. (2007). However, the initial attachment of the parasite immediately post-infection has not previously been studied in detail. The pathology associated with this initial attachment was very localised, mostly affecting the infected gill filament, and was very limited. However, once the parasite entered the host's vascular system a more pronounced effect was observed with thrombus formation and a more pronounced influx of leukocytes and thrombocytes. The identification of host tissue and IgM

within the oral cone of parasites that had initially burrowed into the blood vessels also highlighted the importance of possible immune modulation and evasion mechanisms for the parasite in order to survive. Therefore, the properties of the parasite's cuticle and gut during the infection process deserves further study, such as the adsorption of 'host-like' molecules, as observed in other blood-dwelling parasites. The possibility of the secretion of parasite-derived immunomodulatory products directed at other host immune molecules, and at later stages of the infection, as stated previously, also deserves further study.

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Chapter 5 The exocrine glands of *Lernaeocera branchialis*

5.1 Introduction

The modulation of the host's immune response by terrestrial haematophagous arthropod parasites, such as ticks, has been well studied (Ribeiro, 1987; Wikel *et al*., 1994; Inokuma *et al*., 1997; Astigarraga *et al*., 1997; Ribeiro *et al*., 1992), however such modulation by aquatic arthropods has received little attention with the exception of the parasitic copepod *Lepeophtheirus salmonis* (the salmon louse). *L. salmonis* has been found to secrete products, such as prostaglandin E_2, which are responsible for modulating the immune response of its host (Ross *et al*., 2000, Firth *et al*., 2000; Fast *et al*., 2002a, 2003, 2004, 2007). However, the production site(s) of these secretory products, *i.e.* glands or organs, have not been determined, and further investigations into this area are warranted. *Lernaeocera branchialis* is intimately exposed to the immune response of its host through its location within the blood vessels, and once fully metamorphosed it remains embedded within its host for an extended period of time whilst it feeds. Therefore, whether *L. branchialis* produces similar compounds to *L. salmonis* requires investigation. The secretory / excretory products of *L. branchialis* will be explored in Chapter 6; however, the possible production sites for compounds secreted at the host-parasite interface are dealt with in this chapter.

Studies concerning the actual function of exocrine glands in copepods are very limited, and often their function has been attributed according to their location rather than through in depth histochemistry and secretory product identification. The best studied exocrine glands in copepods to date are those of the buccal cavity (Boxshall, 1982; Arnaud *et al.*, 1988a, b; Zeni & Zaffagnini, 1992; Vaupel Klein & Koomen, 1994), the mucous glands of the capsule-dwelling copepods (Hicks & Grahame, 1979), and the luminous glands of Calanoida (Clarke *et al*., 1962,

Herring, 1988; Bannister & Herring, 1989). However, the location and structure of exocrine gland populations of *L. salmonis* have been studied by Bron (1993) and Bell *et al.* (2000). Bron (1993) found the immunohistochemical stain 3', 3-diaminobenzidine tetrahydrochloride (DAB) to stain some exocrine glands in *L. salmonis*. DAB is a chromogenic substrate which in the presence of hydrogen peroxide reacts with endogenous peroxidases to produce a brown precipitate. DAB however, has also been observed to react with catalases, cytochrome oxidase, sulphated mucopolysaccharides, a diverse range of haeme proteins and possibly other compounds (Bussolati, 1971; Ekes, 1971; Longo *et al.,* 1972; Seligman *et al*., 1973). Bell *et al*. (2000) went on to use this method to highlight the location of exocrine glands at all life stages of *L. salmonis* and another parasitic copepod, *Caligus elongatus*. These authors observed the staining properties of DAB to be a peculiar feature of the exocrine glands of parasitic caligid copepods, but not generally of their free-living counterparts. However, the function of many of the exocrine glands could only be speculated upon and was not precisely determined, except for glands such as those of the frontal filament complex. Knowledge of the exocrine glands of *L. branchialis* is much more limited in comparison to that of *L. salmonis*, with only a single study of the internal morphology of *L. branchialis* carried out by Capart (1948). This author observed multiple gland systems; including a frontal gland and a pair of cephalothoracic glands the author termed the maxillary glands. The ultrastructure of these glands and their exiting pores were not studied however, and the image quality was limited due to the technology available at the time of the study. Investigations of the exocrine glands of other aquatic parasites have also been published, especially for the parasitic monogeneans of fish (Whittington & Cribb, 1998; El-Naggar & Kearn, 1983; El-Nagaar & Kearn, 1989; Kearn & Gowing, 1989; Cribb *et al*., 2004). These studies are more numerous

in comparison to those regarding parasitic copepods and have included descriptions of the structure of the hamulus and haptor glands, and the glands associated with the anterior adhesive areas and body margins. Therefore, the aims of this study were to map and investigate the exocrine glands, and their communicating pores, on the external surface of *L. branchialis* by light microscopy, and transmission and scanning electron microscopy. Those glands associated with the area of the parasite in close-contact with the host *i.e*. the cephalothorax, especially the feeding area, were concentrated on. This was in order to determine possible production sites of parasite–derived compounds involved at the host-parasite interface, such as host–modulating products, and their point of secretion. This would allow future studies to be performed to identify the products secreted from the exocrine glands, especially those at the site of attachment / feeding, of the parasite, and the secretion kinetics of parasite-derived molecules during the infection process.

5.2 Materials and methods

5.2.1 Whole mount staining of exocrine glands

5.2.1.1 DAB staining

Free-swimming and gravid, fully metamorphosed females were stained with 0.667mg.ml^{-1} 3', 3 diaminobenzidine tetrahydrochloride (DAB; Sigma, Poole, UK) in Tris buffered saline, pH 7.6 with 0.026% hydrogen peroxide (Sigma) as a substrate following the methods of Bell *et al*. (2000). The optimum staining time was found to be 3 h at room temperature. Staining was halted by the addition of 70% (v/v) ethanol, and specimens 'cleared' with 87.5% lactic acid (Sigma) for 12 h to improve DAB stain visualisation. Negative controls consisted of cleared females without DAB.

5.2.1.2 Light microscopy and mosaic image reconstruction with increased depth of field

DAB-stained free-swimming females were placed in a cavity slide with a drop of lactic acid, cover-slipped and viewed with an Olympus BX51TF light microscope. Images were taken with a Zeiss AxioCam MRc colour digital camera at different depths of fields, from the dorsal surface to the ventral surface and *vice versa*, by manually adjusting the focus. These images were stacked in the order they were taken, and reconstructed into a mosaic image with an increased depth of field using DeepFocus software version 2.0.0.37 (Stuart Ball), which uses the best focused components from each depth and assembles them into a focussed whole image.

5.2.2 Light microscopy of exocrine glands of *L. branchialis* adult females

5.2.2.1 Fixation and resin processing

Free-swimming female and fully metamorphosed, female *L. branchialis* were fixed and resin embedded in Technovit® 7100 (Heraeus Kulzer, Germany) as described in Chapter 2 Section 2.11.3. Due to the large size of the fully metamorphosed females, the cephalothorax was separated from the genito-abdominal region post-fixing in Davidson's fixative prior to storage in ethanol. The two pieces were embedded in resin blocks separately to allow sectioning.

5.2.2.2 Haematoxylin and eosin (H&E) staining

H&E staining of resin sections was performed as described by Heraeus Kulzer technical support (see website: www.ebsciences.com/histology/gma_he.htm), except that Mayer's haematoxylin was used instead of Gill's haematoxylin.

5.2.2.3 Alcian blue, pH 2.5-periodic acid Schiff's (AB-PAS) staining

Alcian blue (pH 2.5) – periodic acid Schiff's (AB-PAS) staining was performed to highlight the presence of acidic and neutral mucopolysaccharides, using a modification of the method described by Heraeus Kulzer technical support. Sections were stained in 1% (w/v) alcian blue pH 2.5 for 15 min, rinsed in running tap water for 5 min with a final rinse in distilled water prior to PAS staining following the standard protocol (see website: www.ebsciences.com/histology/gma_pas.htm).

5.2.3 Description of the ultrastructure of the exocrine glands by transmission electron microscopy

Whole free-swimming *L. branchialis* females from wild flounder and the cephalothorax of gravid, fully metamorphosed females from wild gadoids were fixed in 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 at 4°C overnight. Specimens were processed for TEM following Hayat (1989). Briefly, specimens were rinsed in 0.1M sodium cacodylate buffer, post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer, rinsed in distilled water, en-bloc stained in 2% uranyl acetate in 30% (v/v) acetone, dehydrated in an acetone series, infiltrated and embedded in agar low viscosity resin (Agar Scientific, Essex, U.K.).

Semi-thin serial transverse and planar sections (0.5µm) were cut for light microscopy with a diamond knife using an ultra-microtome (Reichert Ultracut-E), and stained with 0.5% (w/v) methylene blue and 0.5% (w/v) azur II in 1% (v/v) Borax for orientation and section selection. Ultra-thin transverse and planar sections (70nm) of exocrine glands mapped by light microscopy were also sectioned and stained with 5% uranyl acetate and lead citrate according to the methods of Reynolds (1963) and Hayat (1989) for TEM. Grids were visualised on a Tecnai G2 Spirit Bio Twin transmission electron microscope.

5.2.4 Mapping of external pores on female *L. branchialis* by scanning electron microscopy

Free-swimming *L. branchialis* females from wild flounder and gravid, fully metamorphosed *L. branchialis* females from wild gadoids were fixed in 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate at 4°C for 2 days. Fully metamorphosed females were subjected to an additional step of proteinase K (Promega, Wisconsin, USA) digestion to remove attached host tissue (100µg.ml^{-1} 50mM Tris-HCl, 5mM EDTA, pH 8 for 1 h). Specimens were rinsed in 0.1M sodium cacodylate for a minimum of 4 h, post-fixed in 1% (w/v) osmium tetroxide in 0.1M sodium cacodylate buffer for 2 h, de-hydrated in an ethanol series and critical point dried using a Bal-Tec 030 critical point drier. Females were then attached to aluminium stubs, gold sputter-coated using an Edwards S150B sputter coater and visualised using a Jeol JSM6460LV scanning electron microscope. External pore openings on the dorsal and ventral body surface were mapped using SEM.

5.3 Results

5.3.1 Whole mount staining of *L. branchialis* exocrine glands by DAB

DAB staining of free-swimming females highlighted numerous possible exocrine glandular structures with bilateral symmetry (Figure 5.1a & d). Within the cephalothorax numerous glands were stained along the edges of the dorsal surface, however, of particular interest were the pair of antennal glands (AG) at the base of the antennae (Figure 5.1a), a frontal gland complex (FGC) located anterior to the nauplius eye (Figure 5.1a, b), the circum-oral glands (CG) positioned laterally to the nauplius eye (Figure 5.1a), and the dorsal post-oral glands (DPOG) composed of four glands immediately under the dorsal cuticle posterior to the

nauplius eye (Figure 5.1a). The FGC appeared to be composed of more than one gland (Figure 5.1b) secreting anterior through the midline of the antennae (Figure 5.1a, b). The adult female attaches to the tip of the gill filament of the intermediate host by her antennae, and inserts the frontal filament into the gill tissue to secure attachment, note the positive DAB staining of the inserted filament (Figure 5.1b).

DAB stained structures with bilateral symmetry were also found within the swimming legs, thoracic segments, and the genital segment (Figure 5.1d-e). The negative controls enabled the actual DAB staining of exocrine glandular structures to be distinguished from the pigmented parasite structures, such as the nauplius eye, which remained pigmented following clearing with lactic acid (Figure 5.1c, f).

Whole-mount DAB staining for the identification of exocrine glandular structures within the fully metamorphosed female *L. branchialis* gave no positive staining results.

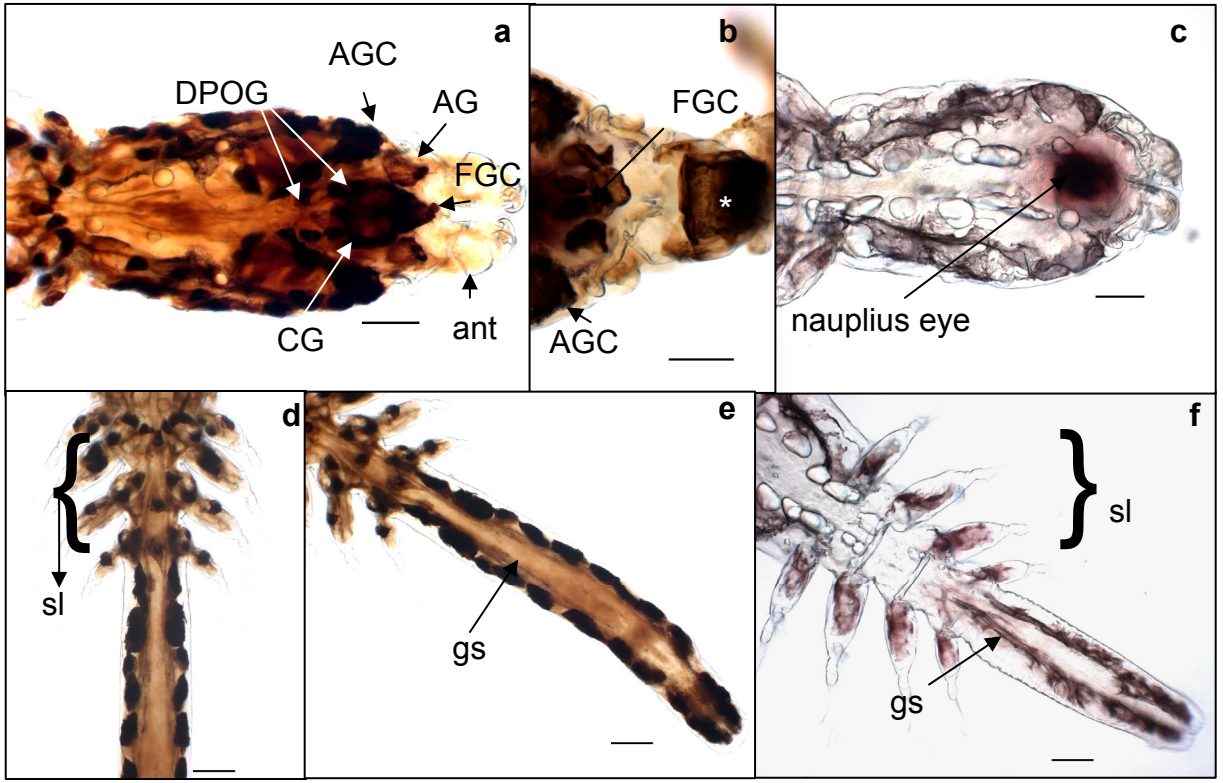

Figure 5.1 Exocrine glandular structures of whole free-swimming female *L. branchialis* (infective stage) stained with DAB and cleared with lactic acid. (a) cephalothorax; (b) anterior tip of cephalothorax from adult female still attached to intermediate host gill tissue by frontal filament (*); (c) negative control cephalothorax cleared with lactic acid; (d) swimming legs, thoracic segments 2-4 and anterior portion of the genital segment; (e) genital segment; (f) negative control thoracic segments 2-4 and genital segment cleared with lactic acid. Ag, antennal gland; AGC, anterior gland complex; ant, antennae; CG, circum-oral gland; DPOG, dorsal post-oral glands; FGC, frontal gland complex; gs, genital segment; sl, swimming legs. Bar = 100µm.

5.3.2 Light microscopy of female *L. branchialis* exocrine glands

The FGC of the free-swimming female was found to be composed of multiple glands or lobes (Figure 5.2a). The FGC did not stain with acidic alcian blue indicating a lack of acidic mucopolysaccharides (Figure 5.2b), and only a slight PAS-positive reaction for neutral carbohydrates was found in discrete areas of the FGC (Figure 5.2c). Therefore, the main secretory component of the FGC was not composed of carbohydrates such as, mucopolysaccharides, glycoproteins or glycolipids.

The paired AGCs were located ventrolaterally to the nauplius eye, and dorsolaterally to the oral cone. They stained intensely with PAS but not acidic alcian blue (Figure 5.3a), suggesting the production of carbohydrates, such as neutral mucopolysaccharides. These complexes were large in comparison to the other gland complexes, and possessed multiple lobules, this being evident from differences in the PAS-staining pattern observed throughout the structure, with the outer-anterior portion staining more intensely with PAS than the inner and posterior portion (Figure 5.3a). The AGC appeared to extend inwards towards the oral region highlighting possibly secretory sites in the vicinity of the 1st or 2nd maxillae (Figure 5.3b). However, the ducts and exact secretory locations of the AGC were not identified. The paired CGs were located laterally to the nauplius eye on either side of the pigment cells (Figure 5.3c, d) extending posteriorly to the cerebrum (Figure 5.3d). They appeared to extend anteroventrally, from the posterior to the anterior of the nauplius eye ventrally towards the oral cone (Figure 5.3a, c, d), however the secretory ducts were not located. The production of neutral carbohydrates was apparent for these glands, evidenced by the positive PAS-staining, however no acidic mucopolysaccharide staining was observed (Figure 5.3a, b).

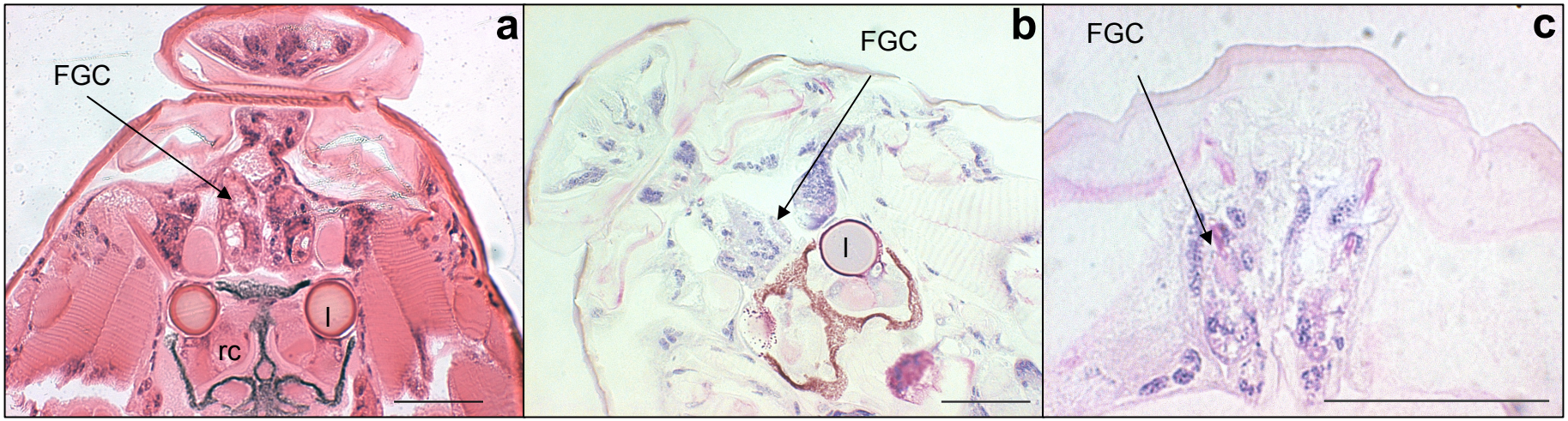

Figure 5.2 Planar sections of the anterior portion of the cephalothorax of the free-swimming female *L. branchialis* stained with (a) H&E or (b & c) alcian blue (pH 2.5)-PAS illustrating the frontal gland complex (FGC) located anterior to the lenses (l) and retinular cells (rc) of the eye. Bar = 50µm

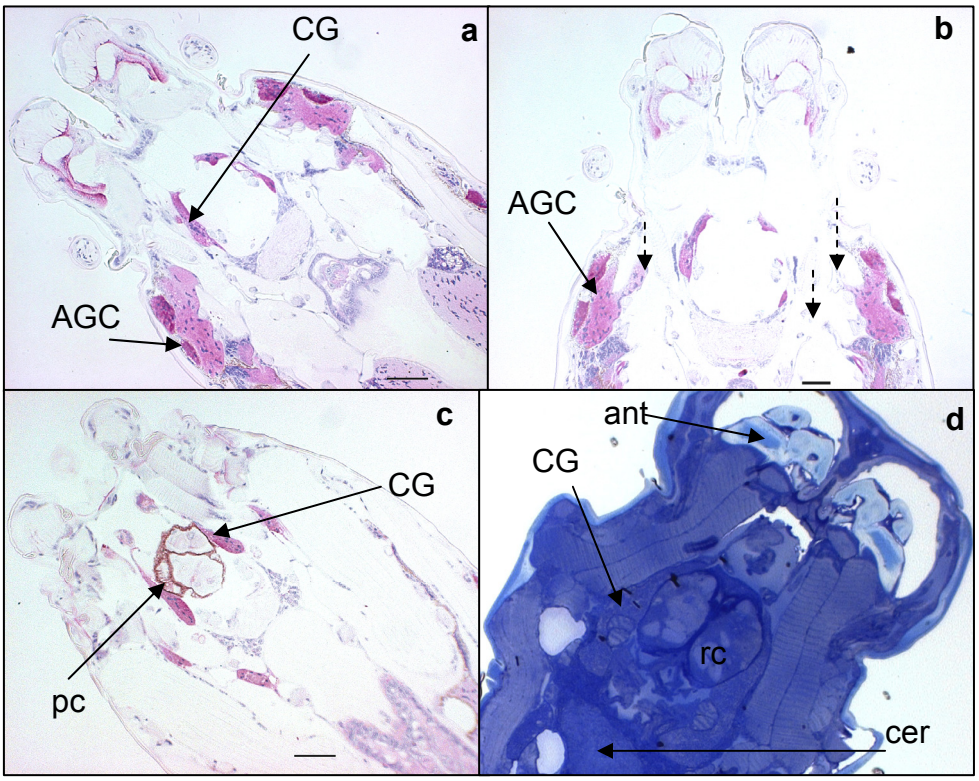

Figure 5.3 Planar sections of the cephalothorax of the free-swimming female *L. branchialis* stained with (a, b, c) alcian blue (pH 2.5)-PAS and (d) methylene blue-azur II, (a) illustrating the anterior gland complex (AGC) and the circum-oral glands (CG) either side of the oral cone, (b) the inward extensions of the AGC towards the oral region (dotted black arrow), (c) the CG located either side of the pigment cells (pc) of the eye, and (d) the CG reaching posterior to the cerebrum (cer). ant, antennae; bar = 50µm

The paired structures of the genital segment of the free-swimming female located on either side of the gut, which stained positively with DAB, were also found to contain neutral carbohydrates, as evidenced by the positive PAS staining (Figure 5.4a). The nuclei were located around the exterior of the structure with an intensely PAS-positive central region (Figure 5.4b). Also, note the unidentified structures at the posterior portion of the genital segment which also stained slightly positive for neutral carbohydrates (Figure 5.4c). They were observed to possess a central lumen containing flocculent material. The paired PAS- and DAB- positive structures

observed in the genital segment appeared to continue anterior with a pair at the anterior portion of the thoracic segments 2 to 4 (Figure 5.4d). They were termed DAB- and PAS- positive thoracic structures (DPTS), and appeared to have a similar morphology to those of the genital segment (Figure 5.4d).

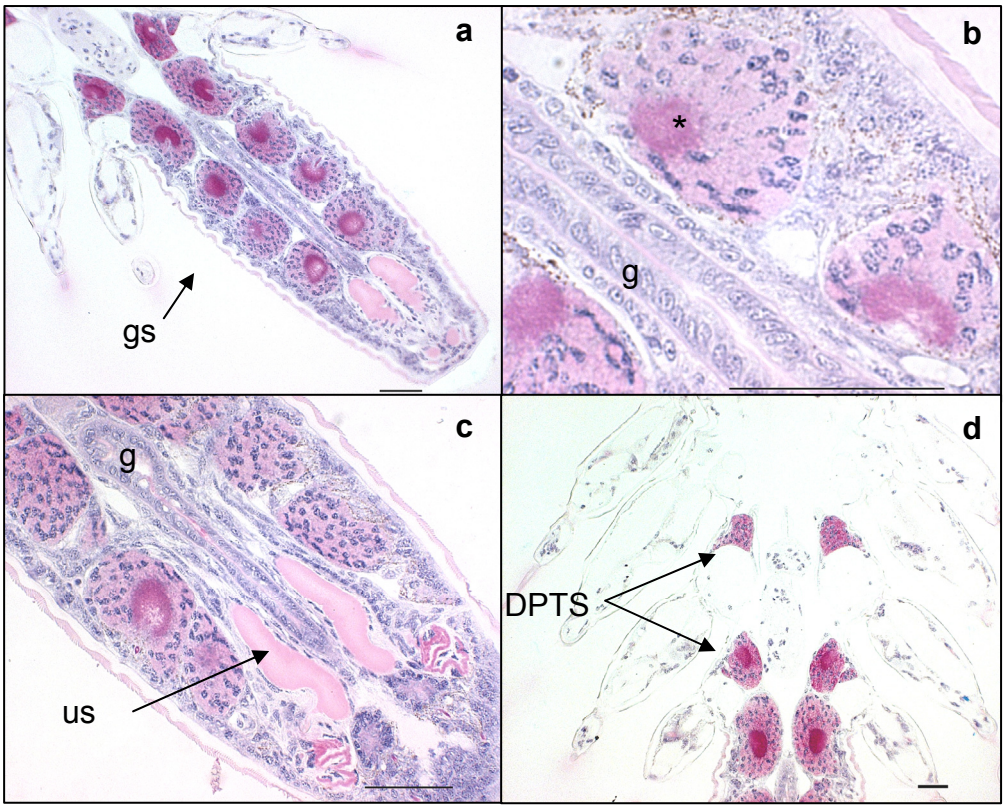

Figure 5.4 Planar sections of the genital and thoracic segments of the free-swimming female *L. branchialis* stained with alcian blue (pH 2.5)-PAS. Illustrating (a) the paired PAS- and DAB- positive structures on either side of the gut along the genital segment, (b) the intense PAS staining on the inner portion of the structures (*), (c) the paired unidentified structures at the posterior portion of the genital segment, and (d) the paired DAB- and PAS- positive thoracic structures (DPTS) located at the anterior of the thoracic segments 2 - 4. g, gut; gs, genital segment; us, unidentified structure. Bar = 50μm

During metamorphosis the cephalothorax extends ventrally with the mouth cone at the tip of the extension, and three antlers grow, one dorsally behind the nauplius

eye and two laterally (Figure 5.5a). The AGC remained multi-lobular, evidenced by differential staining patterns, with a centralised duct (Figure 5.5e), stretching transversally from the entrance of the lateral antler to the gut (Figure 5.5b), extending longitudinally from a position postero-laterally to the antennae (Figure 5.5f) to one posterior to the oral region. They also extended ventrally from the dorsal surface of the cephalothorax to the external edge of the muscles attached to the top of the 'ventral extension' of the oral region (Figure 5.5b, c).

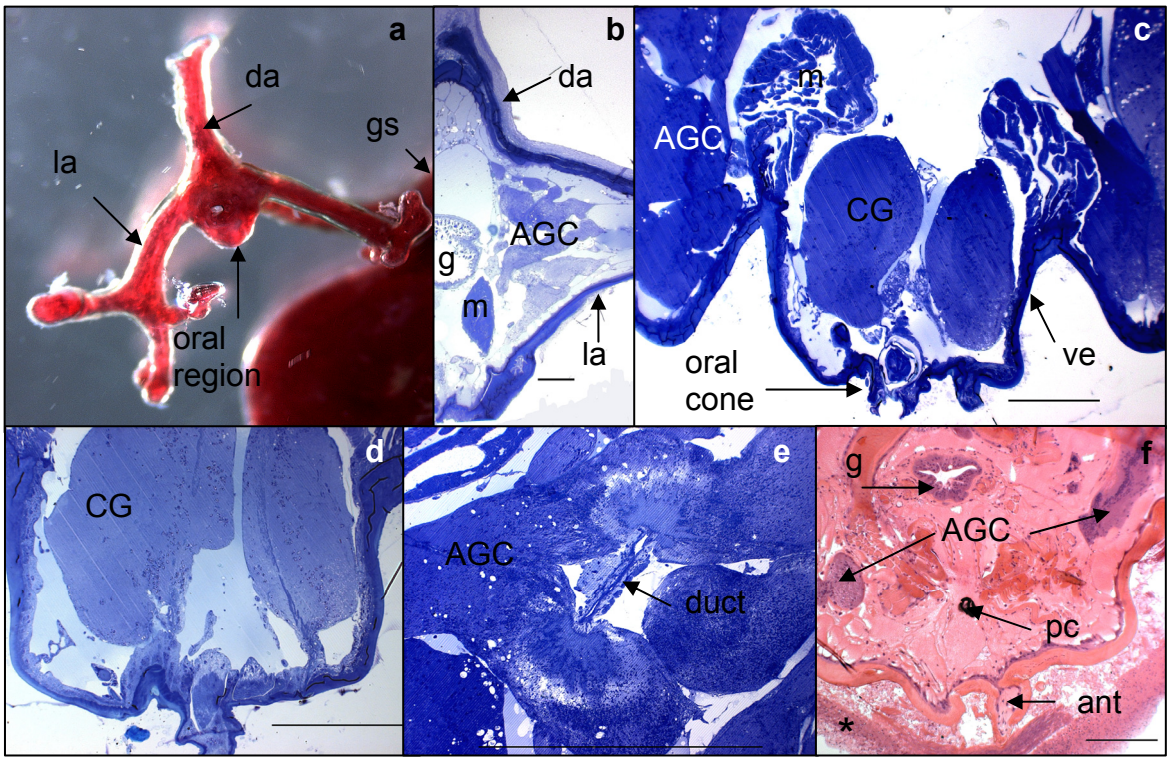

Figure 5.5 Glandular structures within the cephalothorax of fully metamorphosed adult female *L. branchialis*. (a) Fully metamorphosed female illustrating the morphology of the cephalothorax. (b) Transverse section of the cephalothorax illustrating the location of the anterior gland complex (AGC) stretching laterally from the gut (g) to the entrance of the lateral antler (la). (c & d) Transverse section of the oral region illustrating the circum-oral glands (CG) located on either side of the oral cone. (e) Transverse section of the AGC illustrating a central duct (arrowed). (f) Planar section of the cephalothorax illustrating the antennae (ant) attached to host tissue (*) with the AGC located posterolaterally to the antennae as in the free-swimming female (Figure 5.3a), note the lack of CG

either side of the pigment cells (pc) of the eye in contrast to the free-swimming stage (Figure 5.3c). da, dorsal antler; gs, genital segment; m, muscle; ve, 'ventral extension' of the oral region. Bar = 200µm.

The CGs were positioned dorsolaterally to the oral cone positioned within a 'ventral extension' of the oral region (Figure 5.5c, d), which houses the oral appendages and appeared to be extensible and retractable by the muscles attached to either side (Figure 5.5c). The CGs appeared to secrete in the vicinity of the oral cone on the 'ventral extension', however the exact secretory site could not be determined. Post-metamorphosis, the paired CGs and AGCs were much larger in size in comparison to those observed in the free-swimming female, and were not found to be positive for carbohydrates when stained with PAS or acidic alcian blue, except for positive-PAS staining within the duct and the area surrounding the duct in the AGCs. The CGs also seemed to reposition during metamorphosis, as they were no longer observed laterally to the pigment cells of the nauplius eye (Figure 5.5f), as observed in the free-swimming females (Figure 5.3b). However, the AGs and FGC observed posterior to the antennae and anterior to the nauplius eye in free-swimming females (Figure 5.1a and 5.2) were not seen in the sections utilised in this study from metamorphosed females (Figure 5.5f).

5.3.3 Ultrastructure of female *L. branchialis* exocrine glands

The CGs of the free-swimming female contained abundant secretory vesicles, with at least three types of secretory vesicles identified by their different electron densities and homogeneities (Figure 5.6a). The presence of fibrous-type 3 secretory vesicles suggested the production of mucopolysaccharides / glycoproteins, which was corroborated by the positive PAS staining of the CGs

(Figure 5.3b). Two more electron dense secretory vesicles, types 1 and 2, were also present. Rough endoplasmic reticulum (RER) was also abundant between the secretory vesicles (Figure 5.6a). The AGs contained very abundant RER and numerous Golgi apparatus, however secretory vesicles were not observed (Figure 5.6b). On the other hand, the anterior and the posterior portion of the multi-lobed AGC both possessed abundant secretory vesicles. However, the anterior portion contained abundant 1-2μm fibrous-type 4 and 5 secretory vesicles (Figure 5.6c), in comparison to the posterior AGC which comprised mainly 0.3-0.5μm very electron dense type 6 and 1 secretory vesicles. This also correlated with the more intense staining of the anterior portion of the AGC with PAS, compared to that of the posterior portion (Figure 5.3a). The posterior portion of the AGC also possessed large vacuoles to the edges of the cytoplasm (Figure 5.6d). All of these glands possessed a syncytial organisation with Golgi apparatus (Figure 5.6b), scattered mitochondria, and abundant RER.

The fully metamorphosed females did not reveal the same abundance of secretory vesicles in the CGs and the AGC as seen in the pre-metamorphosed, free-swimming stages. This may be due to their release during host invasion / metamorphosis, or due to the feeding stage of the individuals sampled. However, gland syncytial organisation was retained and they remained ‘geared’ for production of vast quantities of protein with abundant RER and Golgi apparatus.

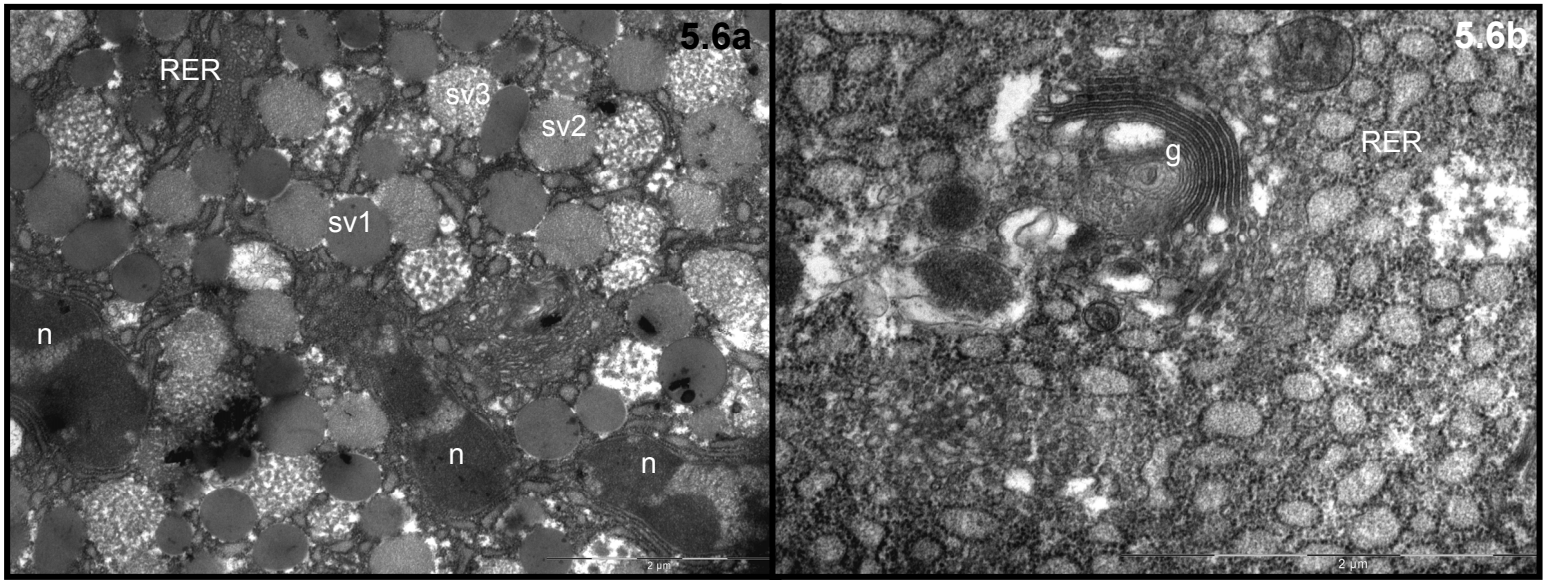

Figure 5.6 Ultrastructure of the glandular structures situated within the cephalothorax of free-swimming female *L. branchialis* (a) circum-oral gland; (b) antennal gland. g Golgi apparatus; n nucleus; RER rough endoplasmic reticulum; sv 1, 2, 3 secretory vesicles of varying electron density and homogeneity.

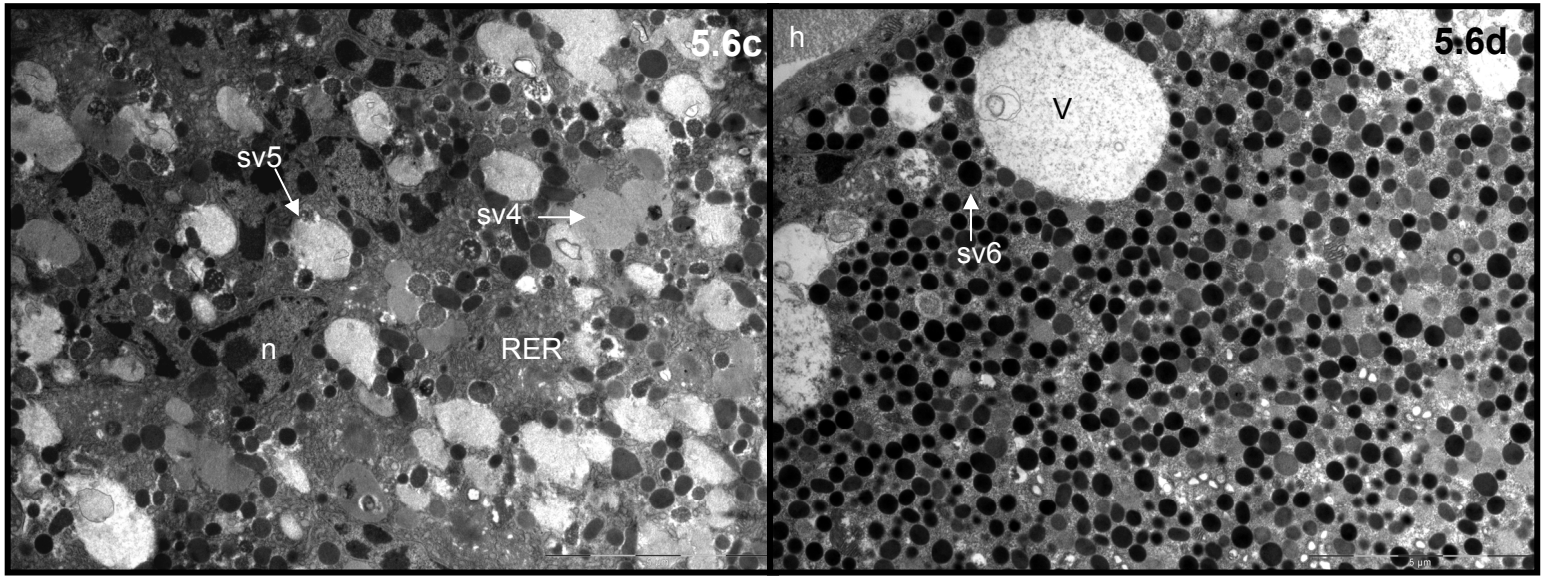

Figure 5.6 continued Ultrastructure of the glandular structures situated within the cephalothorax of free-swimming female *L. branchialis* (c) anterior, anterior gland complex (AGC); (d) posterior AGC, note the large vacuoles, V, at the edge of the cytoplasm. h haemolymph; n nucleus; RER rough endoplasmic reticulum; sv 4, 5, 6 secretory vesicles of varying electron density and homogeneity.

The dorsal portion of the CGs contained numerous vacuoles with abundant and prominent RER (Figure 5.7a). The outer-edge of the central portion of the CGs also possessed abundant RER and well developed Golgi apparatus associated with vesicles leaving the trans face (Figure 5.7b). Numerous mitochondria were observed lining the plasma membrane and plasma membrane invaginations of the CGs (Figure 5.7b and c) with some scattered throughout the cytoplasm. Dilation of the RER was evident in the central and ventral portion of the CGs (Figure 5.7c-f), which usually occurs during periods of high production and storage of secretory products. The ventral portion of the CGs also revealed unusual RER dilation patterns (Figure 5.7e and f).

The ventral portion of the AGC near to the 'ventral extension' of the oral region, and the outer portion of the AGC at the entrance of the lateral antler both revealed prominent and abundant RER (Figure 5.8a and b). The ventral AGC contained abundant secretory vesicles of types 1-5, as described previously in the free-swimming female. However, another secretory vesicle was apparent which was similar to the larger and fibrous type 4 vesicles although, they were more electron dense and termed type 7 vesicles (Figure 5.8a). The outer portion of the AGC contained fewer secretory vesicles in comparison to the ventral portion, and these were similar to the type 4 and 5 vesicles of the free-swimming female (Figure 5.8b). The inner dorsal AGC showed extreme RER dilation, scarce secretory vesicles and mitochondria lined the inner side of the plasma membrane (Figure 5.8c). This highlights apparent functional differences in the multiple lobules of the AGC which varied in their PAS staining and ultrastructure.

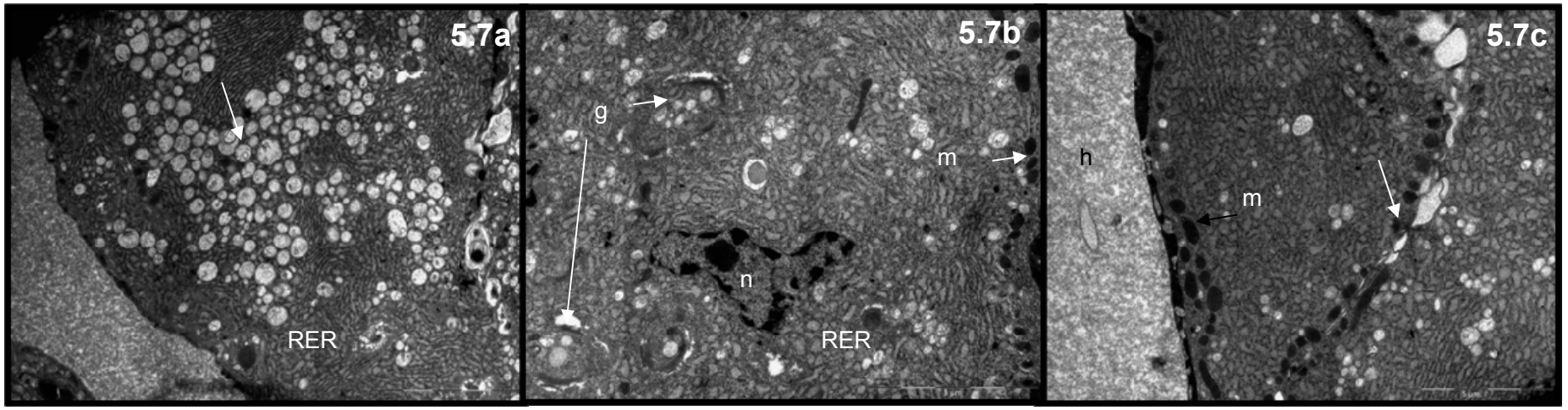

Figure 5.7 Ultrastructure of gravid fully metamorphosed female *L. branchialis* circum-oral glands (CG). (a) dorsal portion of CG showing vacuoles, white arrow; (b) outer-edge of central portion of CG illustrating numerous Golgi apparatus, g, with vesicles leaving the trans face; white arrow; (c) outer-edge of central portion of CG illustrating plasma membrane invaginations lined with mitochondria, white arrow. g, Golgi apparatus; h, haemolymph; m, mitochondria; n, nucleus; RER, rough endoplasmic reticulum.

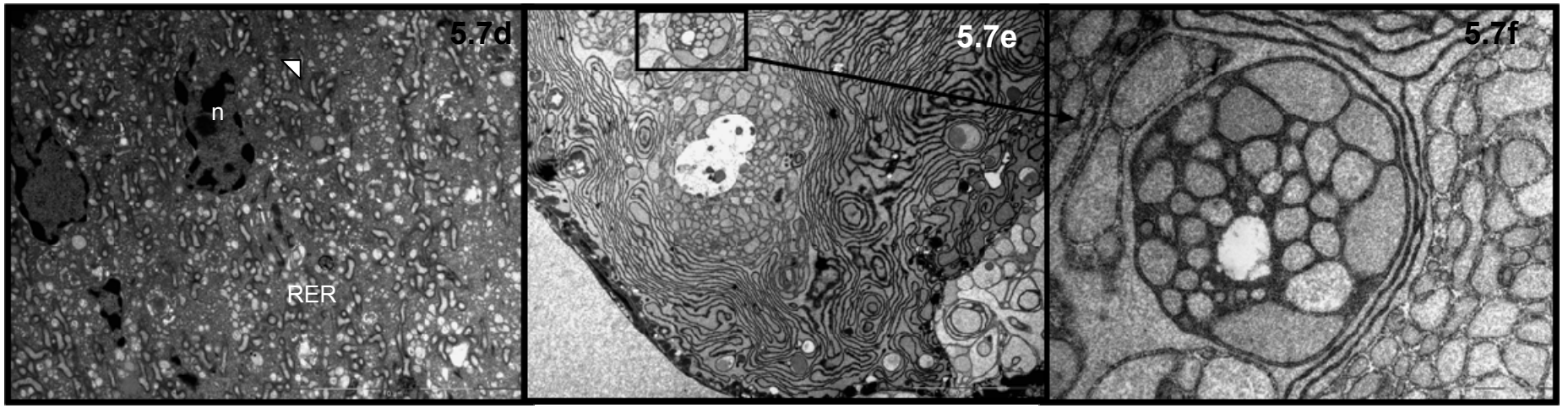

Figure 5.7 continued Ultrastructure of gravid fully metamorphosed female *L. branchialis* circum-oral glands (CG). (d) central portion of CG with dilation of rough endoplasmic reticulum, white arrow head; (e) ventral portion of CG; (f) close-up of e. n, nucleus; RER, rough endoplasmic reticulum.

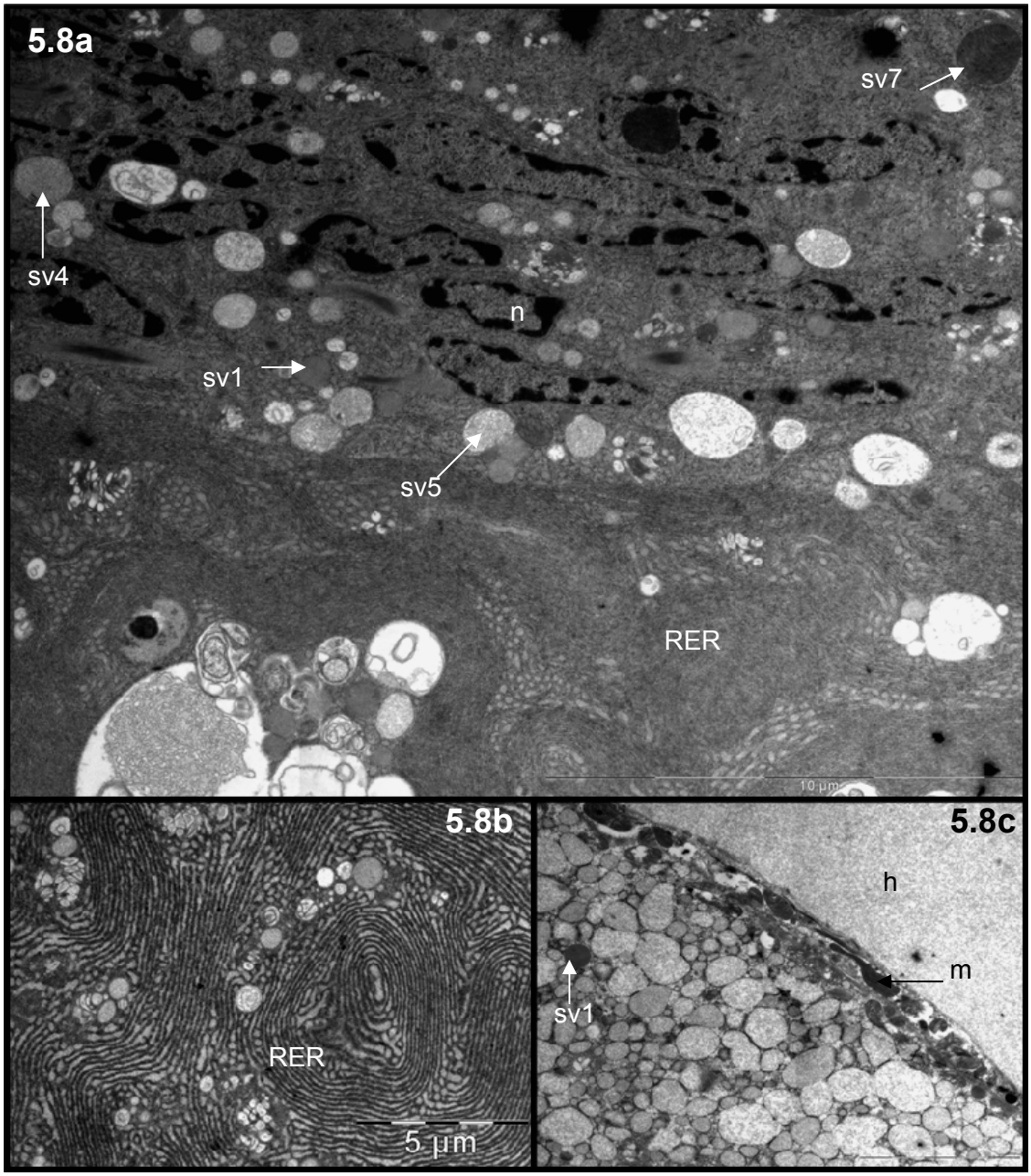

Figure 5.8 Transmission electron micrographs illustrating ultrastructure of gravid fully metamorphosed female *L. branchialis* anterior gland complexes (AGC). (a) ventral portion of AGC, (b) outer portion of AGC at entrance of lateral antler, (c) inner dorsal portion of AGC. h, haemolymph; m, mitochondria; n, nucleus; RER, rough endoplasmic reticulum; sv1, 4, 5, 7, secretory vesicle types.

5.3.4 Mapping of external pores on female *L. branchialis* by SEM

Numerous pore openings with bilateral symmetry on the anterior dorsal surface of the free-swimming female were mapped (Figure 5.9a). Pores 10 – 13 on the dorsal surface, as indicated in Figure 5.9a, co-localised with the four DAB - positive DPOGs (Figure 5.1a), and were therefore likely candidates for the secretory locations of these exocrine glands. The pores on the dorsal surface of the thoracic segments (Figure 5.9a), such as pores 23 and 25 shown in Figure 5.10b and d, 'mirrored' the pattern and location of the DAB – positive exocrine glands located in the thoracic segments (Figure 5.1d). No pores were found to be associated with the paired DPTSs located ventrally to these exocrine glands, and at the anterior portion of the thoracic segments 2 - 4 (Figure 5.1c and 5.4d). Similarly, no pores were observed on the cuticle of the dorsal surface of the genital segment to be associated with the paired DAB and PAS - positive structures running either side of the gut (Figure 5.1d and 5.4a). However, these pores may have been missed due to the anterior part of the thoracic segments being partially covered through overlapping by the anterior segment, and the extreme folding of the genital segment cuticle obscuring possible pores. The pores on the dorsal surface, irrespective of their location, all had a similar morphology; circular with a slightly elevated rim and an approximate diameter of 1µm (Figure 5.10b-f).

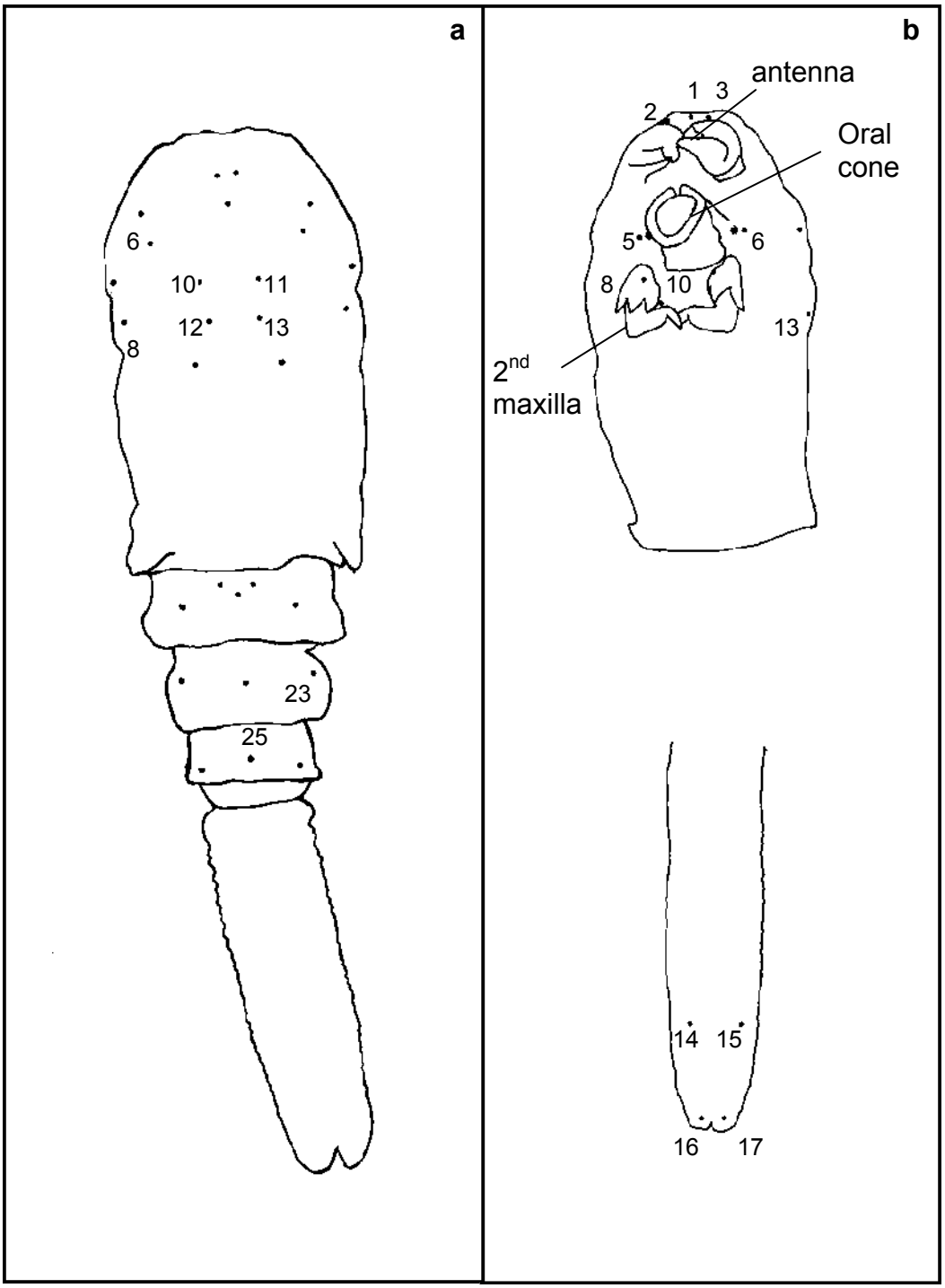

Figure 5.9 Map of pores on (a) dorsal and (b) ventral surface of free-swimming adult female *L. branchialis*. Note the pores on the swimming legs, 1st maxillae, ventral surface of the thoracic segments, antennules and within the oral cone were not mapped due to the difficulty in viewing the entire surface of the appendages or segments. Black circle = pore; black asterisks either side of oral cone = location of the 1st maxillae.

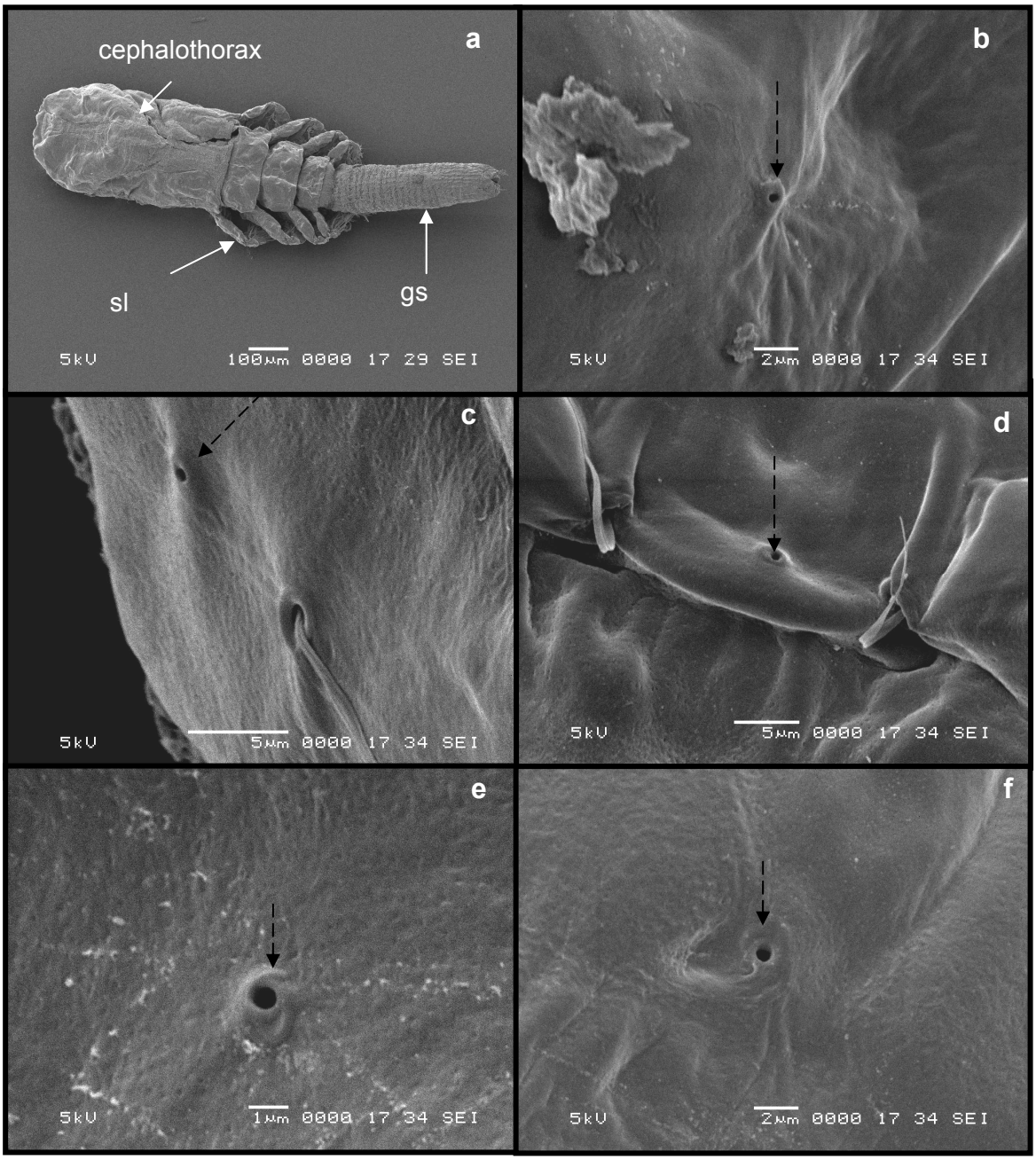

Figure 5.10 Scanning electron micrographs of typical pores mapped on the dorsal surface of the free-swimming adult female *L. branchialis*. (a) dorsal surface of female illustrating parasite morphology. (b) pore 23 opening on right hand side of dorsal surface of thoracic segment 3. (c) pore 8 opening on left hand side of dorsal surface of cephalothorax. (d) pore 25 opening onto the midline surface of thoracic segment 4. (e) pore 6 opening onto dorsal surface of cephalothorax. (f) pore 13 opening onto dorsal surface of cephalothorax. sl, swimming legs; gs, genital segment.

The ventral surface of the free-swimming female possessed fewer pores than the dorsal surface, however, bilateral symmetry was maintained (Figure 5.9b). The most interesting pores regarding this study were those associated with, or located near to the feeding appendages of the parasite. For instance, a pair of pores (pores 5 and 6) situated posterolaterally to the 1st maxillae was observed, allowing the secretion / excretion of gland products either side of the oral cone (Figure 5.9b and, 5.11b and c, as indicated by the black dashed arrow). A further two sets of pores were associated with the 2nd maxillae. One pair (*e.g*. pore 8) was identified on the ventral surface of the 2nd maxillae (Figure 5.9b) with a 'slit-shaped' morphology (Figure 5.11d). The other pair (*e.g.* pore 10) was situated on the ventral surface of the cephalothorax underneath the 2nd maxillae (Figure 5.9b and 5.11e). The exocrine glands discharging through these pores were not identified, however, as the ducts leading to the pores were not identified in this study. The CG and AGC, on the other hand, appeared to secrete in these locations. The pores located near to the 1st maxillae and underneath the 2nd maxillae were of similar morphology to the pores located at the side of the cephalothorax on the ventral surface (*e.g*. pore 13; Figure 5.11g), and those on the dorsal surface (Figure 5.10b-f). On the posterior ventral surface, a pair of pores (pores 14 and 15) with an elongated morphology (Figure 5.11h) was located protruding from the cuticular folds of the genital segment (Figure 5.9b). These pores were co-localised with a pair of unidentified structures observed in the genital segment (Figure 5.4c). Posterior to this, a pair of pores (pores 16 and 17) was observed on the abdominal segment, anterolaterally to the anus (Figure 5.9b). These pores were 'raised', with an elongated canal, and surrounded by cuticular ornamentation (Figure 5.11i).

At the anterior of the cephalothorax a pair of pores (pores 2 and 3) was located at the base of the dorsal surface of the antennae (Figure 5.9b and 5.11f). These are likely candidates for the secretory / excretory site of the AGs (Figure 5.1a). A single pore (pore 1) centrally positioned at the anterior of the cephalothorax, dorsolaterally to pores 2 and 3 (Figure 5.9b and 5.11f), was observed and is thought to be the point from which the frontal filament gland extrudes (Figure 5.1a, b). The mapping of pores on the swimming legs, within the oral cone, on the 1st maxillae, on the antennules and the ventral surface of the thoracic segments could not be achieved due to practical difficulties.

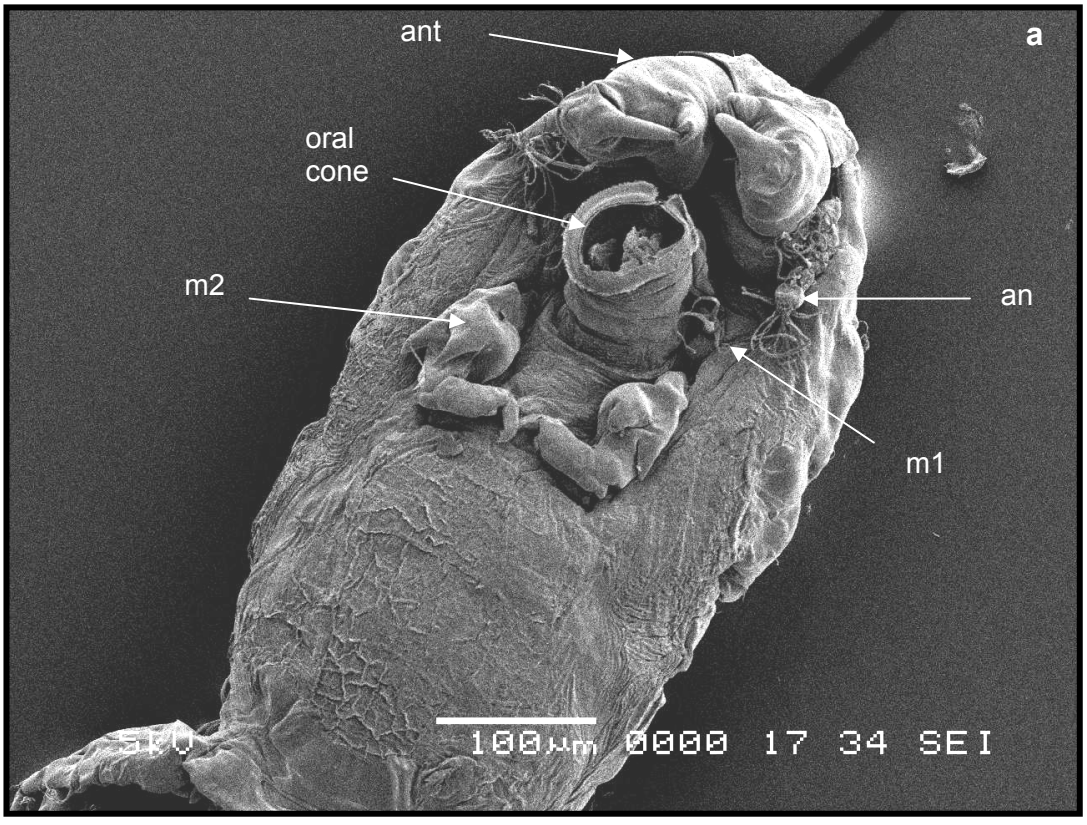

Figure 5.11 Scanning electron micrographs of (a) the ventral surface of the free-swimming female *L. branchialis* cephalothorax illustrating the location of the appendages. an, antennule; ant, antennae; m1, 1st maxillae; m2, 2nd maxillae.

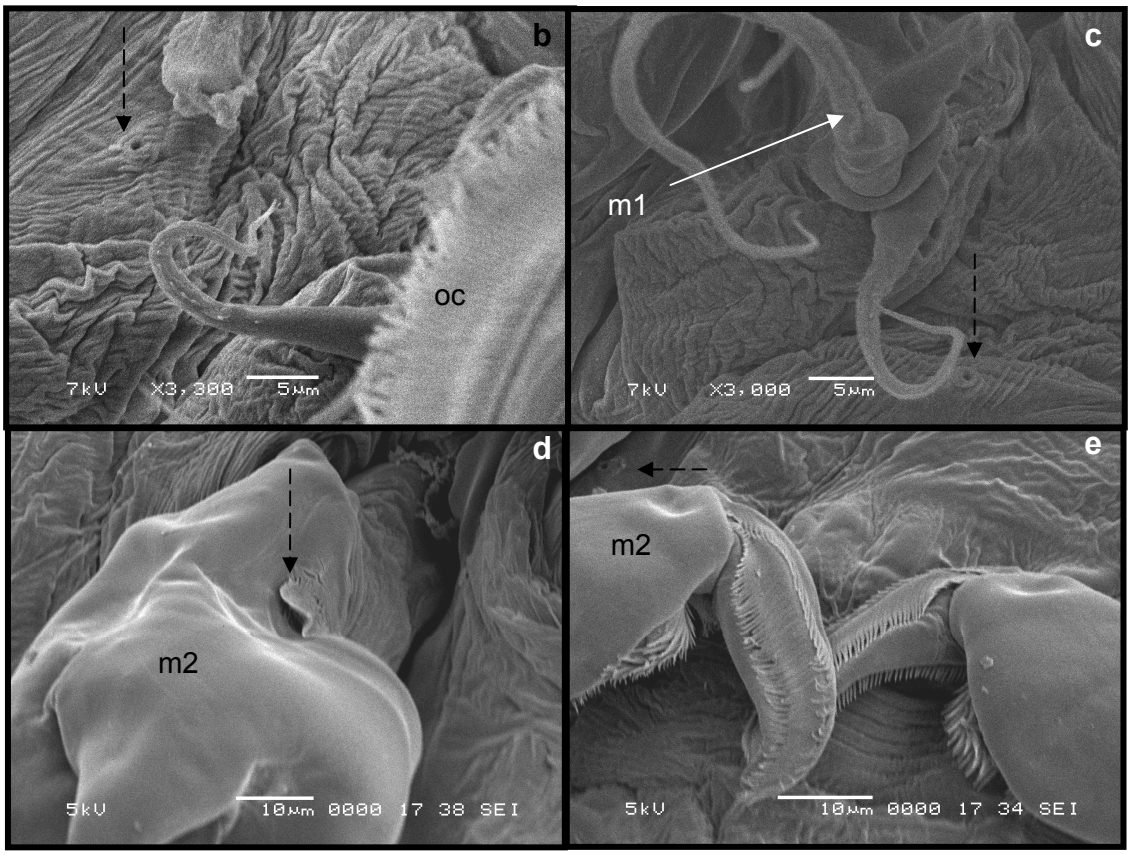

Figure 5.11 continued. Scanning electron micrographs of typical pores mapped on the ventral surface of the free-swimming adult female *L. branchialis*. (b & c) pores 5 & 6 opening onto the ventral surface to the left or right hand side of the oral cone (oc), respectively, and posterolateral to the 1st maxillae (m1). (d) pore 8 opening on the surface of the 2nd maxillae (m2). (e) pore 10 opening on the ventral surface of the cephalothorax under m2. Black dashed arrow = pore location.

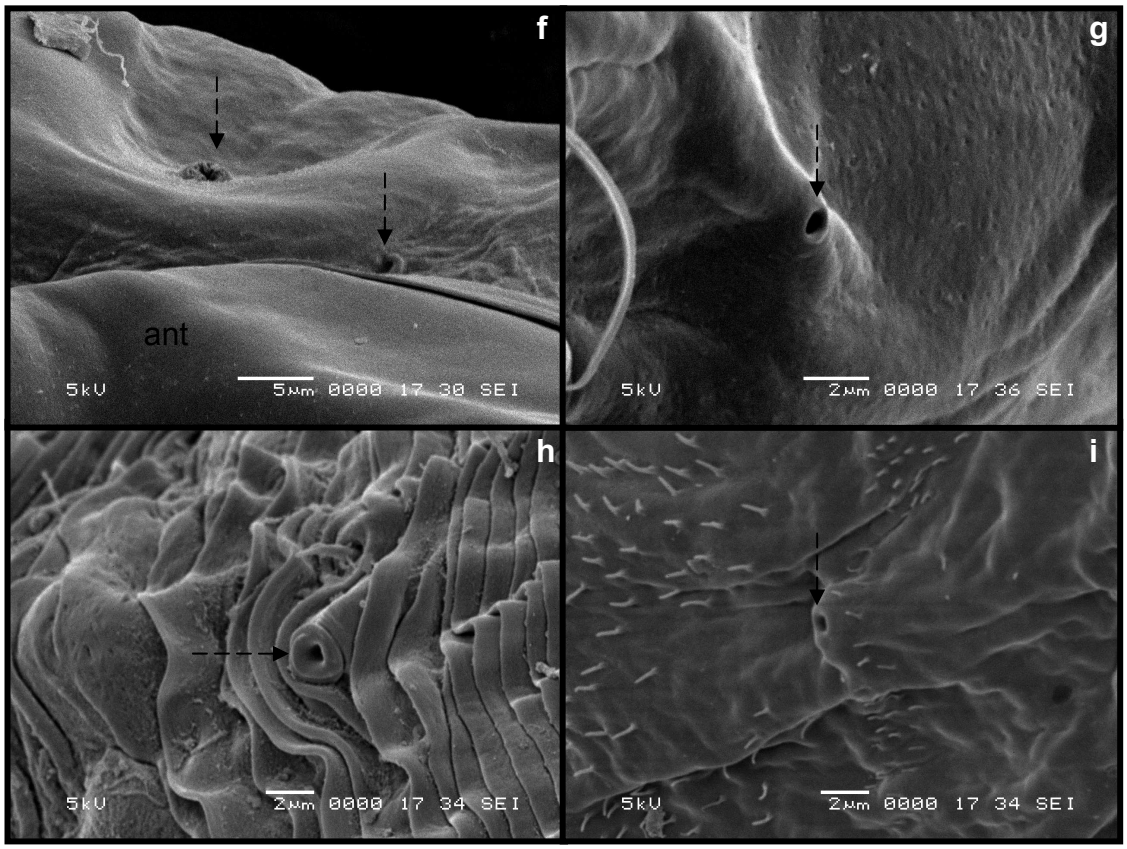

Figure 5.11 continued. Scanning electron micrographs of typical pores mapped on the ventral surface of the free-swimming adult female *L. branchialis*. (f) pore 1 opening onto the middle of the frontal plate and pore 3 opening at the base of the antennae (ant) on the dorsal surface. (g) pore 13 opening laterally from m2 on the side of cephalothorax. (h) pore 14 opening on the genital segment. (i) pore 17 opening on the abdominal segment anterolaterally to the anus. Black dashed arrow = pore location; ant, antennae.

The pores opening onto the cephalothorax and neck of the fully metamorphosed parasite could not be determined due to 1) host tissue still attached to the external surface of the parasite, and 2) the large surface area of the parasite needed to be covered by SEM (Figure 5.12a). The former remained a problem even after a proteinase K pre-treatment to remove host tissue. The latter was addressed by concentrating on the oral region (Figure 5.12a, b). The area within the vicinity of the oral cone was of particular interest as this was where the CGs were thought to

secrete their products. Pore openings similar to those of the free-swimming female positioned laterally to the 1st maxillae (Figure 5.11b, c) and on or underneath the 2nd maxillae (Figure 5.11d and e, respectively) were sought but not observed. The 'folded lobes of cuticle' located laterally to the oral cone (Figure 5.12b, d) may 'hide' the exiting pores of the CGs, and host tissue obscured the view of the 2nd maxillae (Figure 5.12d). However, a large slit in the cuticle dorsal to the antennae and centrally located between the antennules was observed (Figure 5.12c). This differed in morphology, size and location to the pore hypothesised to be the secretory site of the FGC in free-swimming females (Figure 5.11f).

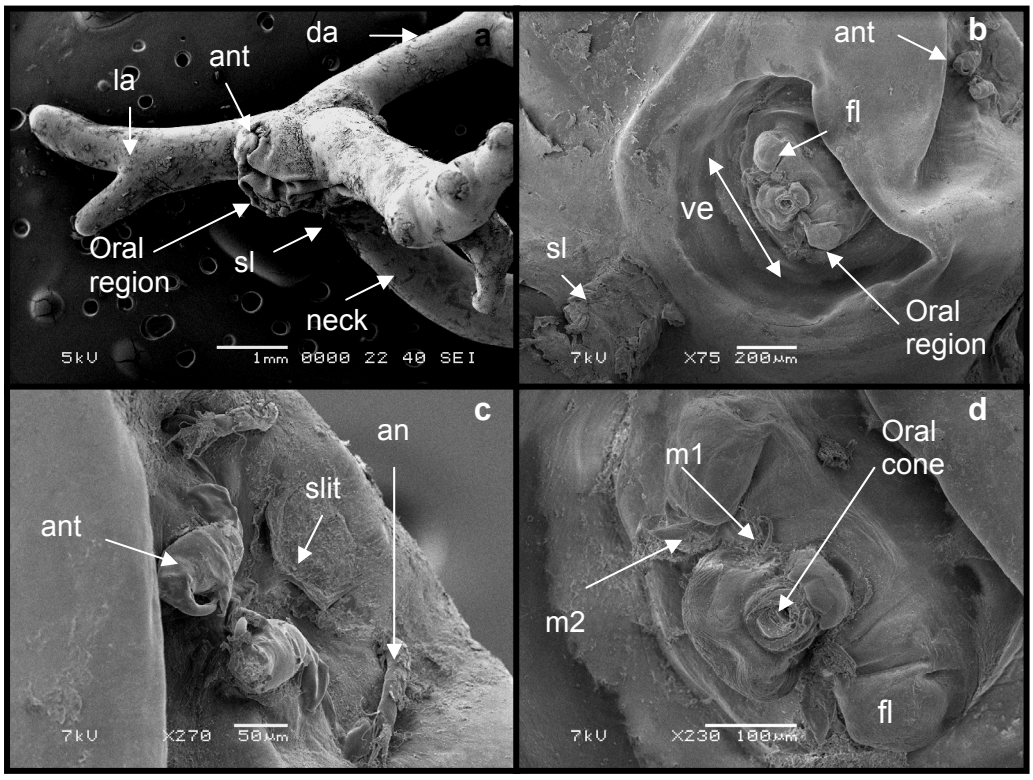

Figure 5.12 Scanning electron micrographs of gravid fully metamorphosed female *L. branchialis* cephalothorax illustrating (a) the location of the appendages and morphology of the cephalothorax; (b) the oral region in relation to the antennae (ant) and swimming legs (sl); note the cavity within which the oral appendages are located on a 'ventral extension' of the oral region (ve); (c) the cuticular slit centrally located between the antennules (an) and dorsal to the antennae (ant); (d) the

location of the oral appendages within the oral region. da, dorsal antler; fl, folded lobe of cuticle; la, lateral antler; m1, 1st maxillae; m2, 2nd maxillae.

5.4 Discussion

5.4.1 Anterior gland complex and circum-oral glands pre- and post- metamorphosis

The DAB staining of *L. branchialis* was found to be successful in highlighting populations of exocrine glands in the free-swimming females, as observed for *L. salmonis* and *C. elongatus* by Bell *et al.* (2000). DAB-positive glands were identified in the vicinity of the oral region, and of particular interest were the CGs and the AGCs. They both possessed neutral carbohydrates due to the positive staining with PAS, suggesting the production of neutral mucopolysaccharides and / or glycoproteins by these glands. This was corroborated by the presence of fibrous-type secretory vesicles, in particular in the outer, anterior portion of the AGC which also stained intensely PAS-positive. Numerous exocrine glands of other copepod species also possess a mucus component (Hicks & Grahame, 1979). Bron *et al.* (1993) suggested that the mucus component of exocrine gland secretions may bind the secretory components, such as enzymes, to prevent their dilution in the external media. They stated that this would aid more efficient contact between the secretions and host tissue or the parasite cuticle. The AGCs and CGs produced multiple types of secretory vesicles suggesting multi-component secretions of which proteins and glycoproteins and / or neutral mucopolysaccharides were a major component. This was evidenced by the abundant and prominent RER, and the presence of Golgi apparatus in the AGCs and the CGs. RER is involved in the synthesis of proteins by ribosomes which line the membranes, and their subsequent storage or transport to the Golgi apparatus. The Golgi apparatus are then involved in the modification of proteins, such as

glycosylation to produce glycoproteins, and their packaging into vesicles which leave the trans face to be distributed to their final location, *e.g*. secretory vesicles which are stored in the cell until their release. Similar membrane-bound, spherical, secretory vesicles of varying electron density have been described in the gland cells associated with the anterior adhesive areas of the parasitic monogeneans *Entobella sp*. and *E. australis* infecting stingrays (Whittington & Cribb, 1998), and of *E. soleae* infecting *Solea solea* (common sole; El Naggar & Kearn, 1983). However, they also described the presence of rod-shaped secretory vesicles, which were not observed in any of the glands studied by TEM for *L. branchialis*. The authors found these spherical secretory vesicles to vary in size from 117nm to 550nm depending on the monogenean species studied, which is similar to the size of the spherical secretory vesicles 1 – 3 and 6 described in the CG and AGC of *L. branchialis*, which were approximately 300 – 500nm in diameter. The positive staining of exocrine glands in parasitic copepods by DAB was hypothesised by Bell *et al.* (2000) to stain the secretory products of the glands and not their ultrastructure. This would imply the production of peroxidases and possibly other compounds stained by DAB, such as catalases and sulphated mucopolysaccharides (Longo *et al.*, 1972; Bussolati, 1971). The production of reactive oxygen species by host phagocytic cells is a well known defense mechanism against parasites. Therefore, many parasites have evolved counteractive measures, such as the secretion of many types of antioxidants including glutathione and thioredoxin peroxidases, and catalases (Hewitson *et al*., 2008; Donnelly *et al*., 2005; Kotze & McClure, 2001). The secretory / excretory products of the blood-feeding nematode *Anguillicola crassus* have also been found to contain glutathione-s-transferase, which the host, *Anguilla anguilla* (European eel), produces a specific antibody response against post-infection (Nielsen &

Buchmann, 1997). Therefore, these glands may produce antioxidants which are secreted at the local feeding area to counteract reactive oxygen species produced by host immune cells infiltrating the infection site, and released by damaged host tissue due to parasite feeding activity. However, DAB has also been found to stain cytochrome oxidase and cytochrome c peroxidase at the ultrastructural level in the mitochondria of cells (Seligman *et al*., 1968; Angermuller & Fahimi, 1981; Enriquez *et al*., 1977). The AGCs and the CGs which stained positive for DAB also possessed numerous mitochondria lining the inner surface of the plasma membrane and scattered throughout the cytoplasm. Therefore, the exact location of the DAB positive reaction requires confirmation by ultrastructural histochemistry studies. However, Bell (2001) found the DAB-positive reaction of exocrine glands in *L. salmonis* to be located in the secretory vesicles, and indicated that they produced a catalase enzyme. On the other hand, the production of acidic sulphated mucopolysaccharides by the CGs and AGC was discounted due to the absence of staining with acidic alcian blue. Bell *et al.* (2000) also stated that peroxidases are involved in the production of prostaglandins from arachidonic acid, such as prostaglandin H synthetase which is involved in the production of prostaglandin E2 (PGE2; Bowman *et al.*, 1996). The secretions of *L. salmonis* have also been found to possess PGE2 (Fast *et al.*, 2004) and it is a common prostanoid found to be secreted by numerous parasites (Belley & Chadee, 1995; Ali *et al*., 1999; Bowman *et al.*, 1996; Ribeiro *et al*., 1985). The importance of PGE_2 in the modulation of the inflammatory response, as a potent vasodilator and in the suppression of the immune response has been stated in numerous studies (Pinge-Filho *et al*., 1999; Williams & Higgs, 1988; Fast *et al.*, 2005; Ribeiro *et al*., 1985; Ham *et al*., 1983). Therefore, the possible production of prostanoids, such as PGE_2, peroxidases and catalases by *L. branchialis* in the exocrine gland

populations highlighted in this study at different metamorphic stages deserves further study.

The paired CGs of the free-swimming female appeared to exit in the vicinity of the oral cone, laterally to the oral cone or possibly around the labrum. However, the labrum was not studied in detail using SEM, especially not the posterior surface. Therefore, the labrum deserves more in depth investigation to highlight any possible pores. Bell *et al*. (2000) also described a pair of circum-oral glands in *L. salmonis*; however, these consisted of two pairs of glands, or a pair of bi-lobed glands, on the ventral surface at the base of the oral cone. The CGs of *L. branchialis* however, resembled more a pair of mucoid glands observed in *L. salmonis* by Bron (1993). The mucoid glands ran from the anterior of the nauplius eye to the start of the cerebrum, possessed PAS-positive vesicles and were hypothesized to discharge either side of the oral cone (Bron, 1993). The CGs of *L. branchialis* could also be similar to the labral glands, which are a common feature of free-living copepods and are often described as the 'salivary glands' (Richards, 1891). However, Arnaud *et al.* (1988a) stated that the described homology with salivary glands was speculative due to the lack of studies on their biochemical composition. These glands have been found to secrete a variety of compounds, which usually exit on the posterior surface of the labrum (Boxshall, 1992). The number of pores through which the labral glands discharge their secretions has been shown to differ, with a single pore reported for harpacticoid copepods and several pores reported in calanoid copepods (Arnaud *et al*., 1988b; Lowe, 1935; Park 1966). The labral glands of copepods have also been found to possess a syncytial organisation with abundant RER, Golgi apparatus and secretory vesicles (Arnaud *et al*., 1988a). These ultrastructural characteristics are consistent with

both the AGCs and the CGs of *L. branchialis*, and highlight their secretory function. The hamulus gland of the parasitic gill monogenean *Cichlidogyrus halli typicus*, thought to secrete at the hamulus hooks with a possible histolytic role enabling penetration of the gill, has also been described by El-Naggar and Kearn (1989) as a large gland possessing syncytial organisation. Whether the pair of glands highlighted in the ventral extension of the oral region of the metamorphosed female, termed the CGs, are the development of the CGs of the free-swimming female, or the ventral extensions of the AGCs remains to be determined. The AGC of the free-swimming female was multi-lobular and appeared to extend inwards from the edge of the cephalothorax towards the oral region. This suggested the possible pores for the secretory site of this gland complex to be either a pore located either side of the oral cone posterolaterally to the 1st maxillae, a pair of pores on the base of the 2nd maxillae or a pair of pores on the ventral surface of the cephalothorax underneath the 2nd maxillae. Post-metamorphosis, the AGCs were enlarged, occupying a large proportion of the cephalothorax, highlighting their probable importance in the metamorphosed female. Capart (1948) described a pair of glands in the metamorphosed female thought to be the AGCs described in the present study. The author described the gland to be multi-lobed, to extend up to the bases of the antlers and to possess two ducts running parallel to the oesophagus and exiting at the base of the 1st maxillae over the oral cone. Although the terminal end of the duct could not be located in the current study, the AGC was observed to extend towards the oral cone, and a pair of pores was located slightly posterolaterally to the 1st maxillae in the free-swimming female. This is therefore, a likely candidate for the secretory site of the AGC. Capart (1948) termed these the maxillary glands, which are usually associated with an excretory function in adult free-living copepods (Boxshall, 1992). However, the AGCs (*i.e*. maxillary glands in

Capart, 1948) were found to possess the 'typical' ultrastructure of secretory glands. Therefore, these glands appear to be secretory and to discharge externally in the vicinity of the oral cone. Bron *et al.* (1993) suggested that the secretion of parasite-derived compounds by exocrine glands directly onto the host tissue could pre-digest host tissue, and / or aid the intake of host tissue in *L. salmonis*. This may be true for *L. branchialis,* enabling efficient feeding on host blood even though a thrombus forms around the parasite as it migrates along the blood vessels (Chapter 4; Smith *et al*., 2007). Other haematophagous parasites have been found to secrete enzymes which dissolute clots to aid blood feeding, such as *Ancylostoma* hookworms which secrete a proteolytic anticoagulant that inhibits both host plasma clotting and promotes fibrin clot dissolution (Hotez & Cerami, 1983). To date, no anticoagulants have been demonstrated in *L. branchialis*. However, it is plausible that in order to obtain a blood meal they secrete compounds to aid clot dissolution and prevent clotting during the feeding process. If this occurs, the anticoagulants are not likely to be continually secreted over the entire infection, as this would be very energetically demanding, and may only be secreted during blood intake. This is only speculative but could explain the observation of intact red blood cells in the parasite gut when thrombi are also observed around the parasite. Other functions of compounds produced by these glands are also likely to be 'key' at the host-parasite interface, such as immunomodulatory roles.

The DAB stain method, however, gave no positive results in the fully metamorphosed female *L. branchialis*. This is likely to be due to a combination of factors: 1) the host tissue remaining on the exterior of the parasite stained intensely with the DAB which may have exhausted the stain prior to penetrating

the glands, 2) the glands no longer contained DAB-positive compounds *e.g*. peroxidases, 3) the thicker cuticle or a difference in the number of pores on the surface per mm^2 cuticle in fully metamorphosed females compared to free-swimming females impeded the penetration of the DAB and / or 4) the concentration of DAB was not optimal for the larger volume of the exocrine glands of fully metamorphosed females. The females were however, cut open along the neck and abdomen in order to allow better penetration of the DAB. This was also not successful due to the deep staining of the damaged area of cuticle. The abundance of secretory vesicles was much lower in the CGs and AGCs of females post-metamorphosis investigated in this study, and may also explain the lack of positive DAB and PAS reaction in these glands post-metamorphosis. However, they possessed more prominent RER, which in some areas, such as the ventral portion of the CGs and the inner dorsal portion of the AGC, had undergone dilation. RER dilation usually occurs when cells are undergoing high protein production and storage, with the accumulation of proteins within the intracisternal spaces (Kristen & Biedermann, 1981). The increased abundance of Golgi apparatus post-metamorphosis, for instance in the central portion of the CGs, also suggests high protein production by the glands post-metamorphosis. This, however, does not corroborate with the scarcity of secretory vesicles in the glands compared to that of free-swimming females. This may be explained by the feeding and / or infection state of the parasite. The abundant secretory vesicles present in the infective free-swimming stage may have been stored ready for the infection process, and secreted at the host-parasite interface to aid the initial attachment and migration to the final infection site. For instance, Wang *et al.* (1999) suggested that the unfed female ixodid ticks, *Rhipicephalus appendiculatus*, produced and stored proteins important for the attachment and / or early feeding stages in the

salivary glands in preparation for the infection. The infective stages of parasites have also often been found to produce proteases to aid their migration in the host tissue to their infection site, which are not secreted by the adult stages. Lackey *et al*. (1989) observed the migratory larval L3 stage of the helminth parasite, *Onchocerca lienalis*, to secrete a serine elastase to enable their migration through the host's cutaneous tissue to the subcutaneous location for adult development. However, they found the adult stages of this parasite to not possess any protease activity corresponding to that of the infective stage. Also, the abundance and composition of the secretory vesicles in these glands in the fully metamorphosed female may depend upon their feeding status. For instance, the protein profile of the salivary glands of the female tick, *R. appendiculatus*, was found to change constantly during the feeding period (Wang *et al*., 1999). Capart (1948) stated that the metamorphosed female does not feed continuously, but takes a blood meal, which can take 5 – 8 days to digest, prior to feeding again. The individuals sampled in this study from wild gadoids were bright red in coloration with intact host red blood cells in their digestive tract, which Capart (1948) described as signs of a parasite which had recently fed, at the early stages of digestion. Therefore, the secretory vesicles may have been partially 'exhausted' during the feeding process prior to digestion. In this respect, El Shoura (1987) found the secretory vesicles of the salivary gland of the adult tick, *Argas persicus*, to be discharged within 5 to 10 minutes after feeding commenced. The abundance and dilation of RER in combination with the presence of Golgi apparatus with vesicles leaving the trans face, suggests that these glands are still highly active in protein synthesis, modification and packaging. Therefore, the glands may be 're-stocking' secretory vesicles prior to the next blood meal, or *de novo* synthesis of proteins may be triggered by the onset of feeding. Oaks *et al*. (1991) demonstrated this in the

salivary glands of feeding ticks, where they found *de novo* gene expression during tick feeding. This was also corroborated by Sauer *et al.* (2000), who stated that the mass and protein content of tick salivary glands increases during feeding. However, this is speculative and to concisely determine this, further ultrastructural and histochemical studies of more individuals at different stages of metamorphosis and feeding state are required.

5.4.2 Frontal gland complex and antennal glands

The FGC of the free-swimming female was positioned dorsally at the anterior of the cephalothorax. This was thought to produce and secrete components involved in the formation and attachment of the frontal filament, used by the female to attach to the 'intermediate' host. Some of these components, presumed to be involved in filament attachment, are thought to be secreted through a pore located at the anterior of the cephalothorax between the antennae. Laterally to the FGC, a pair of glands termed the AGs was also identified, and is thought to secrete via a pair of pores positioned at the dorsal surface of the base of the antennae. Whether these AGs were also involved in the production of components involved in the attachment of the frontal filament needs to be determined due to their secretory site at the base of the antennae. The AGs, FGC and the frontal filament were all positive for the DAB reaction suggesting the secretion of peroxidases, catalases and / or possibly other DAB positive compounds described previously. Bell (2001) found the filament duct, filament and the FGC of the copepodid, chalimus and preadult *L. salmonis* stages, respectively, to be DAB-positive. He suggested that this apparent DAB-positive secretion may be involved in host immune response modulation (Bell, 2001). On the other hand, the AGs were negative and the FGC was mostly negative for neutral carbohydrates by the PAS reaction except for

discrete areas which were positive, suggesting that the main components of the secretions of the FGC and AGs are not mucopolysaccharides or glycoproteins. Capart (1948) described the frontal filament of *L. branchialis* to be injected and diffuse into the host's gill tissue without definite form. This is in contrast to *L. salmonis*, *L. pectoralis* and *C. elongatus* which form well defined frontal filaments composed of a stem and a basal plate (Bron *et al.,* 1991; Johnson & Albright, 1992; Pike *et al.*, 1993; Anstensrud, 1990). The frontal filament organ of *L. salmonis* is composed of three groups of glands, termed A-C (Bron *et al.*, 1991; Gonzalez-Alanis *et al.*, 2001), which were negative with the PAS and alcian blue stain, except for the ducts of the A gland (Bron, 1993). The axial duct which runs down the middle of the stem, and the basal plate of the frontal filament were also found to be positive for the PAS stain, whereas the stem was negative (Bron, 1993). In the present study, the histochemistry of the secretions of these glands in the attached pre-metamorphosed females on the intermediate host should be compared with that of the free-swimming, pre-metamorphosed stages, as the frontal filament is no longer required once the female detaches from the intermediate host. Therefore, changes in these glands may have occurred after detachment from the intermediate host. Capart (1948) also observed a gland system secreting between the antennae of *L. branchialis*, which she termed the frontal gland, and she still observed the frontal gland in the metamorphosed female. However, Capart (1948) did not determine whether this gland was still functional in the metamorphosed adult. In the current study, however, the FGC and AGs were not observed in the fully metamorphosed females. This is possibly due to: 1) repositioning of the glands during metamorphosis; 2) degeneration of these glands due to a lack of requirement, for example the metamorphosed female does not require a frontal filament for attachment, or 3) the size of the metamorphosed

female resulting in the gland complexes being ‘missed’ during sectioning, especially if they remained a similar size to those of the free-swimming female. The lack of DAB-positive reactions and the large size of the fully metamorphosed female made locating exocrine gland populations more difficult than in the free-swimming female. The fact that these glands were not found in the metamorphosed female and that they are principally thought to be involved in the production of the frontal filament, meant that the ultrastructure of these glands was not studied further due to time constraints. However, these gland systems appear to insert their contents into the gill tissue of the host when the parasite is attached by the antennae and frontal filament in the intermediate host, and are also in close proximity to the definitive host’s tissue during the initial attachment and migration of the parasite into the gill filament / arch by the antennae. Therefore, these glands and their secretory products warrant further study, and to establish whether the secretory products possess any host-modulating properties directed at the intermediate and / or the definitive host species and / or components or enzymes to aid the migration of the parasite from the gill filament into the afferent branchial artery. The secretion of proteolytic enzymes to aid parasite migration through the host tissue to their final infection site is a common feature of the infective stages of parasites (Lackey *et al*., 1989; McKerrow *et al*., 1990; Williamson *et al*., 2006). The fact that the glands and the frontal filament inserted into the intermediate host stained DAB-positive may also suggest the secretion of host-modulating compounds, such as peroxidases, by these glands into the host, as described previously for the AGC and CGs.

Other structures within the cephalothorax, thoracic segment, swimming legs, genital segment and abdominal segment stained positively with DAB and PAS,

and were hypothesised to be glandular. However, due to the fact that this study concentrated on the glands of the cephalothorax, in particular those discharging in close proximity to the oral region, the ultrastructure of these structures were not determined by TEM, and were beyond the scope of this study. Therefore, in order to help determine their function, ultrastructural and in-depth histochemical studies are required. However, some of these glands are likely to be integumental glands, such as the DPOGs of the cephalothorax, which were situated just underneath the cuticle discharging on the dorsal surface posterior to the nauplius eye. Numerous functions of integumental glands have been suggested, such as cuticle hardening (Stevenson, 1961), cuticle antifouling (Boxshall, 1982; Bannister, 1993), and pheromone production for the attraction of mates (Gharagozlou-van Ginneken, 1979). Other functions are also likely for some of these glands which were not characterised. For instance, Bell *et al.* (2000) also observed the glands of the thoracic limbs to be deeply stained by DAB in the swimming copepodid and motile adult stages of caligid parasites, suggesting that they were an important aspect of limb function. The unidentified structures observed in the genital segment of the free-swimming female could be the paired seminal receptacles, which store spermatozoa for fertilisation after delivery of the spermatophores by the adult male during copulation, as in adult female caligids (Huys and Boxshall, 1991). However, in order to corroborate this further, detailed histological studies are required.

5.4.3 Conclusions

The infective free-swimming female stage of *L. branchialis* possessed numerous exocrine gland populations with bilateral symmetry, which were positive for the DAB reaction. Of particular interest were the CGs and the AGCs, which were located in the cephalothorax in the vicinity of the oral region. These were found to

possess a syncytial organisation with 'typical' features of secretory glands, such as RER, Golgi apparatus and secretory vesicles. They produce multi-component secretions of which proteins, neutral mucopolysaccharides and / or glycoproteins are thought to be a major component. They were also positive for the DAB reaction suggesting the secretion of peroxidases which may perform defensive mechanisms at the host-parasite interface, such as counteracting the harmful effects of host-derived reactive oxygen species released by host phagocytic cells *e.g*. hydrogen peroxide. The ducts leading to the secretory pores of these glands were not located. However, the CGs appeared to exit near the oral cone (possibly near the labrum), whilst the AGCs appeared to exit laterally to the oral cone, either via a pair of pores located to either side of the 1st maxillae or those located underneath or on the 2nd maxillae. Obviously, more in depth ultrastructural and scanning electron microscope studies are required to determine the exact secretory locations of these gland populations. Post-metamorphosis the AGC remained functional and much enlarged in comparison to that of the free-swimming female. A pair of large glands were also observed either side of the oral cone within the 'ventral extension' of the oral region, which were thought to be homologous to the CGs of the free-swimming female, which had 're-positioned' ventrally from that of the free-swimming female during metamorphosis. However, whether these CGs located in the metamorphosed female were the same as those located laterally to the nauplius eye stretching ventrally to the oral cone in the free-swimming female, or were the ventral extensions of the AGCs remains to be determined. This would involve identifying the exact secretory site of the glands *i.e.* pores, and their secretory ducts. Although further work is required to determine the exact secretory locations of these glands, this study has located gland complexes which may secrete compounds of importance for the parasite at the host-parasite

interface, to allow its migration through the hosts' tissues and its survival whilst feeding on host blood. Further studies could include the detection of different enzyme activities within the gland tissues, such as alkaline phosphatase and acid phosphatase, in order to determine other enzyme activities which may be present. For instance, Buchmann (1998b) observed a strong positive acid phosphatase reaction in the glands of *Gyrodactylus derjavini*, and strong esterase, aminopeptidase and acid/alkaline phosphatase activities in the intestine. They hypothesised that these enzymes are probably released onto the host's epithelium eliciting injury and a host reaction. Future studies would also involve the identification of the secretory products of the AGCs, CGs, FGC and AGs in *L. branchialis* at different stages of metamorphosis / infection and feeding states. This would enable the kinetics of the secretory product production and discharge of these glands at different stages of the infection and feeding to be followed. Such work could be employed to highlight 'key' parasite-derived compounds required for the success of the infection, from the initial attachment of the parasite through to the blood feeding of the metamorphosed female. This would open the door to future studies into vaccine development against parasite-derived compounds for *L. branchialis*, should it become a problem for the aquaculture industry, and possibly for other parasitic copepods, such as *L. salmonis*.

Chapter 6 The effects of *Lernaeocera branchialis* – derived secretory / excretory products on the immune response of *Gadus morhua*

6.1 Introduction

L. branchialis endures a long and intimate contact with its host, owing to its haematophagous, mesoparasitic lifestyle. Many other haematophagous parasites have been found to adapt to similar environments by evading or secreting bioactive molecules capable of modulating their host's inflammatory and immune responses, in order to prolong the infection and feeding period (*e.g.* Ribeiro, 1987; Wikel *et al*., 1994; Inokuma *et al*., 1997; Astigarraga *et al*., 1997; Ribeiro *et al*., 1992; Fast *et al*., 2002a, 2003, 2004 & 2007). The secretions of terrestrial haematophagous arthropods, such as soft and hard ticks, mosquitoes, tsetse flies and sand flies, have received the majority of attention due to the fact that they act as vectors for debilitating diseases, such as Lyme's disease, malaria, sleeping sickness and leishmaniasis, affecting a wide variety of mammalian hosts from humans and pets, to economically important livestock species. This has led to large numbers of researchers trying to develop vaccines against these vectors to prevent them from feeding successfully and hence, conveying the disease agents such as *Leishmania spp*. to their mammalian host through their saliva (Morris *et al*., 2001; Xu *et al*., 2005). The components of these arthropod vectors' saliva and salivary glands have therefore, become of great interest to researchers, many of whom have found saliva and salivary glands to contain a mixture of pharmacologically active complexes, such as anticoagulants, antiplatelet aggregation compounds, analgesics, immunosuppressants and anti-inflammatory compounds (*e.g.* Ribeiro *et al* 1985; Wikel *et al*., 1994; Wikel, 1996; Inokuma *et al*., 1997; Astigarraga *et al*., 1997; Bowman *et al* 1996; Zhu *et al* 1997). These parasite-derived products are thought to be crucial factors in the survival of the parasite allowing them to successfully feed on host blood, especially in ticks which remain attached to their hosts for prolonged

periods of time in comparison to mosquitoes. This prolonged intimate contact at the host-parasite interface is, without parasite intercession, likely to initiate host blood coagulation, vasoconstriction, inflammation and other host immune responses to impede infection.

The modulation or evasion of host immune responses is well characterised in many terrestrial host–arthropod parasite interactions due to their economic and human health implications, however those involving teleost hosts are not as well studied. Crustacean parasites, such as *L. branchialis*, unlike arthropod insects do not possess recognisable 'salivary glands'. They have still however, been found to secrete / excrete products in / onto their hosts. The salmon louse *Lepeophtheirus salmonis* has been most studied to date due to its economic cost to salmonid aquaculture. Trypsin-like proteases (Ross *et al*., 2000, Firth *et al*., 2000; Fast *et al*., 2002a, 2003 and 2007), prostaglandin E_2 (Fast *et al*., 2004) and other compounds (Fast *et al*., 2007), found to be secreted by *L. salmonis* during dopamine incubation are thought to aid feeding and / or host immune response evasion at the site of infection (Fast *et al.* 2002a, 2003 and 2007). Dopamine is a neurotransmitter known to induce salivation in ticks (Kaufman, 1978; Tatchell, 1967) and the release of secretory products by *L. salmonis* (Fast *et al*., 2004), by injection or applying it to the arthropod's cuticle.

When infection and / or injury occur in vertebrates inflammation ensues, and the principal cell types reported at sites of parasitic copepod infection and inflammation in a variety of fish species are neutrophils and macrophages (Joy & Jones, 1973; Boxshall, 1977; Shields & Goode, 1978; Paperna & Zwerner, 1982; Shariff & Roberts, 1989). These phagocytic leukocytes play a key role in this initial response,

resulting in the phagocytosis and subsequent degradation of pathogens and cellular debris by reactive oxygen species (ROS) within the phagosome. The respiratory burst results in the production of ROS such as hydrogen peroxide which is highly toxic to phagocytosed microbes and is metabolised from superoxide ions produced by the activity of NADPH oxidase. The production of nitric oxide (NO) by inducible NO-synthase (iNOS) is also an important cytotoxic effector of macrophages (Neumann *et al*., 2001). Extracellular release of anti-microbial factors may also destroy pathogens without phagocytosis (Neumann *et al*., 2001). Cells infiltrating an infection site therefore produce a range of cytotoxic compounds which are thought to play a role in anti-parasitic activity. Numerous studies have suggested the capacity of macrophage and neutrophil–derived cytotoxic factors to result in parasite tegument damage or death (Whyte *et al*., 1989; Sharp *et al*., 1991; Hamers *et al*., 1992; Nakayasu *et al*., 2005). However, other authors have shown parasites to evade this cytotoxic effect. *Trypanosoma borreli*, for instance, activates granulocyte ROS production thought to allow parasite dissemination, however no cytotoxic effects to the parasite are observed, and high numbers of *T. borreli* have been found to down-regulate neutrophil responses *in vitro*, this being hypothesised to prolong the chronic infection phase (Scharsack *et al*., 2003).

Crude cytokine preparations from mitogen-stimulated leukocytes of numerous fish species have been shown to prime macrophages for enhanced respiratory burst activity, phagocytosis and chemotaxis, and act synergistically with lipopolysaccharide (LPS) increasing NO production *in vitro* (Secombes, 1987; Graham & Secombes, 1988; Neumann & Belosevic, 1996; Neumann *et al*., 1995; Mulero & Meseguer, 1998). These preparations are termed macrophage-activating

factor (MAF) and contain soluble factors with interferon-γ (IFN-γ) like properties (Graham & Secombes, 1990). The major mammalian MAF component is IFN-γ (Nagakawara *et al.*, 1982; Nathan *et al*., 1983) which is primarily produced by T cells and NK cells. However, other MAF may be present in crude mitogen-stimulated leukocyte supernatants, and strong evidence for the existence of tumor necrosis factor-α (TNFα) in crude MAF derived from rainbow trout leukocytes has been made (Hardie *et al.*, 1994; Jang *et al*., 1995; Tahir & Secombes, 1996; Campos-Perez *et al*., 1997).

Humoral innate immunity is also of importance to the clearing of parasitic infections in vertebrate hosts, with complement being highlighted in the literature as a major anti-parasitic component in mammalian and teleost hosts. Complement is part of the innate immune system and consists of serum proteins and cell membrane receptors that act together to kill pathogens. It is activated by three different biochemical pathways (alternative, classical and mannose-binding lectin; Boshra *et al*., 2006; Sunyer *et al.*, 2003) resulting in the production of inflammatory anaphylatoxins, opsonisation of 'non-self' surfaces leading to enhanced phagocytosis and antigen processing, and cell lysis by the membrane attack complex. The functionality of the anaphylatoxins C3a and C5a in teleost fish have recently been studied. C3a was found to strongly enhance rainbow trout head kidney leukocyte respiratory burst activity (Rotlant *et al*., 2004), but not to induce their migration in rainbow trout or carp (Rotlant *et al*., 2004; Kato *et al*., 2004). However, C5a has been shown to be a potent chemo-attractant of leukocytes in rainbow trout and carp (Holland & Lambris, 2004; Boshra *et al*., 2004; Kato *et al*., 2004), and also to induce the respiratory burst in rainbow trout leukocytes (Boshra

et al., 2004). These anaphylatoxins therefore play key roles in inducing the inflammatory response and activating innate immune responses to 'clear' infection (Gasque, 2004; Suyer & Lambris, 1998; Sunyer *et al*., 2005). The complement system has therefore been associated with both teleost and mammalian host immunity to a variety of parasites to date (Wehnert & Woo, 1980; Puentes *et al*., 1987; Forward & Woo, 1996; Harris *et al*., 1998; Buchmann, 1998; Rubio-Godoy *et al*., 2004). However, numerous pathogens including haematophagous parasites have evolved complement evasion strategies in order to establish an infection. This area of research has expanded greatly over the past few years; for more information consult the recent reviews by Zipfel *et al*. (2007) and Schroeder *et al*. (2009).

Parasite survival within a host is, in part, dependent on its ability to evade host immune responses. This can be achieved either by preventing recognition by the host or modulating the immune response to prevent effects which will ultimately lead to parasite death or elimination. Female *L. branchialis,* once attached to the gill filaments of their host, are capable of migrating through the lumen of the branchial afferent artery towards the ventral aorta or the *bulbus arteriosus*. During this process a thrombus forms around the parasite following the parasite's migration within the blood vessels. Smith *et al*. (2007) described this thrombus to become more organized around the cephalothorax with the in-growth of granulation tissue from the arterial or *bulbus arteriosus* wall, and local neo-vascularisation. They also observed the granulation tissue to become more extensive with a more prominent role of the *bulbus arteriosus* endothelium (Smith *et al*., 2007). The cephalothorax of females at later stages of metamorphosis are sequestered within granulation tissue,

becoming embedded within the arterial or *bulbus arteriosus* wall within a connective tissue capsule (Schuurmans Stekhoven, 1936; Capart, 1947). However, the parasite is not eliminated and remains capable of feeding (as red blood cells are found within the gut) and producing eggs. Therefore, the aims of this study were to investigate the effect of *L. branchialis*–derived secretory / excretory products (SEPs) on some key innate cellular and humoral immune responses of naïve Atlantic cod. This was to help address the question of whether SEPs produced by *L. branchialis* are capable of modulating their host environment? To answer this, a number of host immune assays in the presence of dopamine-induced SEPs were performed *in vitro*.

6.2 Materials and methods

6.2.1 Secretory / excretory product (SEP) collection

Secretory / excretory products (SEPs) from *L. branchialis* were collected by the application of dopamine following a protocol described by Fast *et al.* (2004) for *Lepeophtheirus salmonis*. For the collection of SEPs from newly attached female *L. branchialis* (*i.e*. 1-2 days post infection; d.p.i.), 20 naïve Atlantic cod were infected with ten free-swimming female *L. branchialis* per fish by bath challenge as described in Chapter 2. Ten fish were euthanised 1 d.p.i. and the remaining ten 2 d.p.i.. The gills were removed and any attached *L. branchialis* dissected free from host tissue and placed in sterile seawater with aeration. The parasites were then placed into another container of sterile seawater with aeration at 10°C for 1 h to reduce host tissue contamination of SEPs. Parasites were then carefully transferred into 1mM dopamine (Sigma) in sterile seawater at a concentration of 26 females per ml for 40 min at 10°C. The parasites were removed and the solution filtered through a 0.2µm filter. In order to concentrate the SEPs and carry out a buffer exchange for

use in the *in vitro* assays, the solutions were pooled and centrifuged at 3000 xg for 15 min in a 5kDa MWCO Amicon Ultra-15 column (Millipore, Mass., USA) three times with the addition of sterile phosphate buffered saline (PBS), pH 7.2. The protein concentration of the pooled SEP samples was quantified using a BCA protein assay kit (Pierce) following manufacturers' instructions, and the SEPs stored at -70°C.

The same protocol was employed for the collection of SEPs from fully metamorphosed female *L. branchialis*. The latter were dissected from wild gadoids (*Melanogrammus aeglefinus*, *Gadus morhua* and *Merlangius merlangus*) naturally infected with *L. branchialis* sampled by trawling in the North sea and Northeast Atlantic on the wild fish disease survey in May 2007 upon the Fisheries Research Services (Aberdeen) vessel, Scotia. However, in this instance, the fully metamorphosed females were incubated at a density of 1 per ml dopamine in seawater. Parasites incubated at the same densities in sterile seawater without dopamine were used as controls for dopamine induction of SEP production. Aliquots of 1mM dopamine in sterile seawater without parasites were also concentrated as for the SEP samples and used in the *in vitro* assays as a control for dopamine or salt carry-over into SEPs (this is termed DSCtrl). This DSCtrl was used in all assays at the same volume to volume ratio as that of the 20µg.ml^{-1} SEP in order to control for the maximum amount of salt or dopamine carry-over into the assay, *i.e*. if 10µl SEP in 100µl cell suspension gave a 20µg.ml^{-1} [SEP] then 10µl DSCtrl would be added to the appropriate cell suspension as a control.

6.2.2 Validation of SEP production

In order to determine whether SEPs were released by *L. branchialis* in the presence of dopamine, following Fast *et al.* (2004), SEP samples, pre-concentration, were examined for proteins and protease activity by SDS-PAGE and zymography, respectively. The SDS-PAGE was performed as described in Chapter 2.8 and zymograpy was performed following the methods given in Firth *et al.* (2000) and Fast *et al. (*2003). Briefly, SEP samples were diluted 1:1 with 4% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 125mM Tris–HCl at pH 6.8, and equal volumes were loaded onto a 12% polyacrylamide gel containing 0.1% (w/v) gelatine. After electrophoresis, gels were washed three times at 4°C with 2.5% (v/v) Triton X-100, 50mM Tris–HCl, pH 7.5, and incubated for 19h at 30 C in the same buffer containing 50mM $MgCl_2$ and 6.25mM $CaCl_2$. Gels were then stained with 0.25% (w/v) Coomassie blue R-250 (40% (v/v) methanol, 10% (v/v) acetic acid). Kaleidoscope™ Precision Plus protein standards (Bio-rad, California, USA.) and Full Range Rainbow™ molecular weight markers (Amersham Biosciences, Buckinghamshire, UK) were used for calibration of protease molecular weights. The protease and protein profiles of the SEP samples were qualitatively compared to samples from parasites incubated in seawater without dopamine, and samples of dopamine in seawater without parasites.

6.2.3 Naïve leukocyte collection

Head kidney-derived and blood-derived leukocytes were isolated as described in Chapter 2.9 from naïve Atlantic cod originating from Viking Fish Farms Ltd. (Ardtoe, Scotland).

6.2.4 Effect of SEPs on phagocyte respiratory burst activity

Head kidney-derived leukocytes (5 x 10^6 cells.ml^{-1} L-15 medium) from 15 naïve Atlantic cod (mean weight 140.8 ± 24.6g) were pre-incubated for 24 h at 6°C in the dark with 0, 2.5, 5, 10 and 20µg.ml^{-1} 1-2 d.p.i. female *L. branchialis* and fully metamorphosed *L. branchialis* SEPs and DSCtrl. Respiratory burst activity of head kidney-derived phagocytes was then measured following a modification of the flow cytometry method of Richardson *et al*. (1998) suggested by Moritomo *et al*. (2003) as detailed in Chapter 2.10.2. The mean fluorescence intensity on fluorescent channel 1 (FL-1) and percentage of the phagocyte population undergoing respiratory burst were recorded and expressed as an index compared to that of phagocytes incubated without SEPs.

e.g.:

$$\frac{\text{mean FI of phagocytes incubated with 20µg.ml}^{-1}\text{ SEP}}{\text{mean FI of phagocytes incubated with 0µg.ml}^{-1}\text{ SEP}} \times 100$$

The cell viability of the phagocytes after the 24 h incubation was checked following the protocol described in Chapter 2.10.1 using propidium iodide (Sigma) to ensure that any effect on phagocyte respiratory burst activity was not due to the death of cells after incubation with SEPs.

Unfortunately, due to the limited quantity of SEPs collected from 1-2 d.p.i. female *L. branchialis,* there were insufficient SEPs left to use in further analysis. This was a result of the small size of the newly attached female parasite and the impracticalities of infecting large numbers of fish to collect enough parasites at this stage for SEP production. Therefore, only the effect of SEPs from fully

metamorphosed *L. branchialis* on the immune responses detailed below could be examined.

6.2.5 Effect of SEPs on macrophage activating factor (MAF) production

6.2.5.1 MAF production

Macrophage activating factor (MAF) was produced via mitogenic stimulation of mixed head kidney-derived leukocyte cultures according to Graham and Secombes (1988). Head kidney-derived leukocytes from 20 naïve Atlantic cod (mean weight 133.2 ± 22.5g) were isolated according to the methods outlined in Chapter 2.9.2 except that all cell suspensions were layered onto 51% Percoll (Sigma) to isolate all leukocyte populations. The cells at the interface between the medium and Percoll were collected and washed twice in L-15 medium at 150 xg for 15 min at 4°C to remove the remaining Percoll. The cells collected from the 20 fish were pooled together and re-suspended to 5 x 10^6 cells.ml^{-1} L-15 medium supplemented with 5 x 10^{-5} M 2-mercaptoethanol (2ME; Sigma).

The pooled leukocyte suspension was seeded into 19 25cm^2 sterile cell culture flasks (5ml suspension per flask; Nunc, Denmark). The cells were pre-incubated for 1 h at 6°C with 0, 5, 10 and 20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs or DSCtrl (3 flasks per treatment). The cells were then pulsed for 3 h with 10µg.ml^{-1} concanavalin A (ConA; Sigma) and 5ng.ml^{-1} phorbol myristate acetate (PMA, Sigma) at 6°C. The cells were then gently washed 5 times with L-15 medium to remove any residual ConA or PMA, prior to culturing the cells for 48 h in L-15 medium with 1% (v/v) foetal calf serum (FCS) and 5 x 10^{-5} M 2-ME. The supernatants were collected from the flasks and those of the same treatments

pooled together prior to centrifugation at 1000 xg for 10 min at 4°C. The cell-free supernatants were split into 5ml aliquots and frozen at -20°C until further use. Negative controls consisted of supernatants taken from cells incubated without ConA or PMA (cell supernatant control in duplicates), and medium removed from cells incubated with conA and PMA immediately post–washing (wash control in duplicates). The latter control was to ensure that any effect of MAF supernatants on Atlantic cod leukocytes were not due to the carry over of the mitogens ConA and PMA into the supernatant. The cell viability of the pooled leukocytes within triplicate wells of a 24 well culture plate (5 x 10^6 cells.well^{-1}) per treatment was determined after 1 h incubation with SEPs or DSCtrl following the protocol described in Chapter 2.10.1 using propidium iodide to ensure that any effect on MAF production was not due to the death of cells after incubation with SEPs.

6.2.5.2 Effect of MAF on naïve phagocyte respiratory burst activity

The activity of the MAF supernatants was determined by measuring their effect upon the respiratory burst activity of naïve Atlantic cod phagocytic cells. Head kidney leukocytes were isolated from 10 Atlantic cod (mean weight 126.3 ± 20.3g) as described in Chapter 2.9.2. The leukocytes (5 x 10^6 cells.ml^{-1} L-15 medium with 1% (v/v) foetal calf serum) were used for macrophage enrichment following the protocols of Sorensen *et al*. (1997) and Steiro *et al.* (1998). Briefly, the leukocyte suspensions were aliquoted into 24-well sterile tissue culture plates (1ml.well^{-1}; Nunc, Denmark), and incubated overnight at 6°C. Non-adhered cells were washed off gently with three washes of L-15 medium. After the last wash MAF supernatants were applied to the wells in duplicates, *i.e*. MAF produced after pre-incubation of the cells with 0 - 20µg.ml^{-1} SEPs and DSCtrl, at an optimal dilution previously determined to be 1 in 8, as described in Section 6.3.3. The monolayers were

incubated for 48 h at 6°C prior to measuring their respiratory burst activity. Briefly, the cells were washed twice with fresh L-15 medium, gently scraped from the bottom of the well and placed into a flow cytometry tube (BD Biosciences). The cells were left to rest for 1 h at 6°C prior to measuring their respiratory burst activity on the flow cytometer as described in Chapter 2.10.2. Mean fluorescence intensity (FL-1) of the gated phagocyte population, which is proportional to the quantity of oxidised DHR-123 *i.e.* H_2O_2, was recorded. Pre-experiments were carried out in order to determine the optimal MAF dilution (1 in 4 or 8), and the effect of MAF supernatants in comparison to the controls, *i.e*. cell supernatant control and the wash control.

6.2.6 Macrophage nitric oxide release

6.2.6.1 Mitogenic stimulation of Atlantic cod macrophages to produce nitric oxide

A range of LPS concentrations were tested in order to determine the optimal concentration with or without PMA to stimulate NO release from cod macrophage monolayers. Head kidney leukocytes were isolated from 3 naive Atlantic cod (mean weight 161 ± 8.1g) as described in Chapter 2.9.2. The leukocytes were used for macrophage enrichment as described in section 6.2.5.2, except that the leukocyte suspensions were aliquoted into 96-well sterile tissue culture plates (100µl.well^{-1}; Iwaki Glass Co., Japan). After the last wash fresh medium containing 0, 1, 10 and 50µg.ml^{-1} LPS extracted from *Vibrio anguillarum* (A kind gift from Dr. Remi Gratacap; Gratacap *et al*., 2008) with or without 1µg.ml^{-1} PMA was applied to triplicate wells. Nitric oxide (NO) release by head kidney-derived macrophages was then measured at 24, 48 and 72 h post-stimulation using the Griess reaction following Neumann *et al* (1995). This assay quantifies NO indirectly by measuring

the stable breakdown product, nitrite, due to the short lifetime of NO. Briefly, 75µl cell supernatant was transferred to a non-absorbent 96 well microplate (Sterilin), 100µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid was added, and after 5 min 100µl 0.1% N-naphthyl-ethylenediamine (w/v) in 2.5% (v/v) phosphoric acid was also added. The O.D. was read at 540nm on an MRX microplate reader (Dynex technologies). The nitrite concentrations of the supernatants were extrapolated from a standard curve of known sodium nitrite (Sigma) concentrations ranging from 0-100µM, against a reference blank of L15 medium only.

6.2.6.2 The effect of SEPs on nitric oxide production by cod macrophages

Head kidney leukocytes were isolated from 13 naïve Atlantic cod (mean weight 131 ± 18.6g) as described in Chapter 2.9.2. The leukocytes were used for macrophage enrichment as described in Section 6.2.6.1. After the last wash fresh medium was applied to duplicate wells containing 0, 2.5, 5, 10 and 20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs and DSCtrl with or without 50µg.ml^{-1} LPS. The NO release by head kidney-derived macrophages was then measured 72 h post-stimulation using the method described above.

6.2.7 The effect of SEPs on chemotaxis

6.2.7.1 Activation of naïve cod serum with zymosan

Fresh cod serum from six naïve Atlantic cod was pooled prior to activation with zymosan A (Sigma) according to the methods of Buchmann & Bresciani (1999). Briefly, pooled cod serum was incubated with 10mg.ml^{-1} zymosan at 37°C for 1 h. The serum was then centrifuged at 700 xg for 5 min in order to pellet the zymosan. The serum supernatant was collected and used fresh for chemotaxis assays. Prior

to activation, serum was incubated at 12°C for 1 h with 0 - 20µg.ml^{-1} fully metamorphosed SEPs or DSCtrl.

6.2.7.2 Chemotactic capacity of zymosan activated serum (ZAS)

Leukocyte chemotaxis experiments were performed using a transwell 96-well permeable support system with a 3µm pore polycarbonate filter (Corning, New York, USA). Zymosan activated sera (1/10 diluted in L-15 medium), produced after incubation with 0-20µg.ml^{-1} fully metamorphosed SEPs or DSCtrl, were used as chemo-attractants, and L-15 medium used as a negative control. One hundred and fifty µl of each chemo-attractant was placed in the lower wells, in duplicates, separated from the upper chambers by a 3µm pore polycarbonate filter. Head kidney- and blood-derived leukocytes from three naïve Atlantic cod (mean weight 146 ± 19.7g) were re-suspended to 8 x 10^6 cells.ml^{-1} in L-15 medium, and 50µl placed in the top chamber (4 x 10^5 cells). The chemotaxis chamber was incubated for 2 h at 6°C. The filter was removed and the leukocytes which had migrated to the bottom well were allowed to settle for 30 min at 6°C. The leukocytes in the lower well were then re-suspended and cytospins prepared by centrifuging 100µl of the cell suspension onto glass slides at 120 xg with a cushion of 50µl heat inactivated foetal calf serum (Sigma) using a Shandon Cytospin® 3 cytocentrifuge (Thermo Scientific, Maine, USA). The slides were air-dried, fixed in methanol and stained with Rapid Romanowsky stain (Raymond A Lamb, U.K.) following manufacturers' instructions. Migration was quantified by counting the number of leukocytes under a light microscope with a 40x objective in 4 random fields of the cytospun suspensions from the lower wells (*i.e.* the leukocytes that had migrated across the filter into the bottom well). Additional controls included lower wells containing 20µg.ml^{-1} SEPs or DSCtrl in order to make sure that any chemotactic capacity of

ZAS was not due to the presence SEPs. Migration was expressed as a chemotaxis index as described by Huising *et al.* (2003), *i.e*. the number of migrated cells / the number of randomly migrating cells (those that migrated towards the negative control comprising L-15 medium only).

6.2.8 The effect of SEPs on the spontaneous haemolytic activity of cod serum

Serum collected from 10 naïve Atlantic cod (mean weight 140.8 ± 26.0g) were individually pre-incubated with 0, 5, 10 and 20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs and dopamine seawater control (DSCtrl) for 1 h at 12°C. Spontaneous haemolytic activity of the serum was determined following the protocol of Magnadottir *et al.* (2001) except with the modification of no EDTA being present in the assay, and data analysis of the percent SH determined following Magnadottir *et al*. (1999a, b). Briefly, sheep red blood cells (SRBC) 1:1 in Alsevers solution (Oxoid, U.K.) were washed 3 times in 0.85% (w/v) saline solution and diluted in complement fixation test diluent (Oxoid) with 0.1% (w/v) gelatine (CFT-G buffer) until 100µl added to 3.4ml distilled water gave an O.D. of 0.740 at 414nm. This suspension was then further diluted 1:1 with CFT-G buffer (to give a 0.5% SRBC suspension). Serum was diluted 1 in 20 in CFT-G and 100µl added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicate. Fifty µl 0.5% SRBC suspension was added and incubated at 37°C for 1 h. Controls on each plate comprised distilled water replacing serum (100% lysis), CFT-G buffer replacing serum (0% lysis) and serum blanks. Additional controls were implemented to detect if the SEPs had any direct effect on the haemolysis of SRBCs, which involved replacing fish serum with 20µg.ml^{-1} SEPs or DSCtrl. After 1 h the plates were centrifuged at 700 xg for 10 min at 4°C, and 125µl supernatant transferred into a

non-absorbent flat-bottomed 96-well microplate (Sterilin), and the absorbance read at 405nm using a MRX microplate reader (Dynex technologies). The sample absorbance values were corrected by subtraction of the absorbance of the sample blank control. The percentage haemolysis for each sample was calculated from the O.D. value of the 100% lysis control.

6.2.9 Statistical analysis

A General Linear Model (GLM) with Tukey's *post-hoc* test was used to determine significant differences between groups ($p < 0.05$). Non-normal data were normalised by log, reciprocal or square root transformation. All other data was normally distributed with equal variances. The fish number was taken as a random factor in the GLM for all data due to the high variation between fish, as each treatment was performed on one fish with many fish replicates. Graphs depict the mean ± 2x standard error (2SE), and mean fish weights are given ± standard deviation.

6.3 Results

6.3.1 Validation of SEP production by SDS-PAGE and zymography

The higher abundance of proteins and proteases within the dopamine-induced SEP samples suggests that the use of 1mM dopamine proved successful in stimulating the production of SEPs by both 1-2 d.p.i. (Figure 6.1B1) and fully metamorphosed *L. branchialis* (Figure 6.1A1, B2). The 1-2 d.p.i. SEP samples from the females, pre-concentration, did not contain enough proteins to detect them via SDS-PAGE, however, those of fully metamorphosed females showed a higher concentration of proteins with multiple bands ranging from >158kDa to <27kDa (Figure 6.1A1). The protein profiles of SEPs from different fully metamorphosed females induced by

dopamine showed some variability between them (Figure 6.1A1) which is likely to be due to their feeding stage or time post host-attachment, and therefore all SEP samples were pooled prior to concentration. Post-concentration, using the 5kDa MWCO columns, the SEPs from fully metamorphosed females showed very abundant bands of proteins (ranging from approximately 250kDa – 10kDa; Figure 6.1A2) and protease activity (ranging from approximately 250kDa – 15kDa; Figure 6.1B3). The concentrated DSCtrl on the other hand showed no protease activity but faint protein bands at approximately 60 and 70kDa (Figure 6.1A2, B3). This suggests a possible protein contamination within the dopamine seawater (DS), however, due to the fact that the same DS was used for the production of SEPs and the concentration of the DSCtrl, any effect of the SEPs on *in vitro* assay activities could not be attributed to these low abundance proteins.

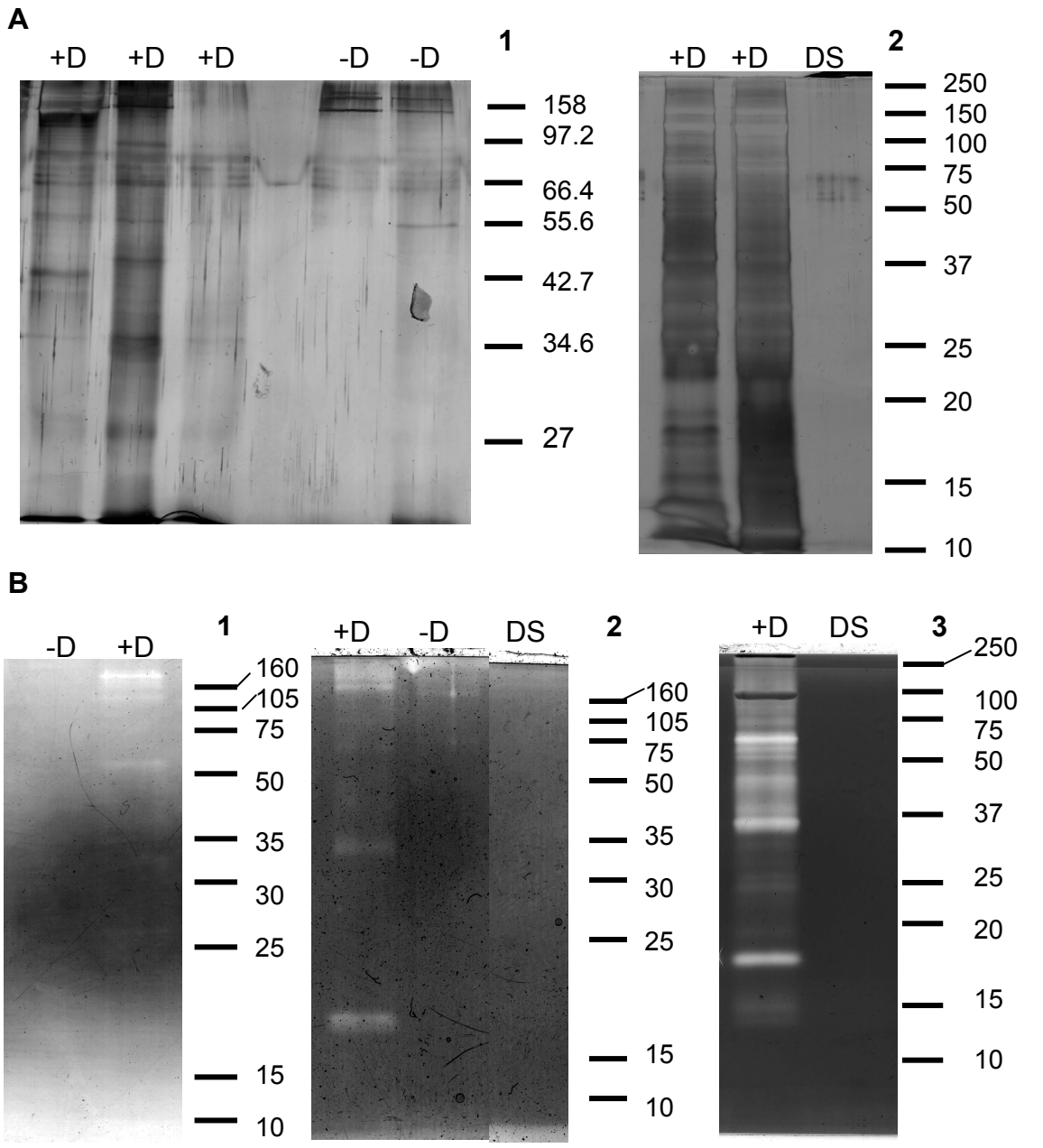

Figure 6.1 (A) SDS-PAGE illustrating the protein profiles of (1) individual pre-concentration and (2) pooled post-concentration SEPs collected from fully metamorphosed female *L. branchialis*. Note the higher protein content in SEPs from 1mM dopamine-exposed (+D) than unexposed (-D) individuals. (B) Zymograms highlighting the protease profiles of (1) pooled pre-concentration SEPs collected from 1-2 d.p.i. and (2) pooled pre-concentration and (3) post-concentration SEPs collected from fully metamorphosed females. Molecular weights are denoted at the side. (DS, dopamine in sterile seawater minus parasites, *i.e*. DSCtrl).

6.3.2 The effect of SEPs on cod phagocyte respiratory burst activity

The SEPs of 1-2 d.p.i. female *L. branchialis* did not significantly affect the respiratory burst activity of head kidney-derived phagocytes in terms of the percent of the population undergoing respiratory burst ($p = 0.713$) or their mean FI *i.e.* H_2O_2 production ($p = 0.468$), regardless of the SEP concentration used (Figure 6.2).

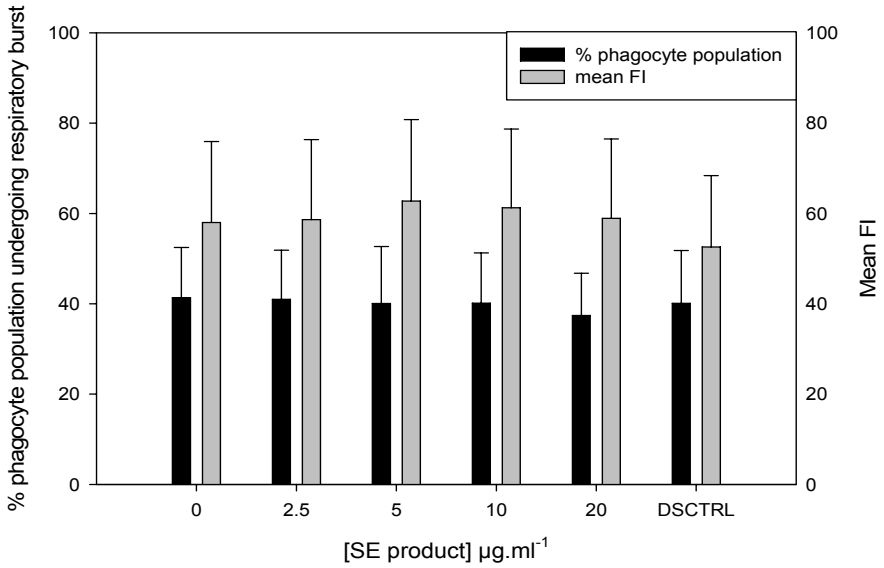

Figure 6.2 Mean percent head kidney phagocytes undergoing respiratory burst and the mean fluorescence intensity (FI) of the phagocyte population after 24 hour incubation with 0-20µg.ml^{-1} 1-2 d.p.i. female *L. branchialis* SEPs or DSCtrl. Error bars = 2SE, n = 15.

Fully metamorphosed female SEPs on the other hand significantly affected the respiratory burst activity of naïve head kidney phagocytes. An increasing trend in the percent of the phagocyte population undergoing respiratory burst was found to occur with increasing concentrations in SEPs (Figure 6.3). The percent of the phagocyte population undergoing respiratory burst with 20µg.ml^{-1} SEPs was significantly higher than that with 0µg.ml^{-1} ($p = 0.034$) or DSCtrl ($p = 0.03$), with a

mean difference of 22.9 ± 14.9% and 18.6 ± 10.7%, respectively (Figure 6.4). The percent of phagocytes undergoing respiratory burst after pre-incubation with 10µg.ml^{-1} SEPs was also found to be significantly higher than that with DSCtrl ($p = 0.045$) but not of phagocytes with 0µg.ml^{-1} SEPs ($p = 0.051$). In contrast, the mean FI of the phagocyte population showed a decreasing trend with increasing SEP concentrations (Figure 6.3). The phagocyte population mean FI was significantly reduced when cells were incubated with 20µg.ml^{-1} SEPs compared to that of cells incubated with 0µg.ml^{-1} ($p = 0.003$) or DSCtrl ($p < 0.001$), with a mean difference of 22.7 ± 5.42% and 27.7 ± 5.4%, respectively (Figure 6.3). The lower SEP concentrations had no significant effect on phagocyte population mean FI compared to when no SEPs were present. However, a significant difference between the mean FI of phagocytes incubated with DSCtrl and 2.5 ($p = 0.0331$), 5 ($p = 0.002$) and 10µg.ml^{-1} ($p = 0.001$) SEPs was found (Figure 6.3). This is probably due to a slight but not significant increase in the mean FI of phagocytes incubated with DSCtrl compared to that of cells incubated with no SEPs (Figure 6.3). Incubation of leukocytes for 24h with the SEPs collected from newly attached or fully metamorphosed females or with DSCtrl had no effect on phagocyte cell viability compared to controls (data not shown). These results suggest that the fully metamorphosed female SEPs increased the percent of the population undergoing respiratory burst, but at the same time reduced their capacity to produce H_2O_2, in a dose-dependent manner. In contrast, the newly attached females had no significant effect on host phagocyte respiratory burst.

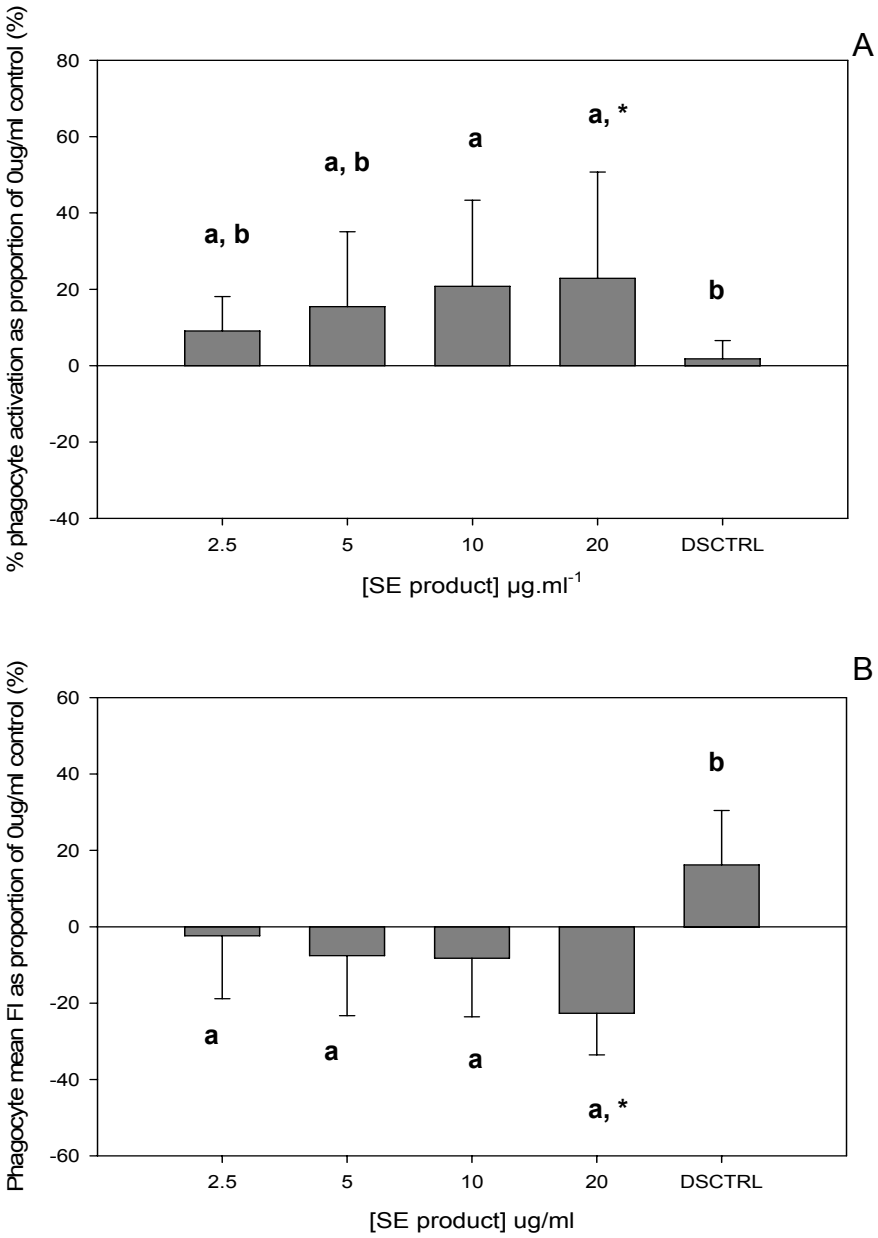

Figure 6.3 Mean percent increase/decrease in (A) the proportion of head kidney phagocytes undergoing respiratory burst and (B) their mean FI after 24 h incubation with 2.5-20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs or DSCtrl relative to those with 0µg.ml^{-1} SEPs, post-PMA stimulation. * denotes significantly different from that of phagocytes incubated with 0µg.ml^{-1} SEPs ($p < 0.05$), and different letters denote significant differences from each other ($p < 0.05$). Error bars = 2SE, n = 15.

6.3.3 The effect of SEPs on MAF production by cod leukocytes

Incubation of cod macrophages with MAF diluted 1 in 8 for 48 h resulted in a significantly higher mean FI *i.e*. H_2O_2 production, compared to macrophages incubated with cell supernatant controls ($p < 0.001$), wash controls ($p = 0.023$), and MAF diluted 1 in 4 ($p = 0.012$; Figure 6.4). Therefore, for all subsequent experiments MAF was diluted 1 in 8 to 'prime' macrophages.

Incubation of macrophages with MAF produced from head kidney leukocytes pre-incubated with 5, 10 and 20µg.ml^{-1} SEPs for 1 h prior to mitogen stimulation, did not result in a significant increase in their mean FI compared to macrophages incubated without MAF ($p = 0.062$, $p = 0.511$ and $p = 0.856$, respectively; Figure 6.4). The 20µg.ml^{-1} SEP concentration resulted in a significantly lower mean FI compared to macrophages incubated with MAF produced without SEPs and after pre-incubation with DSCtrl ($p = 0.0394$ and $p = 0.016$, respectively; Figure 6.4). Macrophages incubated with MAF produced from head kidney leukocytes pre-incubated with 0µg.ml^{-1} SEPs and DSCtrl on the other hand showed a significant increase in their mean FI compared to phagocytes incubated without MAF ($p = 0.0007$ and $p = 0.0002$, respectively; Figure 6.4). The 1 h incubation of leukocytes with SEPs or DSCtrl had no effect on cell viability compared to controls (data not shown). Therefore, the highest concentration of SEPs used seems to have reduced the capacity of leukocytes to produce MAF after mitogen stimulation with a 'priming' effect on the respiratory burst activity of naïve macrophages.

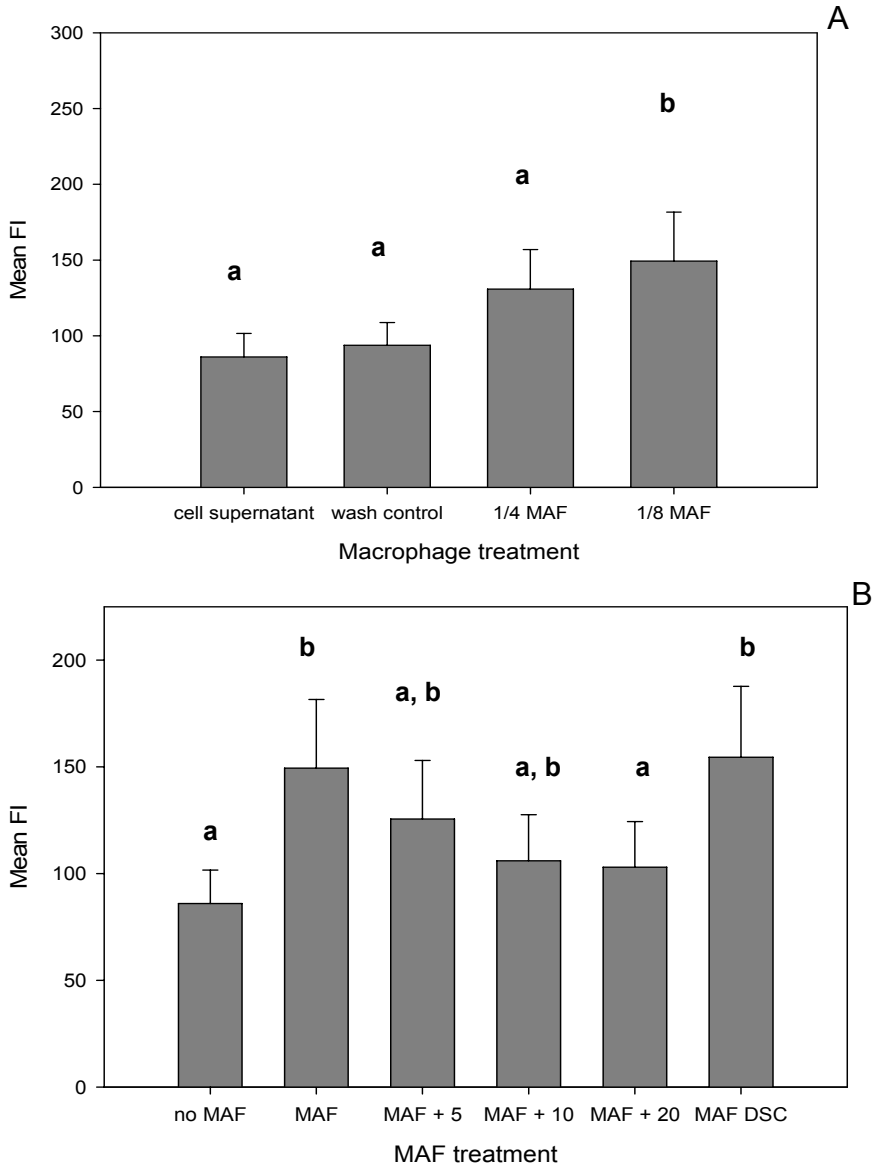

Figure 6.4 (A) Mean FI of head kidney phagocytes after 48 h incubation with macrophage activating factor (MAF) diluted 1/4 and 1/8 or negative controls consisting of cell supernatant and wash controls diluted 1/8. (B) Mean FI of head kidney phagocytes after 48 h incubation with macrophage activating factor (MAF) diluted 1/8 produced after pre-incubation of leukocytes with 0-20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs or DSCtrl for 1h. Different letters denote significant differences from each other ($p < 0.05$). Error bars = 2SE, n = 10.

6.3.4 Macrophage nitric oxide (NO) release by cod macrophages

6.3.4.1 Mitogenic stimulation of macrophages to produce NO

The production of nitric oxide was not detected after stimulation of macrophage monolayers with LPS with or without PMA using the method described, regardless of the concentration used or the stimulation time (data not shown).

6.3.4.2 The effect of SEPs on nitric oxide production by cod macrophages

Incubation of macrophages with fully metamorphosed female SEPs with or without LPS did not result in the production of a detectable amount of nitric oxide using the assay employed in this experiment (data not shown).

6.3.5 The effect of SEPs on cod leukocyte chemotaxis

6.3.5.1 The effect of SEPs on the chemotactic capacity of zymosan activated cod serum

Serum activated with zymosan (ZAS) in the presence of 0µg.ml^{-1} SEPs and DSCtrl induced the migration of head kidney (HK)-derived leukocytes compared to random migration controls significantly ($p = 0.005$ and $p = 0.013$, respectively; Figure 6.5A). In contrast, ZAS produced in the presence of 20µg.ml^{-1} SEPs did not significantly induce HK leukocyte migration compared to random migration controls ($p = 0.923$), and significantly more leukocytes migrated towards ZAS produced with 0µg.ml^{-1} SEPs and DSCtrl compared to ZAS produced in the presence of 20µg.ml^{-1} ($p = 0.018$ and $p = 0.049$; Figure 6.5A). The ZAS seems to be a more potent chemo-attractant for blood–derived leukocytes than those from the head kidney as illustrated by the higher chemotaxis indices of the former (Figure 6.5). The migration of peripheral blood–derived leukocytes towards ZAS revealed a similar trend to that

seen for HK leukocytes (Figure 6.5), with ZAS produced in the presence of 0μg.ml^{-1} SEPs and DSCtrl significantly inducing leukocyte migration compared to random migration controls ($p = 0.006$ and $p = 0.016$, respectively; Figure 6.5B). However, ZAS produced in the presence of 5μg.ml^{-1} SEPs also induced significant migration ($p = 0.036$; Figure 6.5B). ZAS produced in the presence of 10 and 20μg.ml^{-1} SEPs did not induce significant migration compared to media controls ($p = 0.097$ and $p = 0.215$, respectively), however no significant difference was detected in the leukocyte migration at these concentrations with respect to ZAS produced in the presence of 0μg.ml^{-1} SEPs and DSCtrl, probably as a result of the high variation of leukocyte migration between fish (Figure 6.5B). These results suggest that SEPs reduce the capacity of zymosan activated serum to induce migration of naïve head kidney leukocytes in a dose-dependent manner. However, a conclusive result for blood leukocytes could not be made due to the fact that although serum activated post-incubation with 0μg.ml^{-1} and 5μg.ml^{-1} SEPs or DSCtrl showed a significant induction of leukocyte migration and ZAS produced in the presence of 10 and 20μg.ml^{-1} SEPs did not induce migration, no significant difference in the migration of leukocytes towards the latter and the controls was demonstrated. This is likely due to the small number of cod used in this particular assay, and the repetition of this experiment with larger numbers of fish may discriminate more significant differences. The controls of medium with 20μg.ml^{-1} SEPs or DSCtrl did not induce/suppress migration compared to the random migration controls (data not shown).

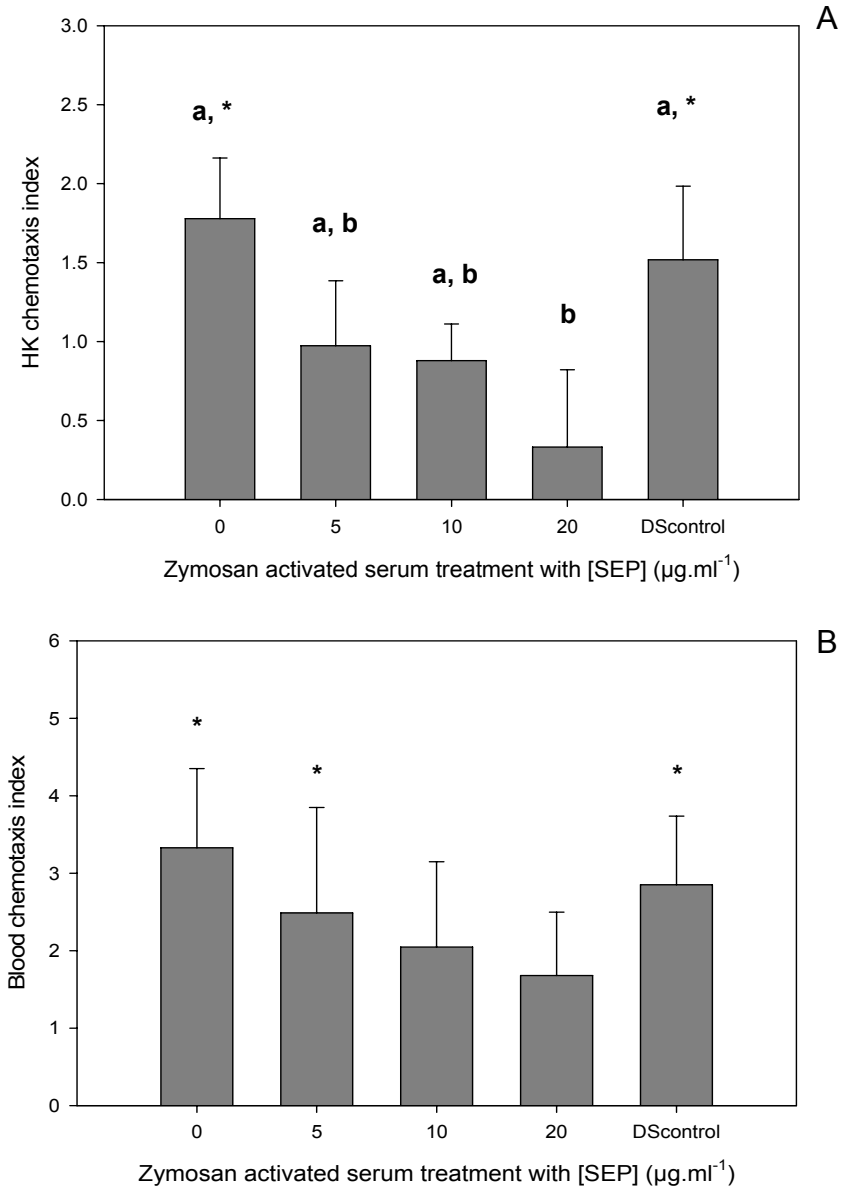

Figure 6.5 Mean chemotaxis index (relative to L-15 media controls) of (A) head kidney leukocytes and (B) blood leukocytes towards zymosan activated serum (1/10) produced after pre-incubation of serum with 0-20µg.ml^{-1} pooled fully metamorphosed *L. branchialis* SEPs or DSCtrl for 1h. * denotes significantly different from L-15 medium control ($p < 0.05$), and different letters denote significant differences from each other ($p < 0.05$). Error bars = 2SE, n = 3.

6.3.6 The effect of SEPs on the spontaneous haemolytic activity of cod serum

The spontaneous haemolytic (SH) activity of cod serum did not significantly change with increasing SEP concentrations, but an increasing trend in SH activity was

observed with increasing SEP concentrations (Figure 6.6). A concentration of 20µg.ml^{-1} SEPs or DSCtrl haemolysis controls did not cause any haemolysis of the SRBCs in comparison to the 0% lysis controls *i.e.* SRBC in assay buffer (data not shown). Therefore, the SEPs did not cause direct haemolysis of SRBCs.

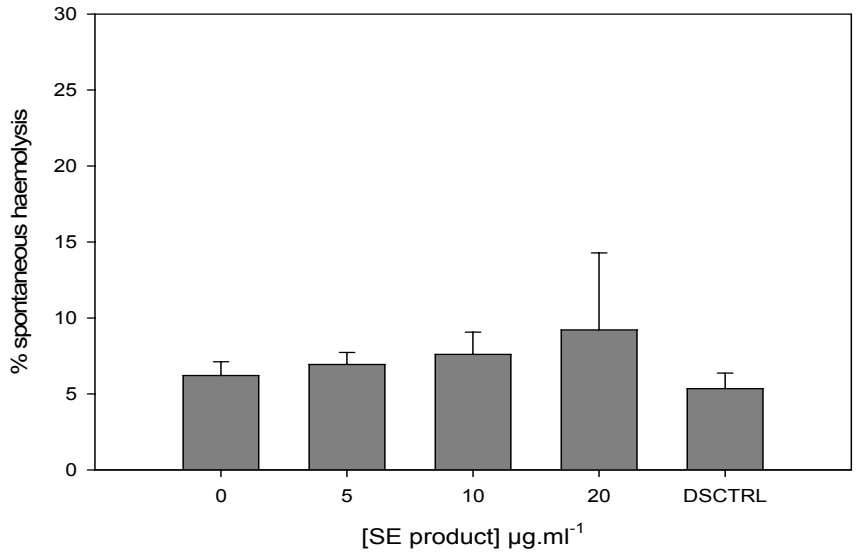

Figure 6.6 Mean percent spontaneous haemolysis activity of serum (1/20) after pre-incubation with 0-20µg.ml^{-1} pooled fully metamorphosed *L. branchialis* SEPs or DSCtrl for 1h. Error bars = 2SE, n = 10.

6.4 Discussion

6.4.1 SEP collection and validation

The application of dopamine to the seawater in which *L. branchialis* were incubated seemed to be successful in inducing SEPs from both the pre- and post-metamorphosed females. This is in line with the results for dopamine stimulation of secretory products from *L. salmonis* demonstrated by Fast *et al*. (2004), who first applied this method to copepod parasites. The fully metamorphosed female *L. branchialis* were found to produce a higher abundance of SEP proteins than the newly attached females, even though they were incubated at 1 female per ml

compared to 26 newly attached females per ml. However, the fact that the fully metamorphosed females are bigger in size than the newly attached females and that due to this they were incubated at different densities, means that no direct comparison could be made between the respective SEP protein concentrations. Following collection, the SEPs were concentrated and buffer-exchanged into phosphate buffered saline and were sterile filtered for use in *in vitro* assays. This was done in order to remove contaminating dopamine and salt, and to allow the measurement of the protein content so as to be able to use different concentrations of SEPs in the various assays. This technique for parasite SEP collection does, however, have some faults which could not be avoided due to the field conditions in which they were collected. The SEPs, as noted by Fast *et al*. (2007), may contain contaminating excretory products of the parasite as the whole body was placed in dopamine. The SEPs were also collected at 10°C over a 40 min period prior to processing at 4°C and long-term storage at -70°C, which, due to the high protease content of the SEPs, may have led to degradation of some parasite-derived proteins. The parasites used for SEP collection were also not cultured in aseptic conditions, which means that other pathogens, such as bacteria, present on the exterior of the parasite may have contributed extracellular products to the SEPs. However, the controls with parasites incubated in seawater alone without dopamine were found to possess much lower protein and protease contents than dopamine-induced SEPs. The concentrated SEPs will also not possess any secreted components smaller than approximately 5kDa due to the use of a 5kDa MWCO column to concentrate them. For example, if any prostaglandins are secreted by *L. branchialis* as in *L. salmonis* (Fast *et al*., 2004) they will not have been represented in the SEPs. However, evidence for the importance of other pharmacologically active components such as proteins of other haematophagous arthropod parasite's

saliva is increasing (Wikel, 1996); with the identification of an increasing number of host-modulating salivary proteins, for example with approximate molecular weights of 5 – 49kDa (Ribeiro *et al*., 1990; Urioste *et al*., 1994; Bergman *et al*., 1998, 2000). Therefore, although the SEPs may not represent a 'whole' picture of the effect of *L. branchialis*-derived products on the immune response of cod, they nevertheless represent a key facet of the parasite's host-modulation capacity.

The range of SEP concentrations (0, 2.5, 5, 10 and 20µg.ml^{-1}) used in the *in vitro* assays were chosen based on studies with other haematophagous arthropod parasites. Studies using saliva and salivary gland extract of the ticks *Ixodes ricinus* and *Rhipicephalus appendiculatus* have shown that total protein concentrations in the range of 2.18 - 30µg.ml^{-1} have immunosuppressive effects on mouse splenocytes, macrophage-like cell lines, BALB/c mouse T cells and human serum activity (Kopecky *et al*., 1999; Gwakisa *et al*., 2001; Mejri *et al*., 2002). Mejri *et al*. (2002) found 15 and 30µg.ml^{-1} *I. ricinus* saliva to cause approximately 40% and 70% inhibition of SRBC lysis by human serum, respectively. They also found 2.18 – 17.5 and 4.37 – 17.5µg.ml^{-1} saliva to significantly decrease primed mouse T cell proliferation and IL-4 production, respectively.

6.4.2 The effect of SEPs on host phagocyte respiratory burst *in vitro*

Pre-metamorphosed, newly attached female SEPs had no effect on the respiratory burst activity of head kidney phagocytic cells *in vitro*, no matter what concentration was used. This may be due to the fact that at this stage the parasite is migrating very quickly from its initial attachment site on the exterior of the gill filament (1 d.p.i.) where it is less exposed to phagocytic leukocytes, to within the branchial afferent artery (2 d.p.i.) (see Chapter 3 and 4). The rapid migration may, of itself, afford the

parasite some protection from phagocytic cell cytotoxic effects. Wagner *et al*., (2008) hypothesised that motile pre-adult and adult *L. salmonis* were more capable of avoiding host inflammatory processes due to their ability to change location on the host, compared to chalimus stages which are attached at a particular location by the frontal filament. The newly attached *L. branchialis* females are also metamorphosing rapidly and migrating through host tissue, which is likely to be a very energetically demanding process. Therefore, the parasite may 'concentrate' on producing compounds for these processes, such as proteases to facilitate their migration to the ventral aorta and *bulbus arteriosus*, and a subsequent 'switch' in compounds produced by the parasite may occur during later metamorphic stages due to changes in their requirements. For example, *Onchocerca lienalis* (a filarial cattle parasite) L3 larvae produce a 43kDa serine elastase thought to play a role in larval migration from the blackfly bite site to the adult worm development site, as the adult stages do not produce this protease (Lackey *et al*. 1989). However, the effects of SEPs from newly attached females on other immune responses (cellular and humoral) were not tested due to the difficulty in collecting adequate quantities of them. This stage of the parasite is nevertheless likely to produce compounds with other host immuno-regulatory effects, especially as the parasite has direct contact with the fish plasma and blood leukocytes. The fact that the newly attached female SEPs did not suppress phagocyte H_2O_2 production does not mean that they do not possess other mechanisms for evading cytotoxic effects of ROS. Many parasitic nematodes produce anti-oxidants to detoxify extracellular ROS like hydrogen peroxide, *e.g. Haemonchus contortus* which produces a catalase (Kotze & McClure, 2001), *Schistosoma mansoni* which produces peroxiredoxin (Kwatia *et al*., 2000), and the eel nematode *Anguillicola crassus* which produces glutathione-s-transferase (Nielsen & Buchmann, 1997).

In contrast, fully metamorphosed female SEPs increased the proportion of the head kidney phagocyte population undergoing respiratory burst. However, at the same time the production of hydrogen peroxide by the phagocytic cells was significantly reduced. Therefore, the SEPs have suppressed the production of hydrogen peroxide produced after PMA stimulation. The SEPs had no effect on the viability of cod leukocytes which correlates with studies by Fast *et al.* (2007) who observed that the SEPs of *L. salmonis* do not affect the viability of Atlantic salmon macrophages or SHK1 cells. Fast *et al*. (2002a) also found the respiratory burst activity of *L. salmonis*-infected Atlantic salmon and rainbow trout (susceptible hosts) macrophages to be reduced by approximately 20% and 10% compared to that of uninfected controls, at 14 – 21 d.p.i. and 21 d.p.i., respectively. This reduction in macrophage activity was also evident in a reduction in their phagocytic capacity and corresponded with the occurrence of multiple bands of LMW proteases in the host's mucus (Fast *et al*., 2002a). These proteases were similar to the trypsins Firth *et al*. (2000) and Ross *et al*. (2000) found in *L. salmonis*-infected salmon mucus, suggesting that these trypsin-like proteases secreted by *L. salmonis* cause host immunosuppression. Many other extracellular parasites have been found to produce saliva or secretory / excretory products which suppress host neutrophil ROS production such as the ticks, *Ixodes dammini* and *Rhipicephalus sanguineus*, and the common liver fluke *Fasciola hepatica* (Ribeiro *et al*., 1990; Inokuma *et al*., 1997; Jefferies *et al*., 1997).

The mechanisms by which these parasites suppress phagocyte function are not completely understood. However, some authors have highlighted the importance of prostaglandin E_2 (PGE_2; Ribeiro *et al*., 1985), for example Ham *et al*. (1983) found that small quantities of PGE_2 inhibited ROS production. However, as stated

previously, if *L. branchialis* produce PGE_2 it would not be represented in the SEPs and therefore, cannot explain the observed suppression of phagocyte respiratory burst activity. Many bacteria and protozoan parasites have been shown to reside within host neutrophils or macrophages evading the production of ROS within the phagosome through the interference with the assembly of NADPH oxidase. Human granulocytic ehrlichiosis bacteria, *Leishmania donovani* promastigotes and the fungus *Aspergillus fumigatus*, for instance, have been found to suppress ROS production by preventing the assemblage of functional NADPH oxidase at the phagosomal membrane (Banerjee *et al*., 2000; Lodge *et al*., 2006). The respiratory burst is catalysed by NADPH oxidase, which is composed of a number of cytosolic ($p67^{PHOX}$, $p47^{PHOX}$, $p40^{PHOX}$ and Rac1/Rac2) and membrane-bound ($gp91^{PHOX}$ and $p22^{PHOX}$ making up cytochrome b_{558}) proteins. PMA stimulates phagocyte respiratory burst via Protein Kinase C (PKC), by mediating its movement from the cytosol to the plasma membrane (Wolfson *et al*., 1985). Nauseef *et al*. (1991) found that PMA led to the association of $p47^{PHOX}$ with the cytoskeleton, translocation of cytosolic $p47^{PHOX}$ and $p67^{PHOX}$ to the plasma membrane and phosphorylation of $p47^{PHOX}$, followed by the assembly of catalytically active NADPH oxidase. Thus, although potential mechanisms are recognised, the question of whether metazoan extracellular parasites such as *L. branchialis* can prevent the formation of ROS-producing NADPH oxidase has not been examined.

A number of parasites have been found to exploit their host's immune regulatory network by up-regulating the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and down-regulating other pro-inflammatory cytokines. *Fasciola hepatica*, for instance, has been found to produce a cathepsin L protease which suppresses the production of interferon-gamma (INF-γ) but induces the

production of IL-10 in mice (O'Neill *et al*., 2001). The saliva and salivary gland extract (SGE) of many species of ticks have also been found to have similar effects, such as *Rhipicephalus sanguineus* saliva, which increased IL-10 and suppressed INF-γ production by mouse splenocytes cultured with *Trypanosma cruzi* (Ferreira & Silva, 1998). The authors also found the saliva to impair macrophage activation by INF-γ resulting in reduced NO production and trypanocidal activity. *Dermacentor andersoni* SGE suppresses INFγ synthesis by conA stimulated murine splenocytes (Ramachandra & Wikel, 1992), and *Ixodes ricinus* SGE reduces INFγ production by mouse splenocytes stimulated by LPS, via the up-regulation of IL-10 (Kopecky *et al*., 1999). IL-10 in mammals has anti-inflammatory properties, such as down-regulating neutrophil ROS production (Dang *et al*., 2006). Seppola *et al* (2008) suggested that IL-10 in Atlantic cod also possess anti-inflammatory activity due it displaying opposite transcriptional levels to the pro-inflammatory cytokines IL1β and IL-8 post-*in vivo* antigen stimulation. The complete sequence for INFγ in cod has also recently been published and it has been suggested to be involved in the innate immune response to both viruses and bacteria (Furnes *et al*., 2009). In the current study the respiratory burst activity of cod phagocytes was measured in a mixed head kidney leukocyte preparation by flow cytometry, so the role of cytokines on the ROS production can not be eliminated.

6.4.3 The effect of SEPs on MAF production by cod head kidney-derived leukocytes *in vitro*

To further investigate the effects of *L. branchialis* SEPs on cytokine production, cod leukocytes were pre-incubated with SEPs prior to MAF production. The SEPs resulted in a dose-dependent suppression of MAF priming capacity on the ROS production of macrophages. This is likely to be due to the SEPs down-regulating the production of MAF by the leukocytes, as has been illustrated in the studies

mentioned earlier (O'Neill *et al*., 2001; Ferreira & Silva, 1998; Ramachandra & Wikel, 1992; Kopecky *et al*., 1999). Teleost MAF contains IFN-γ like properties (Graham & Secombes, 1990) and there is also increasing evidence for the presence of tumor necrosis factor-α (TNFα) in MAF-derived from rainbow trout leukocytes (Hardie *et al*., 1994; Jang *et al*., 1995; Tahir & Secombes, 1996; Campos-Perez *et al*., 1997). Therefore, *L. branchialis* seems to produce SEPs which suppress the production of MAF, possibly through the suppression of IFN-γ and TNFα, suppressing the priming of phagocytic leukocytes. On the other hand, the effects of the SEPs could result from the production of a compound which counteracts the capacity of MAF to prime phagocyte ROS production, or inactivates it post-production *e.g*. proteolysis or competitive binding. Neumann *et al.* (2000) characterised a macrophage deactivating factor of approximately 10-12kDa in mitogen stimulated goldfish leukocytes, which inhibited macrophage NO production. They went on to hypothesise that it could be transforming growth factor beta (TGFβ) due to its similar molecular weight and the fact that mammalian TGFβ suppresses NO production (Ding *et al*., 1990), and the respiratory burst of rainbow trout macrophages (Jang *et al*., 1994). However, until the components of Atlantic cod MAF and its regulation have been verified; the exact mechanism(s) behind the suppression of MAF priming cannot be determined. Studies in this area will be hampered by the lack of recombinant proteins for functional studies and protein markers to quantify cytokines at the protein level. Even though molecular sequences for an increasing number of cytokines are becoming available enabling the detection of their mRNA expression, the 'whole' picture cannot be determined without examining what occurs post-protein production. For instance, Hajnicka *et al*. (2001) illustrated numerous ixodid tick species to produce a protein which binds host IL-8, inactivating its capacity to induce host neutrophil migration. Therefore, the

regulation of the host's immune response has occurred at the post-protein production of IL-8 and not by suppressing the mRNA production of IL-8.

6.4.4 The effect of SEPs on cod macrophage nitric oxide release *in vitro*

In this study the intracellular H_2O_2 production of cod phagocytes was measured in response to *L. branchialis* SEPs, however due to the fact that *L. branchialis* is an extracellular parasite it may have been more useful in hindsight to measure the extracellular release of ROS. Human PMA stimulated neutrophils and Bacille Calmette Guerin-activated macrophages release H_2O_2 which has been found to be cytotoxic to lymphoma cells and fibroblasts (Nathan *et al*., 1979; Simon *et al*., 1981). Therefore, the extracellular release of H_2O_2 could result in damage to the exterior of *L. branchialis*. However, whether the extracellular release of H_2O_2 is correlated to its intracellular production in cod phagocytes is not documented. In light of this, the effect of *L. branchialis* SEPs on the extracellular release of NO by cod macrophage-enriched head kidney leukocytes was investigated. However, using the Griess reagent to quantify NO by its breakdown product nitrite was not successful in detecting any NO release from cod macrophage monolayers. Stimulation with up to 50µg.ml^{-1} *V. anguillarum* LPS with or without 1µg.ml^{-1} PMA, or up to 20µg.ml^{-1} fully metamorphosed *L. branchialis* SEPs did not induce detectable amounts of NO production. The effect of SEPs on macrophage NO production therefore, could not be determined from this study. Martin-Armis *et al.* (2008) also found cod heart atrium scavenger endothelial cells not to produce NO when stimulated with up to 400ng.ml^{-1} LPS (*E. coli* O26;B6), but only when stimulated with 40µg.ml^{-1} Poly IC. Steiro *et al.* (1998) also found cod macrophages to respond to a lesser extent in terms of ROS production to stimulation with a number of compounds, including LPS, than other teleost fish such as Atlantic

salmon and mammals, and no stimulatory effect on phagocytosis was observed. The authors went on to state that the reason behind this reduced sensitivity to LPS by cod *in vitro* is unknown. Whether the culture conditions of cod macrophages are not optimal for NO production remains to be determined. Cod macrophages are very sensitive to temperature, and short-lived in culture, possessing high viability only up to 4-7 days in culture (Steiro *et al*., 1998; Sorensen *et al*., 1997), compared to macrophages from other teleost species reported to give more long lived cultures (Braun-Nesje *et al*., 1981; Wang *et al*., 1995). The lack of detection of NO could also have been due to the assay used, which detects one of the breakdown products, nitrite, whereas more recent assays combine the detection of both nitrate and nitrite by firstly converting nitrate to nitrite.

6.4.5 The effect of SEPs on the chemotactic capacity of zymosan activated cod serum on host leukocytes

The effects of SEPs on the immune response of cod was further analysed by exploring its interactions with the complement system. To begin with, the effect of SEPs on the production of the leukocyte chemo-attractant zymosan activated serum (ZAS) was investigated. Zymosan activates the alternative complement pathway in teleosts and mammals (Sakai, 1983). Therefore, the alternative complement pathway of cod serum was activated by zymosan to promote the production of the anaphylatoxins C3a and C5a, known inflammatory mediators, with or without *L. branchialis* SEPs. The ZAS was then used as a chemo-attractant for head kidney-derived and blood- derived leukocytes, as C5a has been characterized in rainbow trout and carp as a potent chemo-attractant of leukocytes (Holland & Lambris, 2004; Boshra *et al*., 2004; Kato *et al*., 2004). The ZAS of cod resulted in significant blood and head kidney leukocyte migration suggesting the presence of anaphylatoxins. However, when the ZAS was produced in the presence of SEPs it

had a reduced capacity to induce the migration of head kidney leukocytes in a dose dependent manner. This suggests that these ZAS contained less active anaphylatoxins than control ZAS. This could result from either the inhibition of the alternative complement pathway prior to the production of anaphylatoxins, or the inactivation / degradation of the anaphylatoxins post-production by SEPs. A similar trend was found in blood-derived leukocytes, however no significant differences were detected, probably due to the low number of cod sampled and the high variability between individuals. The saliva and SGE of several tick species has been found to inhibit host serum alternative complement activity including those of *Ixodes ricinus*, *Ornithodores erraticus*, *O. moubata*, *I. scapularis*, *I. hexagonas*, and *I. uriae* (Mejri *et al*., 2002; Ribeiro, 1987; Astigarraga *et al*., 1997; Lawrie *et al*., 1999). Ticks achieve this through a wide variety of methods. *O. moubata*, for instance, produces a 16.8kDa protein, omcl, which inhibits C5 cleavage (Nunn *et al*., 2005), and *I. scapularis* produces the protein Salp20, which inhibits alternative complement activity by preventing C3b and factor B deposition and removal of pre-bound factor B (Tyson *et al*., 2007). *Entamoeba histolytica* and *Serratia marcescens* have been found to produce extracellular proteases capable of digesting and inactivating the host anaphylatoxins C3a and C5a (Reed *et al*., 1995; Oda *et al*., 1990).

6.4.6 The effect of SEPs on cod serum spontaneous haemolytic activity, *in vitro*

In order to further investigate whether *L. branchialis* SEPs inhibit serum alternative complement activity, their effect on serum spontaneous haemolysis was measured. The SEPs did not affect the spontaneous haemolytic activity of the cod serum in this assay. However, the spontaneous haemolytic activity measured was extremely low averaging 6.2% spontaneous haemolysis. Recent studies have similarly found serum from cultured Icelandic cod to possess very low or no spontaneous

haemolytic activity compared to that measured in wild cod in the late 1990s (Magnadottir *et al*., 1999a, b; Magnadottir, 2000). The possible reasons for this lack of activity and the unusual nature of cod haemolytic activity have been discussed in Chapter 3. The results from this investigation therefore, suggest that SEPs have no effect on cod serum haemolytic activity. However, the fact that the haemolytic activity of cod serum is so unusual compared to that of other teleosts and that the activity detected was very low means that no final conclusion can be drawn. The discrepancy between the chemotaxis assay results suggesting that the SEPs reduce the production or activity of anaphylatoxins, and those of the haemolysis assay showing no effect of the SEPs on serum haemolytic activity, may be due to the degradation / inactivation of anaphylatoxins post-production, or due to the storage of the serum. The sera used for zymosan activation and subsequently in the chemotaxis assay was prepared fresh the same day, however the sera used in the subsequent haemolysis assay had been stored at -70°C for 6 months prior to measuring haemolytic activity. The storage of serum prior to complement activity measurement depends largely on the species; Lange and Magnadottir (2003) found halibut serum haemolytic activity to be depressed by 90% after only 3 weeks storage at -80°C, whereas sea bass sera maintained their activity after 1 year. However, Magnadottir *et al*. (1999a, b) stated that cod serum spontaneous haemolytic activity was unaffected by storage at -20°C. Whether this has contributed to the low activity measured within cod serum or whether factors similar to those found recently in cultured Icelandic cod are to blame remains to be determined. The concentration of SEPs used in the assay may also have not been optimal to have caused an effect. This is due to the fact that the serum was pre-incubated with SEPs at 0-20μg.ml^{-1} prior to serum dilution 1 in 20 and the addition of SRBC for the assay, rather than at a final concentration of 0-20μg.ml^{-1} per well.

This would have made the highest concentration of SEP protein 0.1µg.well^{-1}. However, in studies on ticks the range of saliva and SGE total protein content used per well were 1.2µg from *I. dammini* (Ribeiro, 1987), approximately 200µg from *O. erraticus* and 20µg from *O. erraticus* (Astigarraga *et al*., 1997) which resulted in a 90% inhibition of host serum haemolysis and an approximately 90% and less than 10% inhibition of serum complement mediated haemolysis, respectively. Therefore, the final concentrations of protein used in this study are markedly lower. Further analysis of the effects of SEPs on haemolytic activity is required using fresh sera and SEPs at higher concentrations. Other elements also require additional investigation, such as purifying the anaphylatoxins of cod to determine their functional activity and the effect of SEPs on their structure and activity. The exact nature of the lytic pathway of cod serum also needs to be further studied using fresh serum to identify the components attributing towards it.

6.4.7 Conclusions

In conclusion, fully metamorphosed female *L. branchialis* produce SEPs with the ability to modulate their host's immune response. In a dose-dependent manner, SEPs were found to suppress phagocyte intracellular ROS production, head kidney leukocyte MAF production with macrophage 'priming' capacities and the leukocyte chemo-attractant activity of ZAS, *in vitro*. This could allow the fully metamorphosed parasite to evade the cytotoxic effects of phagocytic cells, MAF (*e.g*. INFγ) – mediated cellular immune responses and anaphylatoxin–induced phagocyte activation and migration at the local site of infection and feeding. This could be vital to allow survival of the parasite within host granulation tissue, and to prevent damage of the gut whilst feeding on blood. Astigarraga *et al.* (1997) found the tick *O. erraticus* capable of feeding from host sites with artificially induced infiltrates by

injection with IL-8 and C5a. They hypothesised that these parasites had evolved feeding on cellular infiltrates at the bite site. Therefore, the capacity of *L. branchialis* SEPs to inhibit cellular activation could also allow feeding at sites of cellular infiltration. However, the parasite-derived components responsible and the mechanisms by which these SEPs suppress such activities need to be further investigated. The investigation of SEPs by western blot with sera from infected and uninfected gadoids could also be interesting in order to determine if the host produces specific antibodies to particular antigens, for example in individuals found to shed / kill the parasite prior to it becoming gravid. For instance, Nielsen and Buchmann (1997) found *Anguilla anguilla* (European eel) infected by the parasitic nematode *Anguillicola crassus* to produce specific antibodies against glutathione-s-transferase present in the parasites' secretory / excretory products. The identification of the pharmacologically active components of these SEPs could also highlight future vaccine candidates should *L. branchialis* become a problem in gadoid aquaculture.

Chapter 7 General discussion and conclusions

The relatively recent advent of commercial Atlantic cod farming in Canada, Iceland, U.S.A., Norway and Scotland (the sole company farming cod in Scotland, No catch fish – organic cod, however, went into receivership early 2008) has led to an increase in the requirement for studies investigating the immune system of Atlantic cod, the host-pathogen interactions during infection with known and potential cultured-cod-pathogens, and vaccine development. *Lernaeocera branchialis* is a parasitic copepod of wild gadoids, and has been highlighted as a potential threat to farmed marine species by Burt and Mackinnon (1997; cited in Hemmingsen & Mackenzie, 2001). This is likely to be due to its many pathogenic effects observed on wild gadoids, and the fact that it has been observed to result in host mortality in some cases (Sundnes, 1970; Khan, 1988; Kabata, 1970; Kabata, 1984; Van Den Broek, 1978; Hislop & Shanks, 1981; Khan & Lee, 1989; Khan *et al*., 1990; Khan *et al*., 1993). These studies on the pathogenic effect(s) of *L. branchialis* on gadoids however, need to be viewed with caution due to the fact that they use observations of wild fish in the field where other factors which were not controlled for may be present. The majority of studies into the host-parasite interactions between *L. branchialis* and its gadoid host have regarded their pathogenic effects on the host, or more recently, the pathology associated with infection by the early metamorphic stages (Smith *et al*., 2007). The prolonged and intimate contact between *L. branchialis* and its host, as it migrates through the lumen of the afferent branchial artery into the ventral aorta / *bulbus arteriosus*, could result in the reduced feeding and survival of the parasite as a consequence of the host's haemostatic and immune responses without further parasite intercession.

The modulation / evasion of host immune and haemostatic responses are well characterised in numerous terrestrial host-parasite interactions. In comparison, those studies regarding teleost hosts are limited, especially for the numerous crustacean parasites infecting teleosts worldwide. In the last decade however, researchers have increased our understanding of the interactions of the parasitic copepod *Lepeophtheirus salmonis* and its salmonid hosts (Ross *et al*., 2000; Firth *et al*., 2000; Fast *et al*., 2002a; Fast *et al*., 2003; Fast *et al*., 2004; Fast *et al*., 2006; Fast *et al*., 2007). These studies have also highlighted *L. salmonis*-derived compounds, including low molecular weight trypsins (23.6 – 23.7kDa; Johnson *et al*., 2002) and prostaglandin E_2 (PGE_2), which are thought to aid parasite feeding and / or host immune response evasion (Fast *et al*., 2002a; Fast *et al*., 2003; Fast *et al*., 2004; Fast *et al*., 2007). Therefore, the aim of this thesis was to investigate the host-parasite interactions, in terms of the systemic and local immune response of Atlantic cod to *L. branchialis* infection, and the immuno-modulation of Atlantic cod by *L. branchialis*-derived secretory / excretory products (SEPs). This research was undertaken to increase our understanding of the immune response of cod to this metazoan parasite, and of the interactions occurring at the host-parasite interface, including the possible mechanisms adapted by the parasite to evade / modulate their host's immune response. This is necessary as the foundation for future studies to produce parasite control measures, such as vaccine development, should *L. branchialis* become a pest to gadoid culture, as *L. salmonis* has for marine salmonid farming.

In the laboratory infections, the parasite was found to attach to the gill filaments of cultured-cod by the antennae, with pathology limited mostly to the infected filament.

The parasite then proceeded to enter the vascular system, migrating within the lumen of the afferent branchial artery towards the ventral aorta. This led to haemorrhaging at the point of parasite entry and the formation of a thrombus around the parasite which followed its' migration and metamorphosis, and became more organized as the infection developed, as described previously by Smith *et al*. (2007). In the present study, the observation of cod leukocytes expressing interleukin 8 (IL8) mRNA within the free-flowing blood at the periphery of the organising thrombus within the lumen of the ventral aorta, as the parasite reached the penella sub-stage, was speculated to aid the recruitment and activation of leukocytes to the site, and the maturation and neovascularisation of granulation tissue. In this respect, Smith *et al*. (2007) described the infection of cod up to the late U sub-stage of *L. branchialis,* noting the in-growth of granulation tissue from the arterial or *bulbus arteriosus* wall as the thrombus around the parasite became more organised, with local neovascularisation of the granulation tissue resulting in the re-canalisation of the vessel. The exact functions of IL8 and the identification of the leukocytes expressing it however, require further study as our understanding of its role in fish is largely based upon functional studies of IL8 in mammals. However, until species-specific recombinant proteins and antibodies are available for IL8, its exact functions in Atlantic cod cannot be concluded. In the present study, the early thrombus was composed of erythrocytes and thrombocytes with an influx of leukocytes including neutrophils. This corresponded to a reduction in the number of circulating thrombocytes and lymphocytes at 3 d post-infection (d.p.i.) in infected Ardtoe-cultured cod possibly due to their use in thrombus formation and preventing blood loss at the point of parasite entry. On the other hand, wild haddock infected by mature *L. branchialis* possessed an increased number of circulating

thrombocytes which may be due to the increased damage of host tissue during parasite feeding. In the laboratory-infection, the parasite was found to reach the ventral aorta by 7 d.p.i., where it remained and continued metamorphosis into an early penella sub-stage by 14 d.p.i.. The lab-infections in this thesis unfortunately did not follow the infection over the full metamorphosis of the parasite due to an unfortunate loss of stock Machrihanish-cultured cod, and the parasites in the longer infection of Ardtoe-cultured cod dying by 28 d.p.i., and not metamorphosing past the early penella sub-stage. Smith *et al.* (2007) however, hypothesized that the later metamorphic stages are likely to become part of an endothelial swelling of the arterial wall sequestering the parasite in a fibrotic capsule with re-canalisation of the artery lumen. This is in line with the observations of Schuurmans Stekhoven (1936) who observed the *bulbus arteriosus* to form a connective tissue capsule around the cephalothorax of fully metamorphosed *L. branchialis*. The lack of success of the infection in Ardtoe-cultured cod was thought to be due to the innate immune response of the host, although the effect of the infection protocol could not be discounted. Van Damme and Hamerlynck (1992), however, also found a proportion of wild whiting to reject *L. branchialis* at the late penella sub-stage in June and July, and suggested that host immunological factors play an important role in parasite elimination in the summer.

The observed systemic immune response of both wild haddock and cultured-cod post-infection by *L. branchialis* depended on the maturation stage of the parasite, and in the former the infection intensity. The infective stage and early metamorphic sub-stages appeared to have less of an effect on the systemic immune response of laboratory-infected cultured-cod, in comparison to the later metamorphic stages

infecting wild haddock in this thesis. This was likely to be due to the increased size of the parasite and host damage caused by the parasite as it matures and feeds. Haddock infected by multiple mature *L. branchialis* possessed reduced circulating monocytes, and increased circulating thrombocytes and serum anti-trypsin activity. Infection by *L. branchialis* was also associated with suppression in haddock serum spontaneous haemolytic activity. The latter may be due to the consumption of the hosts' haemolytic components as the host tries to counteract the increased damage caused by the massive increase in parasite size and its feeding activity. This was also highlighted due to the fact that the infection of cultured-cod up to the early penella sub-stage had no effect on the serum spontaneous haemolytic activity, suggesting that only when the parasite reaches the mature stages is an effect observed. However, the cultured-cod utilised within this thesis possessed very low serum spontaneous haemolytic activity with high variation between individuals, which is similar to reports by Magnadottir (2000) for Icelandic-cultured cod, since 2000, making it difficult to draw any conclusions about the effect of the early metamorphic stages on this activity. The SEPs of fully metamorphosed *L. branchialis* were also not found to have any effect on the spontaneous haemolytic activity of cod serum *in vitro*. However, the concentrations of SEPs used in this study were low in comparison to other studies on the effect of tick saliva on host complement activity (Ribeiro, 1987; Astigarraga *et al*., 1997). Therefore, the effect of parasite-derived secretions on host spontaneous haemolytic activity can not be concluded until further studies using a wider range of SEP concentrations are carried out, and the true nature of the spontaneous haemolytic activity of cod serum is elucidated. The increased anti-trypsin activity of serum in haddock with multiple parasites was also thought to be due to the host either counteracting parasite-

derived proteases which may increase in quantity with multiple parasites, or host proteases released at the site of infection during tissue injury which is probably more pronounced when multiple parasites are present, or a combination of both. The SEPs of fully metamorphosed *L. branchialis* females, collected within this thesis, were also found to contain numerous proteases highlighted by zymography, and therefore, the increased release of parasite-derived proteases into the host by multiple parasites may be a valid hypothesis.

The parasites infecting Machrihanish-cultured cod did not possess any host IgM or complement component C3 binding on their cuticle up to the early penella sub-stage 14 d.p.i., however, both IgM and C3 binding occurred on the dead and degrading parasites 56 d.p.i. of Ardtoe-cultured cod. This highlights the possible importance of these opsonins in the elimination of *L. branchialis* by some cod, and also the possibility of parasite mechanisms involved in evading host C3 and IgM binding to their surface during the infection. However, the infection of Machrihanish-cultured cod was terminated once the parasite reached the early penella sub-stage with C3 binding in the outer zone of the cellular aggregate surrounding the parasite, prior to the conclusion of the infection, and the earlier stages of the local infection site prior to parasite death were not studied in the Ardtoe-cultured cod. Therefore, the lack of IgM binding on the parasite during the infection studied up to 14 d.p.i. may be due to the fact that the time period post-infection was too short for the development of a specific antibody response to occur against *L. branchialis*. The fact that the infection of the Machrihanish-cultured cod was terminated early also means that the success of the infection in Machrihanish cod also can not be concluded, and therefore, the relevance of the lack of binding or binding of these

opsonins on the cuticle of live and dead parasites, respectively, and the binding of C3 to the periphery of the host cellular aggregate around the parasite on its survival can not be verified. However, this does highlight the importance of studying the cuticle of *L. branchialis* to determine any protective role against host immune responses it may possess. For instance, other parasites have been found to adsorb host molecules to their surface to prevent opsonin binding and complement activation (Rasmussen & Kemp, 1987; Meri *et al*., 2002; Roberts *et al*., 2005; Plouffe & Belosevic, 2004). The binding of C3 was, however, observed at 14 d.p.i. in the outer zone of the host cellular aggregate around the early penella *L. branchialis* within the ventral aorta. This may be bound to parasite-derived products, and involved in opsonisation, and the recruitment and activation of leukocytes at the local infection site by the release of anaphylatoxins (Rotlant *et al*., 2004; Kato *et al*., 2004; Holland & Lambris, 2004; Boshra *et al*., 2004). However, the SEPs of fully metamorphosed females were found to suppress the chemoattractant capacity of zymosan activated cod serum (ZAS) on cod head kidney leukocytes, which was thought to be via the suppression of the production / activity of cod anaphylatoxins by the SEPs. Therefore, if the SEPs of the penella sub-stages possess the same activity as the fully metamorphosed female SEPs, the production or activity of anaphylatoxins post-C3 binding at the local site of live parasites may be suppressed. This, however, awaits *in vivo* verification, as well as the determination of the functions of cod anaphylatoxins and the exact mechanism(s) behind the suppressed chemoattractant capacity of ZAS by SEPs.

The possible importance of the hosts' phagocytic leukocytes and their functions in the elimination of *L. branchialis* was highlighted in this thesis. A suppression of the

proportion of blood phagocytes undergoing respiratory burst and the mean intracellular hydrogen peroxide production by those phagocytic leukocytes was observed as *L. branchialis* migrated into the ventral aorta and metamorphosed into the early penella sub-stage at 14 d.p.i. of Machrihanish-cultured cod. Similar host phagocyte activity suppression has been observed in *L. salmonis*-infected susceptible salmonid hosts, and has been attributed to both stress-related immunosuppression, and modulation by parasite-derived compounds in the absence of a stress response (Mustafa *et al*., 2000a & b; Fast *et al*., 2002a). However, whether this suppression in the present study was due to 1) modulation by parasite-derived compounds, 2) 'consumption' of host phagocyte activity, and / or 3) stress-related immunosuppression, can not be concluded, especially as chronic stress in teleosts generally has a suppressive effect on the immune response (Maule *et al*., 1989; Schreck, 1996; Demers & Bayne, 1997; Ruis & Bayne, 1997; Ortuno *et al.*, 2001; Fast *et al.*, 2008). The SEPs of metamorphosed *L. branchialis* however, were found to suppress the intracellular production of hydrogen peroxide by cod phagocytes *in vitro*, which could result in the suppression of the host phagocyte ROS production at the local infection site by the later metamorphic stages of the parasite. However, whether the SEPs of the early penella sub-stage, like those of the fully mature stage, also possess a suppressive capacity on phagocyte ROS production, and whether this led to the systemic suppression observed in the lab-infected cod *in vivo* remains to be elucidated, and could have been a combination of the factors mentioned above. This is particularly the case with respect to the observation that the incubation of phagocytes with fully metamorphosed SEPs resulted in an increase in the proportion of the phagocyte population undergoing respiratory burst *in vitro*, whereas infection by the early

penella sub-stage led to a decrease in the proportion of blood-derived phagocytes undergoing respiratory burst *in vivo*. This *in vivo* suppression in blood-derived phagocyte respiratory burst activity, however, was not observed in Ardtoe-cultured cod. The parasite was found to be dead in these cod by 28 d.p.i., and therefore, the lack of phagocyte activity suppression may have resulted either from the fact that ther parasite may not have been capable of producing immuno-modulatory compounds, or from the fact that this stock of cod may have been more resistant to the suppressive effects of the infection on phagocyte activity. This may have aided the death of the parasite, as the infiltration of phagocytic cells and their cytotoxic effect on parasites has been noted in other hosts (Whyte *et al.*, 1989; Sharp *et al.*, 1991; Hamers *et al.*, 1992; Nakayasu *et al*., 2005). The differences between the systemic immune response to *L. branchialis*-infection in the cod originating from the two sources may also have been due to the poorer condition of the batch of Machrihanish-cultured cod utilised in this study. However, further studies are required to investigate the immune response to infection over the entire metamorphosis of *L. branchialis* to determine whether the infection was successful or not, and preferably in host populations with varying susceptibility to *L. branchialis*. This would allow the importance of the differences observed in the immune parameters studied, with regard to parasite elimination *i.e.* host resistance, to be elucidated. This, however, will not be possible without further studies into the susceptibility / resistance of different stocks of cultured-cod.

The capacity of macrophage activating factor (MAF) produced in the presence of fully metamorphosed female SEPs to prime the respiratory burst activity of naïve cod macrophage-enriched leukocytes was also suppressed. This was thought to be

due to either the suppression of MAF production by cod leukocytes or the induction of the production of a compound by the leukocytes which counteracts the priming effect of MAF, or inactivates it post-production. However, to further investigate the mechanism(s) involved in this host immunosuppression, the components of cod MAF and its regulation need to be identified. This will depend upon the production of recombinant proteins for functional studies and protein markers to quantify the production of cytokines possibly responsible for the priming activity of MAF supernatant or involved in the regulation of MAF production. The parasite-derived component(s) responsible for this suppression of host ROS production by phagocytes and MAF activity also need to be identified. The regulation of the immune factors studied *in vitro* also require further investigation at the local level *in vivo* post-infection, *e.g*. the production of MAF components and their regulatory cytokines at the feeding area during the infection.

The SEPs of the initial parasitic stages, *i.e.* those at 1-2 d.p.i. of cod, however were found not to suppress intracellular hydrogen peroxide production by host phagocytic leukocytes *in vitro*, which corroborates with the lack of suppression in the respiratory burst activity in Machrihanish-cultured cod *in vivo* at the initial stages of infection. The fact that intracellular ROS production was measured means that these early parasitic stages may still produce compounds capable of detoxifying extracellular ROS production, such as anti-oxidants like glutathione and thioredoxin peroxidases, and catalases. Many parasites, including haematophagous nematodes of teleosts, have been found to secrete numerous types of anti-oxidants to counteract the cytotoxic effects of ROS (Nielsen & Buchmann, 1997; Hewitson *et al.*, 2008; Donnelly *et al*., 2005; Kotze & McClure, 2001). The positive

diaminobenzidine (DAB) staining of the exocrine gland populations of the infective stage of *L. branchialis*, highlighted within this thesis, also suggests the possible production of peroxidases and / or catalases for secretion into / onto the host tissue by the infective stage of the parasite. Bell *et al.* (2000) found similar results in the parasitic copepods *L. salmonis* and *Caligus elongatus*, and also suggested that the secretions of these glands may be involved in the detoxification of reactive oxygen species (ROS) produced by damaged host tissue and leukocytes. These authors also stated that peroxidases have been found to be involved in the production of prostaglandins, such as prostaglandin H synthetase which is required for the production of prostaglandin E_2 (PGE_2). Fast *et al*. (2004) also identified PGE_2 within the secretions of *L. salmonis* and it is a common prostanoid found to be secreted by numerous parasites (Belley & Chadee, 1995; Ali *et al*., 1999; Bowman *et al*., 1996; Ribeiro *et al*., 1985). The importance of PGE_2 in the suppression of the immune response has also been stated in numerous studies (Pinge-Filho *et al*., 1999; Fast *et al*., 2005; Ribeiro *et al*., 1985; Ham *et al*., 1983). In the current study however, *L. branchialis*-derived PGE_2 (if produced) would not be represented in the SEPs, nor other compounds smaller than 5kDa. Therefore, the possible production of prostanoids, such as PGE_2, peroxidases and catalases by *L. branchialis* at different metamorphic stages deserves further study.

The effect of SEPs on the production and activity of host signalling molecules, such as cytokines and anaphylatoxins, also merits further study to elucidate whether the parasite modulates its host's immune response by 'hijacking' the signal molecules of the immune system. The progress of these studies will however, be hampered by the lack of knowledge of these processes in Atlantic cod, such as the MAF and

regulatory cytokines involved and the functionality of the different anaphylatoxins; as well as the lack of protein markers to quantify them *in vitro,* or to detect them at the local infection site *in vivo*. The effect of newly attached female SEPs also requires further investigation, as the histopathology shows parasite entry to be followed by an organizing thrombus formation with the in-growth of granulation tissue from the arterial or *bulbus arteriosus* wall (Smith *et al.*, 2007). Whether the parasite 'harnesses' the immune response to allow its sequestration within host connective tissue to feed off local capillaries without subsequent clearing, needs to be determined. The identification of pharmacologically active components of the SEPs along with immune response studies in individuals or species with differing susceptibilities could, however, help determine the importance of certain host mechanisms at the host-parasite interface involved in parasite clearing (Kocakova *et al.*, 2003).This in turn could allow the identification of vaccine candidates should *L. branchialis* become a problem for gadoid aquaculture.

The effect of SEPs on the immune response of cod, *in vitro*, such as reduced MAF priming activity and ZAS chemoattractant capacity, are likely to act on a local level at the site of infection. The systemic suppression of serum haemolytic activity, phagocytic respiratory burst activity and the number of circulating monocytes in infected gadoids, however, is likely to result in them being more susceptible to secondary infections. For instance, Mustafa *et al.* (2002b) found rainbow trout infected by *L. salmonis* to possess suppressed phagocyte function as the parasite moulted into the pre-adult stages, and these fish to be more susceptible to other infections, as they possessed more xenomas when secondarily challenged with *Loma salmonae* than *L. salmonis*-free hosts. If *L. branchialis* did become a problem

within cultured-cod or haddock this could be problematic for disease control. Further studies regarding the secondary challenge of *L. branchialis*-infected fish with common gadoid pathogens faced in aquaculture therefore need to be carried out.

This thesis identified numerous exocrine glands in the adult female *L. branchialis*, including some associated with the oral region. These included the circum-oral glands (CGs) and the anterior gland complexes (AGC), which were found to produce multi-component secretions of which proteins and glycoproteins and / or neutral mucopolysachharides were major components. The exact secretory sites of these glands were not identified but were thought to be in the vicinity of the oral cone *i.e.* the feeding area. Therefore, the secretory products of these glands are likely to play an important role in the host-parasite interface, possibly enabling efficient blood feeding and parasite survival by host immuno-modulation. This emphasises the need to identify the secretory products of these glands and their functions at the various metamorphic and feeding stages of the parasite, to highlight critical parasite-derived components involved in the success of the infection from the initial infective stage to the feeding of the fully metamorphosed female. This could lead to future studies into vaccine development against parasite-derived components 'key' to their survival. Targets could include secreted proteases involved in the initial host invasion and migration into the blood vessels, or secreted proteins possibly involved in the modulation of the immune or haemostatic response, neutralising them with host-derived antibodies to inhibit parasite migration and establishment. This strategy has been found to have some success in the laboratory for mammalian parasites, such as ticks (Morris *et al*., 2001) and hookworms (Williamson *et al.*, 2006). However, the production of recombinant

protein vaccines is practically and commercially more challenging than the production of those arising from more classical vaccine development strategies, and involves the identification of an appropriate antigenic compound secreted by the parasite. The contribution of the parasite's gut to parasite-derived compounds involved in the host-parasite interface should also not be overlooked. For instance, Agyei *et al.* (1992) found the midgut cells of ixodid ticks to possess both a secretory and digestive function, with the production of granules expressing peroxidase activity coinciding with the digestion of the blood meal. *L. salmonis* has also been found to secrete trypsin in / onto their host (Ross *et al*., 2000, Firth *et al*., 2000; Fast *et al*., 2002a; Fast *et al*., 2007), which is produced in the midgut (Johnson *et al*., 2002; Kvamme *et al*., 2004). This is thought to be involved in the feeding of *L. salmonis* and / or the modulation of the host's immune response to allow efficient feeding and survival (Fast *et al.* 2002a). The midgut of *L. salmonis* is a common target for studies trying to identify possible antigenic compounds to produce a vaccine, for example Roper *et al*. (1995). Therefore, the secretory products of the exocrine glands outlined in this study, as well as those of the gut of *L. branchialis,* warrant further study regarding their characterisation and function at the host-parasite interface.

This study has examined the immune response of Atlantic cod to the parasitic copepod *L. branchialis*, and has highlighted some possible strategies utilised by *L. branchialis* to survive within the hostile environment of its gadoid host, as well as identifying areas that warrant additional research to further understand this host-parasite interaction. Overall, further investigation of the secretory products of the glands highlighted in this study and the SEPs of different metamorphic stages, in

terms of their modulation of the hosts' immune / haemostatic response, in conjunction with experimental infections of cultured-cod with varying susceptibility to *L. branchialis* are required. This could guide the development of future vaccines against *L. branchialis* or possibly other parasitic copepod species, which might be required in a commercial environment.

References

Abelseth, T.K., Stensvag, K., Espelid, S., Nygaard, R., Ellingsen, T., Bogwald, J., Dalmo, R.A. 2003. The spotted wolffish (*Anarhichas minor* Olafsen) complement component C3: isolation, characterisation and tissue distribution. *Fish and Shellfish Immunology*. **15(1)**: 13-27.

Afonso, A., Silva, M.T. 1998. Uptake of neutrophils and neutrophilic components by macrophages in the inflamed peritoneal cavity of rainbow trout (*Oncorhynchus mykiss). Fish and Shellfish Immunology***. 8(5)**: 319-338.

Agyei, A.D., Runham, N.W., Blackstock, N. 1992. Histochemical changes in the midgut of two ixodid tick species *Boophilus microplus* and *Rhipicephalus appendiculatus* during digestion of the blood meal. *Experimental and Applied Acarology*. **13(3)**: 187-212.

Alcorn, S.W., Murray, A.L., Pascho, R.J. 2002. Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). *Fish and Shellfish Immunology*. **12(4)**: 303-334.

Alexander, J.B and Ingram, G.A. 1992. Noncellular nonspecific defence mechanisms of fish. *Annual Review of Fish Diseases*. **2**: 249-279.

Aljamali, M., Bowman, A.S., Dilwith, J.W., Tucker, J.S., Yates, G.W., Essenberg, R.C., Sauer, J.R. 2002. Identity and synthesis of prostaglandins in the lone star tick, *Amblyomma americanum* (L.), as assessed by radio-immunoassay and gas chromatography/mass spectrometry. *Insect Biochemistry and Molecular Biology*. **32(3)**: 331-341.

Allen, J.R., Khalil, H.A., and Graham, J.E. 1979. The location of tick salivary antigens, complement and immunoglobulin in the skin of guinea-pigs infested with *Dermacentor andersoni* larvae. *Immunology*. **38**: 467-472.

Alsuth, S. and Ebeling, E. 1989. A survey of the diet and diseases of *Gadus morhua* L. around Helgoland in the German Bight. *ICES CM1989/G*:**49.**

Alvarez-Pellitero, P. 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology*. **126(3-4)**: 171-198.

Alvarez-Pellitero, P., Palezuela, O., Sitja-Bobadilla, A. 2008. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). *Parasitology International*. **57**: 110-120.

Angermuller, S. and Fahimi, H.D. 1981. Selective cytochemical localistion of peroxidase, cytochrome oxidase and catalase in rat liver with 3.3'-diaminobenzidine. *Histochemistry and Cell Biology*. **71(1)**: 33-44.

Anstensrud, M. 1989. Experimental studies of the reproductive behaviour of the parasitic copepod *Lernaeocera branchialis* (Pennellidae). *Journal of Marine Biological Association, U.K*. **69**: 465-476.

Anstensrud, M. 1990. Effects of mating on female behaviour and allometric growth in the two parasitic copepods *Lernaeocera branchialis* (L., 1767) (Pennellidae) and *Lepeophtheirus pectoralis* (Mueller, 1776) (Caligidae). *Crustaceana*. **59**: 245-258.

Aranishi, F. and Nakane, M. 1997. Epidermal proteases of the Japanese eel. *Fish Physiology and Biochemistry*. **16(6)**: 471-478.

Araujo-Jorge, T.C., Barbosa, H.S., Meirelles, M.N.L. 1992. *Trypanosoma cruzi* recognition by macrophages and muscle cells: perspectives after a 15-year study. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*. **87(V)**: 43-56.

Armstrong, P.B. 2006. Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. *Immunobiology*. **211**: 263-281.

Arnaud, J., Brunet, M., Mazza, J. 1988a. Labral glands in *Centropages typicus* (Copepoda, Calanoida). I. Sites of synthesis. *Journal of Morphology*. **197(1)**: 21-32.

Arnaud, J., Brunet, M., Mazza, J. 1988b. Labral glands in *Centropgaes typicus* (Copepoda, Calanoida) II. Sites of secretory release. *Journal of Morphology*. **197(2)**: 209-219.

Arnesen, S.M., Schrøder, M.B., Dalmo, R.A., and Bøgwald, J. 2002. Antigen uptake and immunoglobulin production in Atlantic cod (*Gadus morhua* L.) after intraperitoneal injection of *Vibrio anguillarum*. *Fish and Shellfish Immunology*. **13**:

159-170.

Arntz, W.E. 1972. Uber das Auftreten des parasitischen Copepoden *Lernaeocera branchialis* in der Kieler Bucht und seine Bedeutung als biologische Markierung. *Archiv Fur FischereiwissEnsChaft*. **23**: 118-127.

Astigarraga, A., Oleaga-Perez, A., Perez-Sanchez, R., Baranda, J.A., Encinas-Grandes, A. 1997. Host immune response evasion strategies in *Ornithodoros erraticus* and *O. moubata* and their relationship to the development of an antiargasid vaccine. *Parasite Immunology*. **19**: 401-410.

Banerjee, R., Anguita, J., Roos, D., Fikrig, E. 2000. Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91PHOX1. *The Journal of Immunology*. **164**: 3946-3949.

Bannister, N.J. 1993. Distribution and structure of sub-culticular glands in the copepod *Temora longicornis. Journal of Marine Biological Association, UK*. **73**: 97-107.

Bannister, N.J. and Herring, P.J. 1989. Distribution and structure of luminous cells in four marine copepods. *Journal of Marine Biological Association, UK*. **69**: 523-533.

Bastide-Guillaume, C., Douellou, L., Romestand, B., Trilles, J.P. 1987. Etude compare des deux *Lernaeocera* heberges respectivement per: *Merluccius merluccius* et *Trisopterus minutus* capelanus dans le Golfe du Lion (Sete, France) morphobiometrie, immunochimie, polymorphisme enzymatique. *Revue des Travaux de l'institut des Peches Maritimes*. **49(3-4)**: 143-154.

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

Bayne, C.J., Gerwick, L., Fujiki, K., Nakao, M., Yano, T. 2001. Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. *Developmental and Comparative Immunology*. **25(3)**: 205-217.

Begg G.S. & Bruno D.W. 1999. The common dab as definitive host for the penellid copepods *Lernaeocera branchialis* and *Haemobaphes cyclopterina*. *Journal of Fish Biology*. **55**: 655–657.

Bell, S. 2001. Exocrine glands of the caligid copepod *Lepeophtheirus salmonis* (Kroyer, 1837). *PhD thesis.* University of Stirling.

Bell, S., Bron, J.E., and Sommerville, C. 2000. The distribution of exocrine glands in *Lepeophtheirus salmonis* and *Caligus elongatus* (Copepoda: Caligidae). *Contributions to Zoology*. **69**: 9-20.

Belley, A. and Chadee, K. 1995. Eicosanoid production by parasites: from pathogenesis to immunomodulation? *Parasitology Today*. **11**: 327-334.

Belperio, J.A., Keane, M.P., Arenberg, D.A., Addison, C.L., Ehlert, J.E., Burdick, M.D., Strieter, R.M. 2000. CXC chemokines in angiogenesis. *Journal of Leukocyte Biology*. **68**: 1-8.

Bengten, E., Stromberg, S., PIlstrom, L. 1994. Immunoglobulin VH regions in Atlantic cod (*Gadus morhua* L.) their diversity and relationship to VH families from other species. *Developmental and Comparative Immunology*. **18(2)**: 109-122.

Bergman, D.K., Ramachandra, R.N, Wikel, S.K. 1998. Characterisation of an immunosuppressant protein from *Dermacentor andersoni* (Acari: Ixodidae) salivary glands. *Journal of Medical Entomology*. **35**: 505-509.

Bergman, D.K., Palmer, M.J., Caimano, M.J., Radolf, J.D., Wikel, S.K. 2000. Isolation and molecular cloning of a secreted immunosuppressant protein from *Dermacentor andersoni* salivary gland. *Journal of Parasitology*. **86(3)**: 516-525.

Bergsson, G., Agerberth, B., Jörnvall, H., and Gudmundsson, G.H. 2005. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). *FEBS Journal.* **272**: 4960-4969.

Bermudez, R., Vigliano, F., Marcaccini, A., Sitja-Bobadilla, A, Quiroga, M.I., Nieto, J.M. 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. *Fish and Shellfish Immunology*. **21(5)**: 501-512.

Boshra, H., Peters, R., Li, J., Sunyer, J.O. 2004. Production of recombinant C5a from rainbow trout (*Oncorhynchus mykiss*): role in leucocyte chemotaxis and respiratory burst. *Fish and Shellfish Immunology*. **17(3)**: 293-303.

Boshra, H., Li, J., and Sunyer, J.O. 2006. Recent advances on the complement system of teleost fish. *Fish and Shellfish Immunology*. **20**: 239-262.

Bowman, A.S. , Dillwith, J.W., and Sauer, J.R. 1996. Tick salivary prostaglandins: presence, origin and significance. *Parasitology Today*. **12**: 388-396.

Boxshall, G.A. 1977. The histopathology of infection by *Lepeophtheirus pectoralis* (Muller) (Copepoda: Caligidae). *Journal of Fish Biology.* **10(4)**: 411-415.

Boxshall, G.A. 1982. On the anatomy of the misophrioid copepods, with special reference to *Bentomisophria palliata*. *Sars. Phil. Trans. Roy. Soc. London, B*. **297**: 125-181.

Boxshall, G.A. 1992. Copepoda. In *Microscopic anatomy of invertebrates*. Eds. F.W. Harrison and A.G. Humes. Wiley-Liss. Inc., New York, U.S.A..

Braun-Nesje, R., Bertheussen, K., Kaplan, G., Seljelid, R. 1981. Salmonid macrophages: separation, *in vitro* culture and characterisation. *Journal of Fish Diseases*. **4**: 141-151.

Bricknell, I.R. Hayes, H.E, Billingsly, P., Vigneau, A.J, Sommerville, C., Raynard, R.S. 2003. Vaccines against the salmon lice (*Lepeophtheirus salmonis*) – a holy grail or just around the corner? *Journal of Parasitology* **89 (Suppl.)**: S149-S157.

Bridle, A.R., Morrison, R.N., Cunningham, P.M.C., Nowak, B.F. 2006a. Quantitation of immune response gene expression and cellular localisation of interleukin-1B mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD). *Veterinary Immunology and Immunopathology*. **114(1-2)**: 121-134.

Bridle, A.R., Morrison, R.N., Nowak, B.F. 2006b. The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during amoebic gill disease (AGD). *Fish and Shellfish Immunology*. **20(3)**: 346-364.

Bricknell, I., Bisset, P., Bowden, T. 2002. The function of Atlantic salmon (*Salmo salar L*.) antibodies under extremes of pH and osmolarity. *Fish and Shellfish Immunology*. **13**: 215-219.

Bron, J.E. 1993. A study of the biology and behaviour of the copepodid larva of the salmon louse *Lepeophtheirus salmonis* (Kroyer, 1837) (Copepoda: Caligidae). *PhD thesis*. University of Stirling.

Bron, J.E., Sommerville, C., Jones, M., and Rae, G.H. 1991. The settlement and attachment of early stages of the salmon louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae) on the salmon host, *Salmo salar*. *Journal of Zoology*. **224**: 201-212.

Bron, J.E., Sommerville, C., Rae, G.H. 1993. The functional morphology of the alimentary canal of larval stages of the parasitic copepod *Lepeophtheirus salmonis. Journal of Zoology*. **230(2)**: 207-220.

Brooker, A.J. 2007. Aspects of the biology and behaviour of *Lernaeocera branchialis* (Linnaeus, 1767) (Copepoda: Pennellidae). University of Stirling PhD thesis. pp 242.

Brunet, M., Arnaud, J., Mazza, J. 1994. Gut structure and digestive cellular processes in marine crustacea. *Oceanography and Marine Biology*. **32**: 335-367.

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

Buchmann, K. 1998b. Histochemical characteristics of *Gyrodactylus derjavini* parasitizing the fins of rainbow trout (*Oncorhynchus mykiss*). *Folia Parasitologica*. **45**: 312-318.

Buchmann, K. 1999. Immune mechanisms in fish skin against monogeneans – a model. *Folia Parasitologica*. **46**: 1-9.

Buchmann, K. and Bresciani, J. 1998. Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*:

association between mucous cell density in skin and site selection. *Parasitology Research*. **84(1)**: 17-24.

Buchmann, K. and Bresciani, J. 1999. Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. *Diseases of Aquatic Organisms*. **35**: 13-22.

Burt, M.D.B. and MacKinnon, B.M. 1997. Parasites and marine aquaculture. *ACC Special Publication*. **2**: 65-68.

Bussolati, G. 1971. Histochemical demonstration of sulfate groups by means of diaminobenzidine. *Journal of Histochemistry*. **3**: 445-449.

Butterworth, A.E. 1984. Cell-mediated damage to helminths. *Advances in Parasitology*. **23**: 143-235.

Caipang, C.M.A., Hynes, N., Puangkaew, J., Brinchmann, M.F., Kiron, V. 2008. Intraperitoneal vaccination of Atlantic cod, *Gadus morhua* with heat-killed *Listonella anguillarum* enhances serum antibacterial activity and expression of immune response genes. *Fish and Shellfish Immunology*. **24(3)**: 314-322.

Caipang, C.M.A., Brinchmann, M.F., Kiron, V. 2009. Profiling gene expression in the spleen of Atlantic cod, *Gadus morhua* upon vaccination with *Vibrio anguillarum* antigen. *Comparative Biochemistry and Physiology*, *Part B*. **153**: 261-267.

Callard, R. and Gearing, A. 1994. The cytokine Factsbook. London: Academic Press.

Campos-Perez, J.J., Ellis, A.E., Secombes, C.J. 1997. Investigation of factors influencing the ability of *Renibacterium salmoninarum* to stimulate rainbow trout macrophage respiratory burst activity. *Fish and Shellfish Immunology*. **7**: 555-566.

Capart, A. 1947. Le Lernaeocera branchialis (L.), copepode parasite des gadides. Journal du Conseil International pour l'Exploration de la Mer **15**: 69-76.

Capart, A. 1948. Le *Lernaeocera branchialis*. Cellule. **52**: 159-212.

Carroll, M.C. 2004. The complement system in regulation of adaptive immunity. *Nature Immunology*. **5**: 981-986.

Casillas, E. and Smith, L.S. 1977. Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology*. **10(5)**: 481-491.

Chang, M.X., Nie, P., Liu, G.Y., Song, Y., Gao, Q. 2005. Identification of immune genes in grass carp *Ctenopharyngodon idella* in response to infection of the parasitic copepod *Sinergasilus major. Parasitology Research*. **96(4)**: 224-229.

Chen, L., He, C., Baoprasertkul, P., Xu, P., Li, P., Serapion, J., Waldbieser, G., Wolters, W., Liu, Z. 2005. Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expresision after infection with *Edwardsiella ictaluri. Developmental and Comparative Immunology*. **29**: 135-142.

Choi-Miura, N.H., Sakamoto, T., Ohtaki, S., Nakamura, H., Ishizawa, S., Takagi, Y. 1993. Elevated compement activities of sera from patients with high density lioprotein deficiency (Tangier disease): the presence of normal level of clusterin and the possible implication in the atherosclerosis. *Clinical Experimental Immunology*. **93**: 242-247.

Clarke, G.L., Conover, R.J., David, C.N., Nicol, J.A.C. 1962. Comparative studies of luminescence in copepods and other pelagic marine animals. *Journal of Marine Biological Association UK*. **42**: 541-564.

Corripio-Miyar, Y., Bird, S., Tsamopoulos, K., Secombes, C.J. 2007. Cloning and expression analysis of two pro-inflammatory cytokines, IL-1B and IL-8, in haddock (*Melanogrammus aeglefinus*). *Molecular Immunology*. **44(6)**: 1361-1373.

Corripio-Miyar, Y., Zou, J., Richmond, H., Secombes, C.J. 2009. Identification of interleukin-22 in gadoids and examination of its expression level in vaccinated fish. *Molecular Immunology*. **46(10)**: 2098-2106.

Covello, J.M., Bird, S., Morrison, R.N., Battaglene, S.C., Secombes, C.J., Nowak, B.F. 2009. Cloning and expression analysis of three striped trumpeter (*Latris lineata*) proinflammatory cytokines, TNF alpha, IL-1B and IL-8, in response to infection by the ectoparasitic, *Chondracanthus goldsmidi. Fish and Shellfish Immunology*. **26(5)**: 773-786.

Cribb, B.W., Armstrong, W.D., Whittington, I.D. 2004. Mechanism of adhesion and detachment at the anterior end of *Merizocotyle icopae* (Monogenea: Monocotylidae) including ultrastructure of the anterior adhesive matrix. *Parasitology*. **129(2)**: 181-190.

Cross, M.L. and Matthews, R.A. 1993. Localized leucocyte response to *Ichthyophthirius multifiliis* establishment in immune carp *Cypinus carpio* L. *Veterinary Immunology and Immunopathology*. **38(3-4)**: 341-58.

Cuesta, A., Salinas, I., Rodriguez, A., Munoz, P., Sitja-Bobadilla, A., Alvarez-Pellitero, P., Meseguer, J., Esteban, M.A. 2006a. Cell-mediated cytotoxicity is the main innate immune mechanism involved in the cellular defence of gilthead seabream (Teleostei: Sparidae) against *Enteromyxum leei* (Myxozoa). *Parasite Immunology*. **28(12)**: 657-665.

Cuesta, A., Munoz, P., Rodriguez, A., Salinas, I., Sitja-Bobadilla, A., Esteban, M.A., Meseguer, J. 2006b. Gilthead seabream (*Sparus aurata* L.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). *Parasitology*. **132(1)**: 95-104.

Dacanay, A., Bentley, B.E., Brown, L.L., Roberts, A.J., Johnson, S.C. 2006. Unique multimeric immunoglobulin crosslinking in four species from the family Gadidae. *Fish and Shellfish Immunology*. **21(2)**: 215-219.

Dalmo, R.A., Ingebrigtsen, K., Bogwald, J. 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *Journal of Fish Diseases*. **20(4)**: 241-273.

Dang, P.M-C., Elbim, C., Marie, J-C., Chiandotto, M., Gougerot-Pocidalo, M-A., El-Benna, J. 2006. Anti-inflammatory effect of interleukin-10 on human neutrophil respiratory burst involves inhibition of GM-CSF-induced p47PHOX phosphorylation through a decrease in ERK1/2 activity. *The FASEB Journal*. **20**: 1504-1506.

Daugschies, A. and Joachim, A. 2000. Eicosanoids in parasites and parasitic infections. *Advances in Parasitology*. **46**: 181-240.

De Brito, T. and Franco, M.F. 1994. Granulomatous inflammation. *Revista do Instituto de Medicina Tropical de Sao Paulo*. **36(2)**: 185-92.

De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., de Vries, J.E. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *Journal of Experimental Medicine*. **174**: 1209-1220.

Debrosses, P. 1948. Le merland (*Gadus merlangus* L.) de côte française de l'Atlantique (deuxieme partie). *Revue des Travaux de l'Institut de Pêches Maritimes, Nantes*. **14**, 71-99.

Delamare-Debouteville, L. 1950. Copepodes parasites de poisons de Banyuls. *Vie et Milieu*. **1**: 108-110.

Demers, N.E. and Bayne, C.J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Developmental and Comparative Immunology*. **21(4)**: 363-373.

Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C., Fearon, D.T. 1996. C3d of complement as a molecular adjuvent bridging innate and acquired immunity. *Science*. **271**: 348-350.

Deuel, T.F., Senior, R.M., Chang, D. 1981. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proceedings of the National Academy of Science , USA*. **78**: 4584.

Deuel, T.F., Senior, R.M., Huang, J.S. 1982. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *Journal of Clinical Investigations*. **69**: 1046.

Dezfuli, B.S., Giari, L., Konecny, R., Jaeger, P., Manera, M. 2003. Immunohistochemistry, ultrastructure and pathology of gills of *Abramis brama* from Lake Mondsee, Austria, infected with *Ergasilus sieboldi* (Copepoda). *Diseases of Aquatic Organisms*. **53**: 257-262.

Dinarello, C.A. 1997. Interleukin-1. *Cytokine Growth Factor Reviews*. **8**: 253-265.

Ding, A., Nathan, C.F., Graycar, J., Derynck, R., Stuehr, D.J., Srimal, S. 1990. Macrophage deactivating factor and transforming growth factors-beta 1 –beta 2 and –beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-

gamma. *The Journal of Immunology*. **145(3)**: 940-944.

Donnelly, S., O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P. 2005. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. *Infection and Immunity*. **73(1)**: 166-173.

Ekes, M. 1971. The use of diaminobenzidine (DAB) for the histochemical demonstration of cytochrome oxidase activity in unfixed plant tissues. *Histochemistry and Cell Biology*. **27(2)**: 103-108.

El Naggar, M.M. and Kearn, G.C. 1983. Glands associated with the anterior adhesive areas and body margins in the skin-parasitic monogenean *Entobdella soleae. International Journal for Parasitology*. **13**: 67-81.

El Naggar, M.M. and Kearn, G.C. 1989. Haptor glands in the gill-parasitic ancyrocephaline monogenean *Cichlidogyrus halli typicus* and the report of a possible prokaryotic symbiont. *International Journal for Parasitology*. **19(4)**: 401-408.

El Shoura, S.M. 1987. Ultrasructural changes in the salivary alveoli of *Argas* (*Persicargas*) *persicus* (Ixodoidea: Argasidae) during and after feeding. *Experimental and Applied Acarology*. **3(4)**: 347-360.

Elarifi, A.E. 1982. The histopathology of larval anisakid nematode infections in the liver of whiting, *Merlangius merlangus* (L.), with some observations on blood leukocytes of the fish. *Journal of Fish Diseases*. **5(5)**: 411-419.

Ellis, A.E. 1977. The leukocytes of fish: A review. *Journal of Fish Biology*. **11**: 453-491.

Ellis, A.E. 1981. Non-specific defense mechanisms in fish and their role in disease processes. *Dev. Biol. Stand*. **49**: 337-352.

Ellis, A.E. 1986. The function of teleost fish lymphocytes in relation to inflammation. *International Journal of Tissue Reactions*. **8(4)**: 263-270.

Ellis, A.E. 1987. Inhibition of the *Aeromonas salmonicida* extracellular proteases by alpha2-macroglobulin in the serum of rainbow trout. *Microbial Pathogenesis*. **3**: 167-177.

Ellis, A.E. 1990. Serum antiproteases in fish. In: *Techniques in Fish Immunology* (eds. J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. van Muiswinkel). SOS Publications, Fair Haven, NJ. pp. 95-99.

Ellis, A.E. 1999. Immunity to bacteria in fish. *Fish and Shellfish Immunology*. **9**: 291-308.

Ellis, A.E. 2001. Innate host defence mechanisms of fish against viruses and bacteria. *Developmental Comparative Immunology.* **25**: 872-939.

Engelsma, M.Y., Stet, R.J., Saeij, J.P., Verburg-van Kemenade, B.M. 2003. Differential expression and haplotypic variation of two interleukin-1 beta genes in the common carp (*Cyprinus carpio* L.). *Cytokine*. **22**: 21-32.

Enriquez, G.L., Ebert, F., Muhlpfordt, H. 1977. Ultrastructural localization of daiminobenzidine reactivity in *leishmania donovani* promastigotes. *Tropenmedizin und Parasitologie*. **28(3)**: 323-32.

Espelid, S., Rødseth, O.M., and Jørgensen, T.Ø. 1991. Vaccination experiments and studies of the humoral immune responses in cod, *Gadus morhua* L., to four strains of monoclonal-defined *Vibrio anguillarum*. *Journal of Fish Diseases*. **14**: 185-197.

Evans, N.A., Whitfield, P.I., Bamber, R.N., and Espin, P.M. 1983. *Lernaeocera lusci* (Copepoda: Pennellidae) on bib (*Trisopterus luscus*) from Southampton waters. *Parasitology*. **86**, 161-173.

Faisal, M., Perkins, P.S., and Cooper, E.L. 1990. Infestation of the pennellid copepod *Phrixocephalus cincinnatus* modulates cell mediated immune responses in the pacific arrowtooth flounder, *Atheresthes stomias*. Eds F.O Perkins. and T.C. Cheng. *Pathology in Marine Science*. San Diego, California., Academic press. pp471-478.

Faliex, E., da Silva, C., Simon, G., Sasal, P. 2008. Dynamic expression of immune response genes in the sea bass *Dicentrarchus labrax* experimentally infected with the monogenean *Diplectanum aequans. Fish and Shellfish Immunology*. **24**: 759-767.

Fange, R., Lundblad, G., Lind, J. 1976. Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. *Marine Biology*. **36(3)**: 277-282.

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

Fast, M.D., Sims, D.E., Burka, J.F., Mustafa, A., Ross, N.W. 2002b. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. *Comparative Biochemistry and Physiology-Part A: Molecular and Integrative Physiology*. **132(3)**: 645-657.

Fast, M.D., Burka, J.F., Johnson, S.C., Ross, N.W. 2003. Enzymes released from *Lepeophtheirus salmonis* in response to mucus from different salmonids. *Journal of Parasitology*. **89(1)**: 7-13.

Fast , M.D., Ross, N.W., Craft, C.A., Locke, S.J., MacKinnon, S.L., Johnson, S.C. 2004. *Lepeophtheirus salmonis*: characterisation of prostaglandin E2 in secretory products of the salmon louse by RP-HPLC and mass spectrometry. *Experimental Parasitology*. **107**: 5-13.

Fast, M.D., Ross, N.W., Johnson, S.C. 2005. Prostaglandin E2 modulation of gene expression in Atlantic salmon (*Salmo salar*) macrophage-like cell line (SHK-1). *Developmental and Comparative Immunology*. **29(11)**: 951-963.

Fast, M.D., Ross, N.W., Muise, D.M., Johnson, S.C. 2006. Differential gene expression in Atlantic salmon infected with *Lepeophtheirus salmonis. Journal of Aquatic Animal Health*. **18**: 116-127.

Fast, M.D., Johnson, S.C., Jones, S.R.M. 2007. Differential expression of the proinflammatory cytokines IL-1B-1, TNF-alpha-1 and IL-8 in vaccinated pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon juveniles. *Fish and Shellfish Immunology*. **22(4)**: 403-407.

Fast, M.D., Hosoya, S., Johnson, S.C., Afonso, L.O.B. 2008. Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long- term stress. *Fish and Shellfish Immunology*. **24(2)**: 194-204.

Feng, S., Zhan, W., Sheng, X., Yang, K., Han, J., Wei, J., Li, J., Qiao, X. 2009. Response of mucosal and systemic sIgM-positive cells in turbot (*Scophthalmus maximus* L.) immunization with *Edwardsiella tarda*. *Veterinary Immunology and Immunopathology*. **129(1-2)**: 108-114.

Fernandez, E.J. and Lolis, E. 2002. Structure, function and inhibition of chemokines. *Annual Review of Pharmacology and Toxicology*. **42**: 469-499.

Ferreira, B.R. and Silva, J.S. 1998. Saliva of *Rhipicephalus sanguineus* tick impairs T cell proliferation and IFN-gamma-induced macrophage microbicidal activity. *Veterinary Immunology and Immunopathology*. **64(3)**: 279-293.

Firth, K.J., Johnson, S.C., Ross, N.W. 2000. Characterisation of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis*) and in whole-body louse homogenate. *Journal of Parasitology*. **86(6)**: 1199-1205.

Fleming, A. M. and Templeman, W. 1951. Discovery of larval forms of *Lernaeocera branchialis* on lumpfish. Fisheries Research Board Canadian Biological Station St. John's, Newfoundland Annual Report. **47**.

Forward, G.M. and Woo, P.T.K. 1996. An *in vitro* study on the mechanism of innate immunity in *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) against *Cryptobia salmositica*. *Parasitology Research*. **82(3)**: 238-241.

Fuchsberger, N., Kita, M., Hajnicka, V., Imanishi, J., Labuda, M., Nuttall, P.A. 1995. Ixodid tick salivary gland extracts inhibit production of lipopolysaccharide-induced mRNA of several different numan cytokines. *Experimental and Applied Acarology*. **19(11)**: 671-676.

Fujiki, K., Shin, D.H., Nakao, M., Yano, T. 2000. Molecular cloning and expression analysis of carp (Cyprinus carpio) interleukin-1B, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. Fish and Shellfish Immunology. 10(3): 229-242.

Furnes, C., Seppola, M., Robertsen, B. 2009. Molecular characterisation and expression analysis of interferon gamma in

Atlantic cod (*Gadus morhua*). *Fish and Shellfish Immunology*. **26(2)**: 285-292.

Garcia-Castillo, J., Pelegrin, P., Mulero, V., Meseguer, J. 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. *Immunogenetics*. **54(3)**: 200-207.

Gasque, P. 2004. Complement: a unique innate immune sensor for danger signals. *Molecular Immunology*. **41(11)**: 1089-1098.

Geldhof, P., Claerebout, E., Knox, D.P., Jagneessens, J., Vercruysse, J. 2000. Proteinases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi. Parasitology*. **121**: 639-647.

Gharagozlou-van Ginneken, I.D. 1979. Etude ultrastructurale et cytochimique de l'activite temporaire des glandes tegumentaires d'un Crustace Copepode. *Ann. Sci. Nat. Zool. Paris*. **13**: 205-212.

Ghose, T., Landrigan, P., Asif, A. 1974. Localisation of immunoglobulin and complement in pulmonary sarcoid granulomas. Chest. 66: 264-268.

Gillespie, R.D., Dolan, M.C., Piesman, J., Titus, R.G. 2001. Identification of an IL-2 binding protein in the saliva of the Lyme Diseas vector tick, *Ixodes scapularis. Journal of Immunology*. **166**: 4319-4326.

Gonzalez, R., Charlemagne, J., Mahana, W., and Avrameas, S. 1988. Specificity of natural serum antibodies present in phylogenetically distinct fish species. Immunology. **63**: 31-36.

Gonzalez, S.F., Buchmann, K., Nielsen, M.E. 2007a. Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory responses caused by the ectoparasite *Ichthyophthirius multifiliis. Fish and Shellfish Immunology*. 22(6): 641-650.

Gonzalez, S.F., Buchmann, K., Nielsen, M.E. 2007b. Complement expression in common carp (*Cyprinus carpio* L.) during infection with *Ichthyophthirius multifiliis. Developmental and Comparative Immunology*. **31(6)**: 576-586.

Gonzalez-Alanis, P., Wright, G.M., Johnson, S.C., Burka, J.F. 2001. Frontal filament morphogenesis in the salmon louse *Lepeophtheirus salmonis. Journal of Parasitology*. **87(3)**: 561-574.

Gordon, A.N., Kelly, W.R., Cribb, T.H. 1998. Lesions caused by cardiovascular flukes (Digenea: Spirorchidae) in stranded green turtles (*Chelonia mydas*). *Veterinary Pathology*. **35(1)**: 21-30.

Graham, S. and Secombes, C.J. 1988. The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. *Immunology.* **65**: 293-297.

Graham, S. and Secombes, C.J. 1990. Do fish lymphocytes secrete interferon-gamma? *Journal of Fish Biology*. **36**: 563-573.

Graves, S.S., Evans, D.L., Dawe, D.L. 1985. Antiprotozoan activity of nonspecific cytotoxic cells (NCC) from the channal catfish (*Ictalurus punctatus*). *Journal of Immunology.* **134(1)**: 78-85.

Grayson, T.H., Jenkins, P.G., Wrathmell, A.B., Harris, J.E. 1991. Serum responses to the salmon louse, *Lepeophtheirus salmonis* (Kroyer, 1838), in naturally infected salmonids and immunized rainbow trout , *Oncorhynchus mykiss* (Walbaum), and rabbits. *Fish and Shellfish Immunology*. **1**: 141-155.

Grayson, T.H., John, R.J., Wadsworth, S., Greaves, K., Cox, D., Roper, J., Wrathmell, A.B., Gilpin, M.L., Harris, J.E. 1995. Immunisation of Atlantic salmon against louse: identification of antigens and effects on louse fecundity. *Journal of Fish Biology*. **47**: 85-94.

Grayson, T.H., Cooper, L.F., Wrathmell, A.B., Roper, J., Evenden, A.J., Gilpin, M.L. 2002. Host responses to *Renibacterium salmoninarum* and specific components of the pathogen reveal the mechanisms of immune suppression and activation. *Immunology.* **106**: 273-283.

Grinde, B. 1989. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. *Journal of Fish Diseases*. **12(2)**: 95-104.

Gudmundsdottir, B.K., Jonsdottir, H., Steinthorsdottir, V., Magnadottir, B., and Gudmundsdottir, S. 1997. Survival and humoral antibody response of Atlantic salmon, *Salmo salar* L., vaccinated against *Aeromonas salmonicida ssp.*achromogenes. *Journal of Fish Diseases*. **20**: 351-360.

Guillaume C., Douellou L., Romestand B. & Trilles J.P. 1983. Influence of a haematophagenous parasite: *Lernaeocera branchialis* (L., 1767) (Crustacea, Copepoda, Penellidae), on some erythrocytic constants of the host fish: *Merluccius merluccius*. *Revue des Travaux de l'Institut des Peches Maritimes Nantes* **47**: 55–61.

Gwakisa, P., Yoshihara, K., Long To, T., Gotoh, H., Amano, F., Momotani, E. 2001. Salivary gland extract of *Rhipicephalus appendiculatus* ticks inhibits *in vitro* transcription and secretion of cytokines and production of nitric oxide by LPS-stimulated JA-4 cells. *Veterinary Parasitology*. **99(1)**: 53-61.

Hajnicka, V., Kocakova, P., Slavikova, M., Slovak, M., Gasperik, J., Fuchsberger, N., Nuttall, P.A. 2001. Anti-interleukin-8 activity of tick salivary gland extracts. *Parasite Immunology*. **23(9)**: 483-489.

Ham, E.A., Soderman, D.D., Zanetti, M.E., Dougherty, H.W., McCauley, E., Kuehl, F.A. 1983. Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *Proceedings of the National Academy of Sciences of the USA.* **80(14)**: 4349-4353.

Hamburger, S.A. and McEver, R.P. 1990. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood*. **75(3)**: 550-554.

Hamers, R., Lehmann, J., STurenberg, F.J., Taraschewski, H. 1992. *In vitro* study of the migratory and adherent responses of fish leukocytes to the eel-pathogenic acanthocephalan *Paratenuisentis ambiguus* (van Cleave, 1921) Bullock and Samuel, 1975 (Eoacanthocephala: Tenuisentidae). *Fish and Shellfish Immunology*. **2**: 43-51.

Hamilton, K.H., Zhao, J., Sims, P.J. 1993. Interaction between apolipoproteins A-I and A-II and the membrane attack complex of caomplement. Affinity of the apolipoproteins for polymeric C9. *Journal of Biological Chemistry*. **268**: 3632-8.

Hammerberg, B. and Williams, J.F. 1978a. Interaction between *Taenia taeniaeformis* and the complement system. *Journal of Immunology*. **120**: 1033-1038.

Hammerberg, B. and Williams, J.F. 1978b. Physicochemical characterisation of complement-interacting factors from *Taenia taeniaeformis. Journal of Immunology*. **120**: 1039-1045.

Hardie, L.J., Chappell, L.H., Secombes, C.J. 1994. Human tumor necrosis factor alpha influences rainbow trout *Oncorhynchus mykiss* leucocyte responses. *Veterinary Immunology and Immunopathology*. **40(1)**: 73-84.

Harris, J.E. 1972. The immune response of a cyprinid fish to infections of the acanthocephalan *Pomphorhynchus laevis. International Journal of Parasitology*. **2(4)**: 459-469.

Harris, J.E., Soleng, A., Bakke, T.A. 1998. Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) mediated by host complement. *Parasitology.* **117**: 137-143.

Hattingh, J. and Van Pletzen, J.J. 1974. The influence of capture and transportation on some blood parameters of fresh water fish. *Comparative Biochemistry and Physiology*. **49A**: 607-609.

Hayat, M.A., 1989. Principles and techniques of electrom microscopy – biological applications. 3rd edition. Macmillan Press Scientific and Medical, Basingstoke and London.

Hemmingsen, W. and Mackenzie, K. 2001. The parasite fauna of the Atlantic cod, *Gadus morhua* L. *Advances in Marine Biology*. **40**: 1-80.

Henry, R.L. 1961. Leukocytes and thrombosis. *Thrombosis Diath. Haemorrh.* **13**: 35.

Herring, P.J. 1988. Copepod luminescence. In: *Biology of Copepods*. Eds G.A. Boxshall and H.K. Schminke. *Hydrobiologia.* **167-168**: 183-195.

Hewitson, J.P., Harcus, Y.M., Curwen, R.S., Dowle, A.A., Atmadja, A.K., Ashton, P.D., Wilson, A., Maizels, R.M. 2008. The secretome of the filarial parasite, *Brugia malayi*: proteomic profile of adult excretory-secretory products. *Molecular and*

Biochemical Parasitology. **160(1)**: 8-21.

Hicks, G.R.F. and Grahame, J. 1979. Mucus production and its role in the feeding behaviour of *Diarthrodes nobilis* (Copepoda: Harpacticoida). *Journal of Marine Biological Association*. *UK*. **50**: 321-330.

Hill, D.J. and Rowley, A.F. 1996. The thromboxane mimetic, U-46619, induces the aggregation of fish thrombocytes. *British Journal of Haematology*. **92**: 200-211.

Hislop, J.R.G. and Shanks, A.M. 1981. Recent investigations on the reproductive biology of the haddock, *Melanogrammus aeglefinus*, of the northern North Sea and the effects on fecundity of infection with the copepod parasite *Lernaeocera branchialis. Journal du Conseil Permanent International par l'Exploration de la Mer* **39**: 244-251.

Holland, M.C.H. and Lambris, J.D. 2002. The complement system in teleosts. *Fish and Shellfish Immunology*. **12(5)**: 399-420.

Holland, M.C.H. and Lambris, J.D. 2004. A functional C5a anaphylatoxin receptor in a teleost species. *The Journal of Immunology*. **172**: 349-355.

Hoole, D. and Arme, C. 1986. The role of serum in the leucocyte adherance to the plerocercoid of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *Parasitology*. **92**: 413-424.

Hoole, D. and Arme, C. 1988. *Ligula intestinalis* (Cestoda: Pseudophyllidea): phosphorylcholine inhibition of fish leucocyte adherance. *Diseases of Aquatic Organisms*. **5**: 29-33.

Hordvik, I., Thevarajan, J., Samdal, I., Bastani, N., Krossoy, B. 1999. Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. *Scandinavian Journal of Immunology*. **50(2)**: 202-210.

Horn, F., Coutinho dos Santos, P., Temignoni, C. 2000. *Boophilus microplus* anticoagulant proetin: A antithrombin inhibitor isolated from the cattle tick saliva. *Archives of Biochemistry and Biophysics***. 384(1)**: 68-73.

Hotez, P.J. and Cerami, A. 1983. Secretion of a proteolytic anticoagulant by *Ancyclostoma* hookworms. *Journal of Experimental Medicine*. **157**: 1594-1603.

Huising, M.O., Stolte, E., Flik, G., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L. 2003. CXC chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology*. **27(10)**: 875-888.

Hurst, M.H., Lola, S.G., Lindberg, R. 2006. Immunomodulation of the hepatic egg granuloma in *Schistosoma japonicum*-infected pigs. *Parasite Immunology*. **28(12)**: 681-686.

Huys, R., Boxshall, G.A. 1991. Copepod evolution. Ray Society, London

Igawa, D., Sakai, M., Savan, R. 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. *Molecular Immunology*. **43(7)**: 999-1009.

Ingram, G.A. 1980. Substances involved in the natural resistance of fish to infection: a review. *Journal of Fish Biology*. **16**: 23-60.

Inokuma, H., Hara, Y., Aita, T., Onishi, T. 1997. Effect of infestation with *Rhipicephalus sanguineu*s on neutrophil function in dogs. *Medical and Veterinary Entomology*. **11(4)**: 401-403.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H., Nakanishi, T. 2002. A new method for fish leucocyte counting and partial differentitaion by flow cytometry. *Fish and Shellfish Immunology*. **13(5)**: 379-390.

Israelsson, O., Petersson, A., Bengten, E., Wiersma, E.J., Anderson, J., Gezelius, G., Pilstrom, L. 1991. Immunoglobulin concentration in Atlantic cod, *Gadus morhua* L., serum and cross-reactivity between anti-cod antibodies and immunoglobulins from other species. *Journal of Fish Biology*. **39(2)**: 265-278.

Jang, S.I., Hardie, L.J., Secombes, C.J. 1994. Effects of transforming growth factor beta 1 on rainbow trout *Oncorhynchus*

mykiss macrophage respiratory burst activity. *Developmental and Comparative Immunology*. **18(4)**: 315-23.

Jang, S.I., Hardie, L.J., Secombes, C.J. 1995. Elevation of rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst with macrophage-derived supernatants. *Journal of Leukocyte Biology*. **57(6)**: 943-947.

Jaso-Friedmann, L., Leary, J.H., Evans, D.L. 2000. Role of nonspecific cytotoxic cells in the induction of programmed cell death of pathogenic protozoans: participation of the Fas ligand-Fas receptor system. *Experimental Parasitology*. **96(2)**: 75-88.

Jefferies, J.R., Turner, R.J., Barrett, J. 1997. Effect of *Fasciola hepatica* excretory-secretory products on the metabolic burst of sheep and human neutrophils. *International Journal for Parasitology*. **27(9)**: 1025-1029.

Jenne, D.E., Lowin, B., Peitsch, M.C., Bottcher, A., Schmitz, G., Tschopp, J. 1991. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. *Journal of Biological Chemistry*. **266(17)**: 11030-6.

Jimenez, N., Coll, J., Salguero, F.J., Tafalla, C. 2006. Co-injection of interleukin-8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*). *Vaccine*. **24(27-28)**: 5615-5625.

Johnson, S.C. and Albright, L.J. 1992. Comparative susceptibility and histopathology of the response of naïve Atlantic, chinook and coho salmon to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Diseases of Aquatic Organisms*. **14(3)**: 179-193.

Johnson, S.C., Ewart, K.V., Osborne, J.A., Delage, D., Ross, N.W., Murray, H.M. 2000. Molecular cloning of trypsin cDNAs and trypsin gene expression in the salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Parasitology Research*. **88(9)**: 789-796.

Joiner, K.A. 1988. Complement evasion by bacteria and parasites. *Annual Review of Microbiology*. **42**: 201-230.

Jolles, P. and Jolles, J. 1984. What's new in lysozyme research? *Molecular and Cellular Biochemistry*. **63(2)**: 165-189

Jones, S.R.M. 2001. The occurrence and mechanisms of innate immunity against parasites in fish. *Developmental and Comparative Immunology*. **25**: 841-852.

Jones, M. E. B. and Taggart, C. T. 1998. Distribution of gill parasite (*Lernaeocera branchialis*) infection in Northwest Atlantic cod (*Gadus morhua*) and parasite induced host mortality: inferences from tagging data. *Canadian Journal of Fisheries and Aquatic Science* **55**, 364-375.

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

Joy, J.E. and Jones, L.P. 1973. Observations on the inflammatory response within the dermis of a white bass, *Morone chrysops* (Rafinesque), infected with *Lernaea cruciata* (Copepoda: Caligidea). *Journal of Fish Biology*. **5(1)**: 21-23.

Kaattari, S.,Evans, D., and Klemer, J. 1998. Varied redox forms of teleost IgM: an alternative to isotopic diversity? *Immunological Reviews*. **166**: 133-142.

Kabata, Z. 1957. Lernaeocera obtusa n. sp., a hitherto undescribed parasite of the haddock (Gadus aeglefinus L.). *Journal of the Marine Biological Association of the United Kingdom.* **36** (3):569-592

Kabata, Z. 1958. Lernaeocera obtusa n. sp. Its biology and its effect on the haddock. *Marine Research Department of Ariculture and Fisheries for Scotland*. **3**: 1-26.

Kabata, Z. 1960. On the specificity of Lernaeocera (Copepoda parasitica). *The Annuls and Magazine of Natural History, Ser*. **13(3)**:133-139.

Kabata, Z. 1961. *Lernaeocera branchialis* (L.) a parasitic copepod from the European and the American shores of the Atlantic. *Crustaceana*. **2**, 243-249.

Kabata, Z. 1969. *Phrixocephalus cincinnatus* Wilson, 1908 (Copepoda: Lernaeoceridae): Morphology, metamorphosis, and host-parasite relationship. *Journal of the Fisheries Research Board of Canada*. **26**: 921-934.

Kabata, Z. 1970. Crustacea as enemies of fishes. In S.F. Sniesko & H.R. Axelrod Diseases of Fishes. T.F.H. Publications, Jersey City, NJ, USA.

Kabata, Z. 1979. Parasitic Copepoda of British fishes. London: The Ray Society.

Kabata, Z. 1984. Diseases caused by metazoans: crustaceans. In O. Kinne. *Diseases of Marine Animals*, **Vol. IV,** Part 1. Introduction, Pisces. Biologische Anstatt Helgoland, Hamburg. pp. 321-399.

Kabata, Z. 1988. Copepoda and Branchiura. In L. Margolis and Z. Kabata eds. Guide to the parasites of fishes of Canada. Part II - Crustacea. Canada, Canadian Special Publication of Fisheries and Aquatic Sciences **101**: 3-127.

Karagouni, E., Athanassopoulou, F., Lytra, A., Komis, C., Dotsika, E. 2005. Antiparasitic and immunomodulatory effect of innovative treatments aginst *Myxobolus sp*. Infection in *Diplodus puntazzo. Veterinary Parasitology*. **134(3-4)**: 215-228.

Karvonen, A., Paukku, S., Seppala, O., Valtonen, E.T. 2005. Resistance against eye flukes: naïve versus previously infected fish. *Parasitology Research*. **95(1)**: 55-59.

Kato, Y., Nakao, M., Shimizu, M., Wariishi, H., Yano, T. 2004. Purification and functional assessment of C3a, C4a and C5a of the common carp (*Cyprinus carpio*) complement. *Developmental and Comparative Immunology*. **28(9)**: 901-910.

Kaufman, W.R. 1978. Actions of some transmitters and their antagonists on salivary secretion in a tick. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology.* **235**: R76-R81.

Khan, R.A. 1988. Experimental transmission, development, and effects of a parasitic copepod, *Lernaeocera branchialis* , on Atlantic cod, *Gadus morhua*. *Journal of Parasitology*. **74**: 586-599.

Khan, R.A. and Lacey, D. 1986. Effect of concurrent infections of *Lernaeocera branchialis* (Copepoda) and *Trypanosoma murmanensis* (Protozoa) on Atlantic cod, Gadus morhua. *Journal of Wildlife Diseases* **22**: 201-208.

Khan R.A. & Lee E.M. 1989. Influence of *Lernaeocera branchialis* (Crustacea: Copepoda) on growth rate of Atlantic cod, (*Gadus morhua*). *Journal of Parasitology* **75**: 449–454.

Khan, R.A., Lee, E.M., and Barker, D. 1990. *Lernaeocera branchialis*: a potential pathogen to cod ranching. *Journal of Parasitology*. **76**: 913-917.

Khan, R. A., Barker, D. E. and Lee, E. M. 1993. Effect of a single *Lernaeocera branchialis* (Copepoda) on growth of Atlantic cod. *Journal of Parasitology* **79**: 954-958.

Kirk, R.S. and Lewis, J.W. 1998. Histopathology of *Sanguinicola inermis* infection in carp, *Cyprinus carpio*. *Journal of Helminthology*. **72**: 33-38.

Knudsen, K.K. and Sundnes, G. 1998. Effects of salinity on infection with *Lernaeocera branchialis* (L.) (Copepoda: Pennellidae). *Journal of Parasitology.* **84**: 700-704.

Kocakova, P., Slavikova, M., Hajnicka, V., Slovak, M., Gasperik, J., Vancova, I., Fuchsberger, N., Nuttal, P.A. 2003. Effect of fast protein liquid chromatography fractionated salivary gland extracts from different ixodid tick species on interleukin-8 binding to its cell receptors. *Folia Parasitologica*. **50**: 79-84.

Koie, M. 1999 Metazoan parasites of flounder *Platichthys flesus* (L.) along a transect from the southwestern to the northeastern Baltic Sea. *ICES Journal of Marine Science* **56**: 157-163.

Köllner, B., Fischer, U., Rombout, J.H.W.M., Taverne-Thiele, J.J., Hansen, J.D. 2004. Potential involvment of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. *Developmental and Comparative Immunology*. **28**: 1049-1062.

Kopecky, J., Kuthejlova, M., Pechova, J. 1999. Salivary gland extract from *Ixodes ricinus* ticks inhibits production of

interferon-gamma by the upregulation of interleukin-10. *Parasite Immunology*. **21(7)**: 351-356.

Kopf, K., Naser, K., van der Heijden, M.H.T., Taraschewski, H. 2000. Humoral immune response of European eel *Anguilla anguilla* experimentally infected with *Anguillicola crassus. Diseases of Aquatic Organisms*. **42**: 61-69.

Kotze, A.C. and McClure, S.J. 2001. *Haemonchus contortus* utilises catalase in defence against exogenous hydrogen peroxide *in vitro. International Journal of Parasitology*. **31(14)**. 1563-1571.

Kristen, U. and Biedermann, M. 1981. Ultrastructure, origin and composition of the protein bodies in the ligule of *Isoetes lacustris L. Annals of Botany*. **48**: 655-633.

Kurlansky, M. 1998. Cod – A biography of the fish that changes the world. Jonathan Random House, London. pp 282.

Kvamme, B.O., Frost, P., Nilsen, F. 2004. The cloning and characterisation of full-length trypsins from the salmon louse *Lepeophtheirus salmonis. Molecular and Biochemical Parasitology*. **136(2)**: 303-307.

Kwatia, M.A., Botkin, D.J., Williams, D.L. 2000. Molecular and enzymatic characterisation of *Schistosoma mansoni* thioredoxin peroxidase. *Journal of Parasitology*. **86(5)**: 908-915.

Kwon, S.R., Kim, C.S., Chung, J.K., Lee, H.H., Kim, K.H. 2002. Inhibition of chemiluminescent response of olive flounder *Paralichthys olivaceus* phagocytes by the scuticociliate parasite *Uronema marinum. Diseases of Aquatic Organisms*. **52**: 119-122.

Lackey, A., James, E.R., Sakanari, J.A., Resnick, S.D., Brown, M., Bianco, A.E., McKerrow, J.H. 1989. Extracellular proteases of *Onchocerca. Experimental Parasitology*. **68(2)**: 176-85.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Laing, K.J., Wang, T., Zou, J., Holland, J., Hong, S. Bols, N., Hirono, I., Aoki, T., Secombes, C.J. 2001. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha. *FEBS Journal*. **268(5)**: 1315-1322.

Laing, K., Bols, N., Secombes, C.J. 2002. A CXC chemokine sequence isolated from rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11. *European Cytokine Network*. **13**: 462-473.

Lang, T. 1989. *Lernaeocera branchialis* in cod (*Gadus morhua*) of the Baltic Sea. *ICES CM1989/J:* **23**.

Lange, S. and Magnadodottir, B. 2003. Spontaneous haemolytic activity of Atlantic halibut (*Hippoglssus hippoglossus* L.) and sea bass (*Dicentrarchus labrax*) serum. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. **136(1)**: 99-106.

Lange, S.,Dodds, A. W., Magnadottir, B. 2004. Isolation and characterization of complement component C3 from Atlantic cod (*Gadus morhua L.*) and Atlantic halibut (*Hippoglossus hippoglossus L.*). *Fish & Shellfish Immunology*. **16**: 227-239.

Langston, A.L., Johnstone, R., Ellis, A.E. 2001. The kinetics of the hypoferreamic response and changes in levels of alternative complement activity in diploid and triploid Atlantic salmon, following injection of lipopolysaccharide. *Fish and Shellfish Immunology*. **11(4)**: 333-345.

Larsen, G., Hemmingsen, W., MacKenzie, K., and Lysne, D.A. 1997. A population study of cod, *Gadus morhua* L., in northern Norway using otolith structure and parasite tags. *Fisheries Research*. **32**: 13-20.

Lawrie, C.H., Randolph, S.E., Nuttall, P.A. 1999. *Ixodes* ticks: serum species sensitivity of anticomplement activity. *Experimental Parasitology*. **93**: 207-214.

Lee, N.S., Nomura, Y., Miyazaki, T. 1999. Gill lamellar pillar cell necrosis, a new birnavirus disease in Japanese eels. *Diseases of Aquatic Organisms.* **37**: 13-21.

Lee, E.Y., Park, H.H., Kim, Y.T., Chung, J.K., Choi, T.J. 2001. Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceous*). *Gene*. **274**: 237-243.

Leef, M.J. & Lee, P.S. 2008. Preliminary investigation into the killing effect of kingfish (*Seriola lalandi*) serum and mucus against the monogenean parasites *Benedenia seriolae* and *Zeuxapta seriolae. Aquaculture international.* DOI 10.1007/s 10499-008-9228.0.

Lehane, M.J. 1997. Peritrophic matrix structure and function. *Annual Review of Entomology*. **42**: 525-550.

Leid, R.W. 1988. Parasites and complement. *Advances in Parasitology*. **27**: 131-168.

Leiro, J., Piazzon, M.C., Budino, B., Sanmartin, M.L., Lamas, J. 2008. Complement-mediated killing of *Philasterides dicentrarchi* (Ciliophora) by turbot serum: relative importance of alternative and classical pathways. *Parasite Immunology*. **30(10)**: 535-543.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., Sunyer, J.O. 2006. B lymphocytes from early vertebrates have potent phagocytic and microbial abilities. *Nature Immunology*. **7**: 1116-1124.

Li, J., Barreda, D.R., Zhang, Y-A., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., Sunyer, J.O. 2007. Complement and B cell cooperation in teleost fish: role in phagocytosis and inflammation. *Molecular Immunology*. **44(1-3)**: 205.

Lindenstrom, T. and Buchmann, K. 1998. Dexamethasone treatment increases susceptibility of rainbow trout, *Onchorhynchus mykiss* (Walbaum), to infections with *Gyrodactylus derjavini* Mikailov. *Journal of Fish Diseases*. **21(1)**: 29-38.

Lindenstrom, T., Buchmann, K., Secombes, C.J. 2003. *Gyrodactylus derjavini* infection elicits IL-1B expression in rainbow trout skin. *Fish and Shellfish Immunology*. **15(2)**: 107-155.

Lindenstrom, T., Secombes, C., Buchmann, K. 2004. Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. *Veterinary Immunology and Immunopathology*. **97(3-4)**: 137-148.

Lindsay, G.J.H. and Gooday, G.W. 1985. Chitinolytic enzymes and the bacterial microflora in the digestive tract of cod, *Gadus morhua. Journal of Fish Biology*. **26(3)**: 255-265.

Lodge, R., Diallo, T.O., Descoteaux, A. 2006. *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cellular Microbiology*. **8(12)**: 1922-1931.

Logtenberg T, Schutte M.E., Ebeling S.B., Gmelig-Meyling, F.H.J., Van Es, J.H. 1992. Molecular approaches to the study of human B-cell and (auto) antibody repertoire generation and selection. *Immunological Review*. **128**:24-47.

Longo, G.P., Dragonetti, C., Longo, C.P. 1972. Cytochemical localization of catalase in glyoxysomes isolated from maize scutella. *Plant Physiology*. **50**: 463-468.

Lovy, J., Wright, G.M., Speare, D.J. 2006. Morphological presentation of a dendritic-like cell within the gills of chinook salmon infected with *Loma salmonae. Developmental and Comparative Immunology*. **30**: 259-263.

Lovy, J. Savidant, G.P., Speare, D.J., Wright, G.M. 2009. Langerin/CD207 positive dendritic-like cells in the haemopoietic tissues of salmonids. *Fish and Shellfish Immunology*. **27**: 365-368.

Lowe, E. 1935. On the anatomy of a marine copepod *Calanus finmarchicus* (Gunnerus). *Trans. Roy. Soc. Edinb*. **58**: 561-603.

Lysne, D.A. and Skorping, A. 2002. The parasite *Lernaeocera branchialis* on caged cod: infection pattern is caused by differences in host susceptibility. *Parasitology* **124**: 69-76.

Madhavi, R. and Anderson, R.M. 1985. Variability in the susceptibility of the fish host, *Poecilia reticulata*, to infection with *Gyrodactylus bullatarudis* (Monogenea). *Parasitology*. **91**: 531-544.

Magnadottir, B. 1998. Comaprison of immunglobulin (IgM) from four fish species. *Icelandic Agriculture Sciences*. **12**: 47-

59.

Magnadottir, B. 2000. The spontaneous haemolytic activity of cod serum: Heat insensibility and other characteristics. *Fish & Shellfish Immunology*. **10**: 731-735.

Magnadottir, B. 2006. Innate immunity of fish (overview). *Fish & Shellfish Immunology*. **20**: 137-151.

Magnadottir, B. and Gudmundsdottir, B.K. 1992. A comparison of total and specific immunoglobulin levels in healthy Atlantic salmon (*Salmo salar* L.) and in salmon naturally infected with *Aeromonas salmonicida subsp. achromogenes*. *Veterinary Immunology and Immunopathology*. **32**: 179-189.

Magnadottir, B. and Lange, S. 2004. Is Apolipoprotein A-I a regulating protein for the complement system of cod (*Gadus mohua* L.)? *Fish & Shellfish Immunology*. **16**: 265-269.

Magnadottir, B.,Jonsdottir, H.,Helgason, S.,Bjornsson, B.,Jorgensen, T., and Pilstrom, L. 1999a. Humoral immune paramters in Atlantic cod (*Gadus morhua* L.) I: The effects of environmental temperature. *Comparative Biochemistry and Physiology, Part B*. **122**: 173-180.

Magnadottir, B.,Jonsdottir, H.,Helgason, S.,Bjornsson, B.,Jorgensen, T., and Pilstrom, L. 1999b. Humoral immune parameters in Atlantic cod (*Gadus morhua* L.) II. The effects of size and gender under different environmental conditions. *Comparative Biochemistry and Physiology, Part B*. **122**: 181-188.

Magnadottir, B.,Jonsdottir, H.,Helgason, S.,Bjornsson, B.,Solem, S. T., and Pilstrom, L. 2001. Immune parameters of immunised cod (*Gadus morhua* L.). *Fish & Shellfish Immunology*. **11**: 75-89.

Magnadottir, B.,Bambir, S. H.,Gudmundsdottir, B. K.,Pilstrom, L., and Helgason, S. 2002. Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. *Journal of Fish Diseases*. **25**: 583-597.

Mann H. 1952. *Lernaeocera branchialis* (Copepoda parasitica) und seine Schadwirkung bei einigen Gadiden. *Archiv Fischerei Wissenschaft* **4**: 133–144.

Mann, H. (1964). Vorkommen, Verbreitung und schadwirkung von *Lernaeocera minuta* T. Scott (Copepoda parasitica). Veroff. Inst. Meeresforsch. *Bremershafen.* **9**: 79-83.

Mann, H. 1970. Copepods and isopods as parasites of marine fishes. In: A Symposium on Diseases of Fishes and Shell Fishes. S. F. Snieszko (Ed.). American Fisheries Society, Washington, D.C., Special Publication **5**: 177-188.

Manson, F.D.C., Fletcher, T.C., Gooday, G.W. 1992. Localisation of chitinolytic enzymes in blood of turbot, *Scophthalmus maximus*, and their possible roles in defence. *Journal of Fish Biology*. **40(6)**: 919-927.

Margos, G., Navarette, S., Butcher, G., Davies, A., Willers, C., Sinden, R.E., Lachmann, P.J. 2001. Interaction between host complement and mosquito-midgut-stage *Plasmodium berghei. Infection and Immunity*. **69(8)**: 5064-5071.

Martin-Armis, M., Zykova, S., Smedsrod, B. 2008. Effects of CpG-oligonucleotides, poly I:C and LPS on Atlantic cod scavenger endothelial cells (SEC). *Developmental and Comparative Immunology*. **32(2)**: 100-107.

Matsuyama, T., Iida, T. 1999. Degranulation of eosinophilic granular cells with possible involvement in neutrophil migration to site of inflammation in tilapia. *Developmental and Comparative Immunology*. **23(6)**: 451-457.

Matzinger, P. 1994. Tolerance, danger and the extended family. *Annual Review of Immunology*. **12**: 991-1045.

Maule, A.G., Tripp, R.A., Kaattari, S.L., Schreck, C.B. 1989. Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Endocrinology*. **120**: 135-142.

McKerrow, J.H. 1989. Minireview: Parasite proteases. *Experimental Parasitology*. **68**: 111-115.

McKerrow, J.H., Brindley, P., Brown, M., Gam, A.A., Staunton, C., Neva, F.A. 1990. *Strongyloides stercoralis*: identification of a protease that facilitates penetration of skin by the infective larvae. *Experimental Parasitology*. **70(2)**: 134-43.

McVicar, A.H. and Fletcher, T.C. 1970. Serum factors in *Raja radiata* toxic to *Acanthobothrium quadripartitum* (Cestoda: Tetraphyllidea), a parasite specific to *R. naevus*. *Parasitology*. **61**: 55-63.

Mejri, N., Rutti, B., Brossard, M. 2002. Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. *Parasitology Research*. **88(3)**: 192-197.

Mellergaard, S. and Lang, T. 1999. Diseases and parasites of Baltic cod (*Gadus morhua*) from the Macklenburg Bight to the Estonian coast. *ICES Journal of Marine Science*. **56**: 164-168.

Meri, T., Jokiranta, S., Hellwage, J., Bialonski, A., Zipfel, P.F., Meri, S. 2002. *Onchocerca volvulus* microfilariae avoid complement attack by direct binding of factor H. *The Journal of Infectious Diseases.* **185**: 1786-1793.

Metzger, A. 1868. Ueber das Männchen und Weibchen der Gattung *Lernaea* vor den Eintritt der sogenannten rüchschreitenden Metamorphose. *Translation in Annals and Magazine of Natural History* **4**: 154-157.

Milev-Milovanovic, I., Long, S., Wilson, M.,Bengten, E., Miller, N.W., Chinchar, V.G. 2006. Identification and expression analysis of interferon gamma genes in channel catfish. *Immunogenetics*.**58(1)**: 70-80

Moritomo, T.,Serata, K.,Teshirogi, K.,Aikawa, H.,Inoue, Y.,Itou, T., and Nakanishi, T. 2003. Flow cytometric analysis of the neutrophil respiratory burst of ayu, *Plecoglossus altivelis*: Comparison with other fresh water fish. *Fish & Shellfish Immunology*. **15**: 29-38.

Morris, D.J., Adams, A., Richards, R.H. 2000. *In situ* hybridisation identifies the gill as a portal of entry for PKX (Phylum Myxozoa), the causative agent of proliferative kidney disease in salmonids. *Parasitology Research*. **86**: 950-956.

Morris, R.V., Shoemaker, C.B., David, J.R., Lanzaro, G.C., Titus, R.G. 2001. Sandfly maxadilan exacerbates infection with *Leshmania major* and vaccinating against it protects against *L. major* infection. *Journal of Immunology*. **167**: 5226-5230.

Morrison, C.M., Cornick, J.W., Shum, G., Zwicker, E. 1984. Histopathology of atypical *Aeromonas salmonicida* infection in Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases*. **7(6)**: 477-494.

Morrison, R.N., Koppang, E.O., Hordvik, I., Nowak, B.F. 2006. MHC class II+ cells in the gills of Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease. *Veterinary Immunology and Immunopathology*. **109**: 297-303.

Morrison, R.N., Zou, J., Secombes, C.J., Scapigliati, G., Adams, M.B., Nowak, B.F. 2007. Molecular cloning and expression analysis of tumour necrosis factor-alpha in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology*. **23(5)**: 1015-1031.

Moyer, K.E., Saggers, G.C., Allison, G.M., Mackay, D.R., Ehrlich, H.P. 2002. Effects of interleukin-8 on granulation tissue maturation. *Journal of Cellular Physiology*. **193(2)**: 173-179.

Mulder, I.E., Wadsworth, S., Secombes, C.J. 2007. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida. Fish and Shellfish Immunology*. **23(4)**: 747-759.

Mulero, V. and Meseguer, J. 1998. Functional characterisation of a macrophage-activating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.). *Fish and Shellfish Immunology*. **8(2)**: 143-156.

Muller H. 1983. The effects of Lernaeocera infestation on cod (Gadus morhua). *Bulletin of the European Association of Fish Pathologists* **3**: 21–22.

Munoz, P., Cuesta, A., Athanassopoulou, F., Golomazou, H., crespo, S., Padros, F., Sitja-Bobadilla, A., Albinana, G., Esteban, M.A. 2007. Sharpsnout sea bream (*Diplodus puntazzo*) humoral immune response against the parasite *Enteromyxum leei* (Myxozoa). *Fish and Shellfish Immunology*. **23(3)**: 636-645.

Murphy, D.M., Forrest, I.A., Corris, P.A., Johnson, G.E., Jones, D., Cawston, T.E., Lordon, J.L., Ward, C. 2008. 459: Azithromycin Attenuates Lipopolysaccharide-Mediated Release of Interleukin-8 and GM-CSF from Primary Bronchial Epithelial Cells Derived from Stable Lung Transplant Recipients. *The Journal of Heart and Lung Transplants*. **27(2)**: S225-226.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G.A., Burka, J.F. 2000a. Effects of sea lice

(*Lepeophtheirus salmonis* Kroyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar*L.). *Fish and Shellfish Immunology*. **10(1)**: 47-59.

Mustafa, A., Speare, D.J., Daley, J., Conboy, G.A., Burka, J.F. 2000b. Enhanced susceptibility of seawater cultured rainbow trout, *Onchorhynchus mykiss* (Walbaum), to the microsporidian *Loma salmonae* during a primary infection with the sea louse, *Lepeophtheirus salmonis*. *Journal of Fish Diseases*. **23(5)**: 337-341.

Nagakawara, A., de Saints, N.M., Nogueira, N., Nathan, C.F. 1982. Lymphokines enhance the capacity of human monocytes to secrete reactive oxygen intermediates. *Journal of Clinical Investigations*. **70**: 1042-1048.

Nakao, M. and Yano, T. 1998. Structural and functional identification of complement components of the bony fish, carp (*Cyprinus carpio*). *Immunological Reviews*. **166**: 27-38.

Nakayasu, C., Tsutsumi, N., Oseko, N., Hasegawa, S. 2005. Role of cellular response in elimination of the monogenean *Neoheterobothrium hirame* in Japanese flounder *Paralichthys olivaceus. Diseases of Aquatic Organisms*. **64**: 127-134.

Nathan, C.F., Silverstein, S.C., Brukner, L.H., Cohn, Z.A. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II hydrogen peroxide as a mediator of cytotoxicity. *Journal of Experimental Medicine*. **149**: 100-113.

Nathan, C.F., Murray, H.W., Wiebe, M.E., Rubin, B.Y. 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *Journal of Expreimental Medicine*. **158**: 670-689.

Nauseef, W.M., Volpp, B.D., McCormick, S., Leidal, K.G., Clark, R.A. 1991. Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *Journal of Biological Chemistry*. **266(9)**: 5911-5917.

Neumann, N.F. and Belosevic, M. 1996. Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). *Developmental and Comparative Immunology*. **20(6)**: 427-439.

Neumann, N.F., Fagan, D., Belosevic, M. 1995. Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. *Developmental and Comparative Immunology*. **19(6)**: 473-482.

Neumann, N.F., Stafford, J.L., Belosevic, M. 2000. Biochemical and functional characterisation of macrophage stimulating factors secreted by mitogen-induced goldfish kidney leukocytes. *Fish and Shellfish Immunology*. **101(2)**: 167-186.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M. 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental and Comparative Immunology*. **25(8-9)**: 807-825.

Nielsen, M.E. and Buchmann, K. 1997. Glutathione-s-transferase is an important antigen in the eel nematode *Anguillicola crassus. Journal of Helminthology*. **71**: 319-324.

Nikoskelainen, S.,Kjellsen, O.,Lilius, E.-M., and Schroder, M. B. 2006 . Respiratory burst activity of Atlantic cod (*Gadus morhua* L.) blood phagocytes differs markedly from that of rainbow trout. *Fish & Shellfish Immunology*. **21**: 199-208.

Nolan, D.T., Reilly, P., Wendelaar Bonga, S.E. 1999. Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in postmolt Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*. **56(6)**: 947-959.

Nolan, D.T., Ruane, N.M., van der Heijden, Y., Quabius, E.S., Bonga, S.E.W. 2000. Juvenile *Lepeophtheirus salmonis* (Kroyer) affect the skin and gills of rainbow trout *Oncorhynchus mykiss* (Walbaum) and the host response to a handling procedure. *Aquaculture Research*. **31(11)**: 823-833.

Nonaka, M. and Smith, S.L. 2000. Complement system of bony and cartilaginous fish. *Fish and Shellfish Immunology*. **10(3)**: 215-228.

Nunn, M.A., Sharma, A., Paesen, G.C., Adamson, S., Lissina, O., Willis, A.C., Nuttall, P.A. 2005. Complement inhibitor of C5 activation from the soft tick *Ornithodoros moubata. Journal of Immunology*. **174**: 2084-2091.

Oaks, J.F., McSwain, J.L, Bantle, J.A., Essenberg, R.C., Sauer, J.R. 1991. Putative new expression of genes in ixodid tick salivary gland development during feeding. *Journal of Parasitology*. **77(3)**: 378-383.

Oda, T., Kojima, Y., Akaike, T., Ijiri, S., Molla, A., Maeda, H. 1990. Inactivation of chemotactic activity of C5a by the serratial 56-kiloDalton protease. *Infection and Immunity*. **58(5)**: 1269-1272.

O'Neill, S.M., Mills, K.H.G., Dalton, J.P. 2001. *Fasciola hepatica* cathepsin L cysteine proteinase suppresses *Bordetella pertussis*-specific interferon-gamma production *in vivo. Parasite Immunology*. **23(10)**: 541-547.

Ortuno, J., Esteban, M.A., Meseguer, J. 2001. Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innat eimmune response. *Fish and Shellfish Immunology*. **11(2)**: 187-197.

Panikkar, N.K. and Sproston, N.G. 1941. Osmotic relations of some metazoan parasites. *Parasitology*. **33**: 214-223.

Papatheodorou, V. and Brossard, M. 1987. C3 levels in the sera of rabbits infested and reinfested with *Ixodes ricinus* L. and in midguts of fed ticks. *Experimental and Applied Acarology*. **3**: 53-59.

Paperna, I. and Zwerner, D.E. 1982. Host-parasite relationship of *Ergasilus labracid* Kroyer (Cyclopidea, Ergasilidae) and the striped bass, *Morone saxatilis* (Walbaum) from the lower Chesapeake Bay. *Annales de Parasitologie Humaine et Comparee*. **57(4)**: 393-405.

Park, T.S. 1966. The biology of a calanoid copepod, *Epilabidocerca amphitrites* McMurrish. *La Cellule*. **66**: 129-251.

Pedashenko, D.D. 1898. Die Embryonalentwickelung und Metamorphose von *Lernaea branchialis. Travaux de la Societe Imperiale des Naturalistes de St. Petersbourg* **26**: 247-307.

Peddie, S., Zou, J., Secombes, C.J. 2002. A biologically active IL-1B derived peptide stimulates phagocytosis and bacterial activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), head kidney leucocytes *in vitro. Journal of Fish Diseases*. **25(6)**: 351-360.

Perkins, P.S. (1994). Ultrastructure of the holdfast of *Phrixocephalus cincinnatus* (Wilson), a blood-feeding parasitic copepod of flatfishes. *Journal of Parasitology*. **80(5)**: 797-804.

Persson, A., Stet, R.J.M., Pilstrom, L. 1999. Characterisation of MHC class I and B2-microglobulin sequences in Atlantic cod reveals an unusually high number of expressed class I genes. *Immunogenetics*. **50(1-2)**: 49-59.

Pettersen, E.F., Bjerknes, R., Wergeland, H.I. 2000. Studies of Atlantic salmon (*Salmo salar* L.) blood, spleen and head kidney leucocytes using specific monoclonal antibodies, immunohistochemistry and flow cytometry. *Fish and Shellfish Immunology*. **10(8)**: 695-710.

Pettersen, E.F., Ulvenes, M., Melingen, G.O., Wergeland, H.I. 2003. Peripheral blood and head kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and seawater transfer of Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunulogy.* **15(5)**: 373-385.

Peveri, P., Walz, A., Dewald, B., Baggiolini, M. 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *Journal of Experimental Medicine*. **167**: 1547.

Pike, A.W., MacKenzie, K., Rowand, A. 1993. Ultrastructure of the frontal filament in chalimus larvae of *Caligus elongatus* and *Lepeophtheirus salmonis* from Atlantic salmon, *Salmo salar*. In: *Pathogens of Wild and Farmed Fish: sea lice*. Eds. G.A. Boxshall and D. Defaye. Ellis Horwood Limited, Chichester, UK.

Pilström, L. and Petersson, A. 1991. Isolation and partial characterisation of immunoglobulin from cod (*Gadus morhua* L.). *Developmental and Comparative Immunology*. **15(3)**: 143-152.

Pilström, L., Warr, G.W., and Strömberg, S. 2005. Why is the antibody response of Atlantic cod so poor? The search for a genetic explanation. *Fisheries Science*. **71**: 961-971.

Pinge-Filho, P., Tadokoro, C.E., Abrahamsohn, I.A. 1999. Prostaglandins mediate suppression of lymphocyte proliferation and cytokine synthesis in acute *Trypanosoma cruzi* infection. *Cellular Immunology*. **193(1)**: 90-98.

Piraret, N., Katagiri, T., Maita, M., Endo, M., Sailasuta, A. 2007. Distribution of *Edwardsiella tarda* antigens and IgM containing cells in tilapia immune organs during septacaemia: an immunohistochemical study. *TJVM*. **38(2)**: 45-52.

Plouffe, D.A. and Belosevic, M. 2004. Enzyme treatment of *Trypanosoma danilewskyi* (Laveran & Mesnil) increases its susceptibility to lysis by the alternative complement pathway of goldfish, *Carassius auratus* (L.). *Journal of Fish Diseases*. **27(5)**: 277-285.

Polzer, M. and Taraschewski, H. 1993. Identification and characterisation of the proteolytic enzymes in the developmental stages of the eel-pathogenic nematode *Anguillicola crassus. Parasitology Research*. **79(1)**: 24-27.

Pressley, M.E., Phelan, P.E., Witten, P.E., Mellon, M.T., Kim, C.H. 2005. Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish. *Developmental and Comparative Immunology*. **29(6)**: 501-513.

Puentes, S.M., Sacks, D.L., and Joiner, K.A. 1987. Complement binding to two developmentally distinct stages of *Leishmania major* promastigotes. *Complement*. **4**: 215.

Purcell, M.K., Kurath, G., Garver, K.A., Herwig, R.P., Winton, J.R. 2004. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish and Shellfish Immunology*. **17**: 447-462.

Ramachandra, R.N. and Wikel, S.K. 1992. Modulation of host immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. *Journal of Medical Entomology* **29**: 818-826.

Ramos, A., Remedi, M.S., Sanchez, C., Bonacci, G., Vides, M.A., Chiabrando, G. 1997. Inhibitory effects of human a2-macroglobulin on *Trypanosoma cruzi* epimastigote proteinases. *Acta Tropica*. **68(3)**: 327-337.

Ramsay, J.M., Speare, D.J., Dawe, S.C., Kent, M.L. 2002. Xenoma formation during microsporidial gill disease of salmonids caused by *Loma salmonae* is affected by host species (*Oncorhynchus tshawtscha, O. kisutch, O. mykiss*) but not by salinity. *Diseases of Aquatic Organisms*. **48**: 125-131.

Rasmussen, K.R. and Kemp, W.M. 1987. *Schistosoma mansoni*: interactions of adult parasites with the complement system. *Parasite Immunology*. **9(2)**: 235-248.

Reed, S.L., Ember, J.A., Herdman, D.S., DiScipio, R.G., Hugli, T.E., Gigli, I. 1995. The extracellular neutral cysteine proteinase of *Entamoeba histolytica* degrades anaphylatoxins C3a and C5a. *Journal of Immunology*. **155(1)**: 226-274.

Reite, O.B. and Evensen, Ø. 2006. Inflammatory cells of teleostean fish: A review focusing on mast cells / eosinophilic granule cells and rodlet cells. *Fish and Shellfish Immunology*. **20**: 192-208.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology*. **17**: 208.

Ribeiro, J.M.C. 1987. Role of saliva in blood-feeding by arthropods. *Annual Review of Entomology*. **32**: 463-478.

Ribeiro, J.M.C., Makoul, G.T., Levine, J., Robinson, D.R., and Spielman, A. 1985. Anti-hemostatic, anti-inflammatory, and immunosuppresive properties of the saliva of a tick, *Ixodes dammini*. *Journal of Experimental Medicine*. **161**: 332-344.

Ribeiro, J.M., Weis, J.J., Telford, S.R. 1990. Saliva of the tick *Ixodes dammini* inhibits neutrophil function. *Experimental Parasitology*. **70(4)**: 382-8.

Ribeiro, J.M.C., Evans, P.M., McSwain, J.L., and Sauer, J.R. 1992. *Amblyomma americanum*: Characterisation of salivary prostaglandin E2 and F2α by RP-HPLC and gas chromatography mass spectrometry. *Experimental Parasitology*. **74:** 112-116.

Richards, D.T., Hoole, D., Lewis, J.W., Ewens, E., Arme, C. 1994a. Changes in the cellular composition of the spleen and pronephros of carp *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae). *Diseaseas of Aquatic Organisms*. **19(3)**: 173-179.

Richards, D.T., Hoole, D., Lewis, J.W., Ewens, E., Arme, C. 1994b. Ultrastructural observations on the cellular response of

carp, *Cyprinus carpio* L., to eggs of the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae). *Journal of Fish Diseases*. **17(5)**: 439-443.

Richards, D.T., Hoole, D., Lewis, J.W., Ewens, E., Arme, C. 1996. *In vitro* polarization of carp leucocytes in response to the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae). *Parasitology*. **112(5)**: 509-13.

Richardson, M. P.,Ayliffe, M. J.,Helbert, M., and Davies, E. G. 1998. A simple flow cytometry assay using dihydrorhodamine for the measurement of the neutrophil respiratory burst in whole blood: Comparison with the quantitative nitrobluetetrazolium test. *Journal of Immunological Methods*. **219**: 187-193.

Rimstad, E., Falk, K., Mikalsen, A.B., Teig, A. 1999. Time course tissue distribution of infectious salmon anaemia virus in experimentallyinfected Atlantic salmon *Salmo salar. Diseases of Aquatic Organisms*. **36**: 107-112.

Roberts, M.L., Lewis, J.W., Wiegertjes, G.F., Hoole, D. 2005. Interaction between the blood fluke, *Sanguinicola inermis* and humoral components of the immune response of carp, *Cyprinus carpio*. *Parasitology*. **131**: 261-271.

Rokicki, J. 1988. Anatomical and functional effects of the parasitic copepod *Lernaeocera branchialis* (L.) on *Pollachius pollachius* (L.) (Pisces: Gadidae). *Acta Ichthyologica et Piscatoria*. **Vol. XVIII** Fasc.2: 73-79.

Rombout, J., Taverne, N., Van de Kamp, M., Taverne-Thiele, A.J. 1993. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology*. **17(4)**: 309-317.

Ronneseth, A.,Wergeland, H. I., and Pettersen, E. F. 2007. Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). *Fish & Shellfish Immunology*. **23**: 493-503.

Roper, J., Grayson, T.H., Jenkins, P.G., Hone, J.V., Wrathmell, A.B., Russell, P.M., Harris, J.E. 1995. The immunocytochemical localisation of potential candidate vaccine antigens from the salmon louse *Lepeophtheirus salmonis* (Kroyer, 1837). *Aquaculture*. **132(3-4)**: 221-232.

Rosenkilde, M.M. and Schwartz, T.W. 2004. The chemokine system – a major regulator of angiogenesis in health and disease. *Ampis*. **112(7-8)**: 481-95.

Ross, N.W., Firth, K.J., Anping, W., Burka, J.F., Johnson, S.C. 2000. Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. *Diseases of Aquatic Organisms*. **41(1)**: 43-51.

Rotlant, J., Parra, D., Peters, R., Boshra, H., and Sunyer, J.O. 2004. Generation, purification and functional characterisation of three C3a anaphylatoxins in rainbow trout: Role in leukocyte chemotaxis and respiratory burst. *Developmental and Comparative Immunology*. **28**: 815-828.

Rowley, A.F., Hunt, T.C., Page, M., Mainwaring, G. 1988. Fish. In: *Vertebrtae Blood Cells*. Eds A.F. Rowley and N.A. Ratcliffe. New York: Cambridge University Press. pp 19-127.

Rowley, A.F., Hill, D.J., Ray, C.E., Munro, R. 1997. Haemostasis in fish – an evolutionary perspective. *Thrombosis and Haemostasis*. **77**: 227-233.

Rubio-Godoy, M., Porter, R, Tinsley, R.C. 2004. Evidence of complement-mediated killing of *Discocotyle sagittata* (Platyhelminthes, Monogenea) oncomiracidia. *Fish and Shellfish Immunology*. **17(2)**: 95-103.

Ruis, M.A.W. and Bayne, C.J. 1997. Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout. *Journal of Aquatic Animal Health*. **9**: 190-195.

Russell, D.G. 1987. The macrophage-attachment gylcoprotein gp63 id the predominant C3-acceptor site on *Leishmania mexicana* promastigotes. *European Journal of Biochemistry*. **164(1)**: 213-221.

Saeij, J.P.J., de Vries, B.J., and Wiegertjes, G.F. 2003. The immune response of carp to *Trypanoplasma borreli*: kinetics of immune gene expression and polyclonal lymphocyte activation. *Developmental and Comparative Immunology*. **27**: 859-874.

Sakai, D.K. 1983. The activation of alternative pathway by pronase, LPS and zymosan in the complement system of

rainbow trout serum. *Bulletin of the Japanese Society of Scientific Fisheries*. **49(3)**: 347-351.

Sakai, D.K. 1984. Induction of non-specific immuno-suppression in a primitive teleost, rainbow trout (*Salmo gairdneri*). *Developmental and Comparative Immunology*. **8(3)**: 569-78.

Sakai, D.K. 1992. Repertoire of complement in immunological defense mechanisms of fish. *Annual Review of Fish Diseases*. **2**: 223-247.

Salin, M.L. and Brown-Peterson, N.J. 1993. Dealing with active oxygen intermediates: A halophilic perspective. *Cellular and Molecular Life Sciences.* **49(6-7)**: 523-529.

Salte, R., Gjoen, H.M., Norberg, K., Gjedrem, T. 1993. Plasma protein levels as potential marker traits for resistance to furunculosis. *Journal of Fish Diseases.* **16(6)**: 561-568.

Sauer, J.R., Essenberg, R.C., Bowman, A.S. 2000. Salivary glands in ixodid ticks: control and mechanism of secretion. *Journal of Insect Physiology*. **46(7)**: 1069-1078.

Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasca, C., Prugnoli, D., Secombes, C.J. 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1B. *Fish and Shellfish Immunology*. **11(8)**: 711-726.

Scapini, P., Lapinet-Vera, J.A., Gasperini, S., Calzetti, F., Bazzoni, F., Cassatella, M.A. 2000. The neutrophil as a cellular source of chemokines. *Immunological Reviews*. **177**: 195-203.

Scharsack, J.P., Steinhagen, D., Kleczka, C., Schmidt, J.O., Korting, W., Michael, R.D., Leibold, W., Schuberth, H.J. 2003. Head kidney neutrophils of carp (*Cyprinus carpio* L.) are functionally modulated by the haemoflagellate *Trypanoplasma borreli. Fish and Shellfish Immunology*. **14(5)**: 389-404.

Scharsack, J.P., Kalbe, M., Derner, R., Kurtz, J., Milinski, M. 2004. Modulation of granulocyte responses in three-spined sticklebacks *Gasterosteus aculeatus* infected with the tapeworm *Schistocephalus solidus*. *Diseases of Aquatic Organisms*. **59**: 141-150.

Schram, T.A. 1979. The life history of the eye-maggot of the sprat, *Lernaeenicus sprattae* (Sowerby) (Copepoda, Lernaeoceridae). *Sarsia* **64**: 279-316.

Schreck, C.B. 1996. Immunomodulation: endogenous factors. *Fish Physiology*. **15**: 311-337.

Schroder, J.M., Mrowietz, U., Morita, E., Christophers, E. 1987. Purification and partial biochemical charcterisation of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. *Journal of Immunology*. **139(10)**: 3474-3483.

Schroder, M. B.,Espelid, S., and Jorgensen, T. O. 1992. Two serotypes of *Vibrio salmonicida* isolated from diseased cod (*Gadus morhua* L.): Virulence, immunological studies and vaccination experiments. *Fish & Shellfish Immunology*. **2**: 211-221.

Schroder, M. B.,Flano, E.,Pilstrom, L., and Jorgensen, T. O. 1998. Localisation of Ig heavy chain mRNA positive cells in Atlantic cod (*Gadus morhua* L.) tissues; identified by *in situ* hybridisation. *Fish & Shellfish Immunology*. **8**: 565-576.

Schroeder, H., Skelly, P.J., Zipfel, P.F., Losson, B., Vanderplasschen, A. 2009. Subversion of complement by haematophagous parasites. *Developmental and Comparative Immunology*. **33(1)**: 5-13.

Schuurmans Stekhoven, J.H.J. 1936. Beobachtungen zur Morphologie und Physiologie der *Lernaeocera branchialis* L. und der *Lernaeocera lusci* Bassett- Smith (Crustacea parasitica). *Zeitschrift fur Parasitenkunde* **8**: 659-698.

Schuurmans Stekhoven, J.H. and Punt, A. 1937. Weitere beitrage zur morphologie und physiologie der *Lernaeocera branchialis*. *Zeitschrift für Parasitenkunde.* **9**, 648-668.

Scott, T. 1900. Notes on some crustacean parasites of fishes. *Report of the Fishery Board of Scotland*. **19**: 144-188.

Scott, T. and Scott, A. 1913. In: The British Parasitic Copepoda **Vol. I & II**. The Ray Society, London.

Secombes, C.J. 1987. Lymphokine-release from rainbow trout leucocytes stimulated with Concanavalin A. Effects upon macrophage spreading and adherance. *Developmental and Comparative Immunology*. **11**: 513.

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

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., Zou, J. 2001. Cytokines and innate immunity of fish. *Developmental and Comparative Immunology*. *25(8-9)*: 713-723.

Seligmen, A.M., Karnovsky, M.J., Wasserkrug, H.L., Hanker, J.S. 1968. Nondroplet ultrasructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). *The Journal of Cell Biology*. **38**: 1-14.

Seligmen, A.M., Shannon, W.A., Hoshino, Y., Plapinger, R.E. 1973. Some important principles in 3,3'-diaminobenzidine ultrasructural cytochemistry. *Journal of Histochemistry and Cytochemistry*. **21(8)**: 756-758.

Seppola, M., Larsen, A.N., Steiro, K., Robertsen, B., Jensen, I. 2008. Characterisation and expression analysis of the interleukin genes, Il-1B, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). *Molecular Immunology*. **45(4)**: 887-897.

Seternes, T., Dalmo, R.A., Hoffmann, J. Bogwald, J., Zykova, S., Smedsrod, B. 2001. Scavenger-receptor-mediated endocytosis of lipopolysacharride in Atlantic cod (*Gadus morhua L.*). *The Journal of Experimental Biology*. **204**: 4055-4064.

Shariff, M. 1981. The histopathology of the eye of big head carp, Aristichthys noblis (Richardson), infested with Lernaea piscinae Harding, 1950. *Journal of Fish Diseases*. **4(2)**: 161-168.

Shariff, M., and Roberts, R.J. 1989. The experimental histopathology of *Lernaea polymorpha* Yu, 1938 infection in naïve *Aristichthys nobilis* (Richardson) and a comparison with the lesion in naturally infected clinically resistant fish. *Journal of Fish Diseases*. **12(5)**: 405-414.

Sharp, G.J.E., Pike, A.W., Secombes, C.J. 1991. Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) leukocyte interactions with metacestode stages of *Diphyllobothrium dendriticum* (Nitzsch, 1824) (Cestoda, Pseudophyllidea). *Fish and Shellfish Immunology*. **1**: 195-211.

Shephard, K.L. 1994. Functions for fish mucus. *Reviews in Fish Biology and Fisheries.* **4**: 401-429.

Sherman, K. and Wise, J.P. 1961. Incidence of the cod parasite *Lernaeocera branchialis* L. in the New England area, and its possible use as an indicator of cod populations. *Limnology and Oceanography*. **6**: 61-67.

Shields, R.J. and Goode, R.P. 1978. Host rejection of *Lernaea cyprinacea* L. (Copepoda). *Crustaceana*. **35(3)**: 301-307.

Sigh, J., Lindenstrom, T., Buchmann, K. 2004a. Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with *Ichthyophthirius multifiliis. Fish and Shellfish Immunology*. **17(1)**: 75-86.

Sigh, J., Lindenstrom, T., Buchmann, K. 2004b. The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*. **27(7)**: 409-417.

Sitja-Bobadilla, A. 2008. Living off a fish: a trade-off between parasites and the immune system. *Fish and Shellfish Immunology*. **25(4)**: 358-372.

Sitja-Bobadilla, A., Redondo, M.J., Bermudez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quioga, I., Nieto, J.M., Alveraz-Pellitero, P. 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). *Fish and Shellfish Immunology*. **21(5)**: 485-500.

Sitja-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Alveraz-Pellitero, P., Perez-Sanchez, J. 2008. Chronic exposure to the parasite *Enteromyxum leei (*Myxozoa: Myxosporea) modulates the immune repsonse and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. *Fish and Shellfish Immunology*. **24(5)**: 610-619.

Silva-Souza, A.T., Almeida, S.C., Machado, P.M. 2000. Effect of the infestation by *Lernaea cyprinacea* Linnaeus, 1758 (Copepoda, Lernaeidae) on the leococytes of *Schizodon intermedius* Garavello & Britski, 1990 (Osteichthyes, Anostomidae). *Revista Brasileira de Biologia*. **60(2)**: 217-220.

Simon, R.H., Scoggin, C.H., Patterson, D. 1981. Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. *Journal of Biological Chemistry*. **256(14)**: 7181-7186.

Slinn D.J. 1970. An infestation of adult Lernaeocera (Copepoda) on wild sole, Solea solea, kept under hatchery conditions. *Journal of the Marine Biological Association of the UK*. **50**: 787–800.

Smedsrod, B., Olsen, R., Sveinbjornsson, B. 1995. Circulating collagen is catabolized by endocytosis mainly by endothelial cells of the endocardium in cod (*Gadus morhua*). *Cell and Tissue Research*. **280(1)**: 39-48.

Smith, J.A. and Whitfield, P.J. 1988. Ultrastructural studies on the early cuticular metamorphosis of adult female *Lernaeocera branchialis* (L.) (Copepoda, Pennellidae). *Hydrobiologia.* **167**, 607-616.

Smith, J.L., Wootten, R. and Sommerville, C. 2007. The pathology of the early stages of the crustacean parasite, *Lernaeocera branchialis* (L.), on Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases* **30**: 1-11.

Sorensen, K. K.,Sveinbjornsson, B.,Dalmo, R. A.,Smedsrod, B., and Bertheussen, K. 1997. Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases*. **20**: 93-107.

Sproston, N.G. 1942. The developmental stages of *Lernaeocera branchialis. Journal of the Marine Biological Association of the UK* **25**: 441-446.

Sproston, N.G. and Hartley, P.H.T. 1941. The ecology of some parasitic copepods of gadoids and other fishes. *Journal of the Marine Biological Association of the UK*. **25**: 361-392.

Srivastava, A.K. 1969. Studies on the haematology of certain freshwater teleosts. V. Thrombocytes and clotting of blood. *Anatomischer Anzeiger*. **124(4)**: 368-74.

Steinhagen, D. and Rombout, J.H. 1994. Response of Ig-positive cells to *Goussia carpelli* (Protozoa: Apicomplexa) infections in carp (*Cyprinus carpio* L.). *Folio Parasitologica*. **41(3)**: 173-6.

Steiro, K.,Johansen, A.,Gildberg, A., and Bogwald, J. 1998. Optimising of culture conditions and stimulation of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases*. **21**: 335-344.

Stenvik, J. and Jorgensen, T. O. 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. *Immunogenetics*. **51**: 452-461.

Stenvik, J., Lundback, A., Jorgensen, T.O., Pilstrom, L. 2000. Variable region diversity of the Atlantic cod (*Gadus morhua* L.) immunoglobulin heavy chain. *Immunogenetics*. **51**: 670-680.

Stenvik, J., Schrøder, M.B., Olsen, K., Zapata, A., and Jørgensen, T.Ø. 2001. Expression of immunoglobulin heavy chain transcripts (VH-families, IgM, and IgD) in head kidney and spleen of the Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology*. **25**: 291-302.

Stevenson, J.R. 1961. Polphenol oxidase in the tegumental glands in relation to the molting cycle of the isopod crustacean *Armadillidium vulgare. The Biological Bulletin*. **121**: 554-560.

Strieter, P., Polverini, P., Kunkel, S., Arenberg, D., Burdick, M., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., Chan, S., Roczniak, S., Shanafelt, A. 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *Journal of Biological Chemistry*. **270**: 27348-27357.

Sundnes, G. 1970. *Lernaeocera branchialis* (L.) on cod in Norwegian waters. Institute of Marine Research, Bergen, Norway, pp. 70.

Sundnes, G., Mork, J., Solemdal, P., and Solemdal, K. 1997. *Lernaeocera branchialis* (L., 1767) on cod in Baltic waters. *Helgolander Meeresuntersuchungen*. **51**, 191-196.

Sunyer, O. J. and Lambris, J. D. 1998. Evolution and diversity of the complement system of poikilothermic vertebrates. *Immunological Reviews*. **166**: 38-57.

Sunyer, J.O., Zarkadis, K., Sahu, A., Lambris, J.D. 1996. Multiple forms of complement C3 in trout that differ in binding to complement activators. *Proceedings of the National Academy of sciences of the USA.* 8546-8551.

Sunyer, J. O.,Tort, L., and Lambris, J. D. 1997. Diversity of the third form of complement, C3, in fish: Functional characterization of five forms of C3 in the diploid fish *Sparus aurata. Biochemical Journal (London)*. **326**: 877-881.

Sunyer, J.O., Boshra, H., Lorenzo, G., Parra, D., Freedman, B., Bosch, N. 2003. Evolution of complement as an effector system in innate and adaptive immunity. *Immunologic Research*. **27(2-3)**: 549-564.

Sunyer, J.O., Boshra, H., Li, J. 2005. Evolution of anaphylatoxins, their diversity and novel roles in innate immunity: insights from the study of fish complement. *Veterinary Immunology and Immunopathology*. **108(1-2)**: 77-89.

Swain, M. and Ross, N. W. 1995. A silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecyl sulfate-polyacrylamide gels. *Electrophoresis*. **16**: 948-951.

Tafalla, C., Coll, J., Secombes, C.J. 2005. Expression of genes related to the early immune response in rainbow trout (*Oncorhynchus mykiss*) after viral haemorrhagic septicemia virus (VHSV) infection. *Developmental and Comparative Immunology*. **29(7)**: 615-626.

Tahir, A. and Secombes, C.J. 1996. Modulation of dab (*Limanda limanda* L.) macrophage respiratory burst activity. *Fish and Shellfish Immunology*. **6**: 135-146.

Tatchell, R.J. 1967. Salivary secretion in the cattle tick as a means of water elimination. *Nature, London*. **213**: 940-941.

Templeman, W., Hooder, V.M., and Fleming, A.M. 1976. Infection of lumpfish *(Cyclopterus lumpus*) with larvae and of Atlantic cod (*Gadus morhua*) with adults of the copepod, *Lernaeocera branchialis*, in and adjacent to the Newfoundland area, and inferences therefrom on inshore-offshore migrations of cod. *Journal of the Fisheries Research Board of Canada.* **33**: 711-731.

Terasaki, M.F., Fujiwara, K. 1992. Fluorescent labeling of membranes: Method for ER staining. *Seimeinokagaku.* **43**:. 632–637.

Thelen, M., Peveri, P., Kernen, P., von Tscharner, V., Walz, A., Baggiolini, M. 1988. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. *The FASEB Journal*. **2**: 2702-2706.

Thomas, P.T. and Woo, P.T.K. 1989a. An *in vitro* study on the haemolytic components from *Cryptobia salmositica* (Sarcomastigophora: Kinetoplastida). *Journal of Fish Diseases*. **12(4)**: 389-393.

Thomas, P.T. and Woo, P.T.K. 1989b. Complement activity in *Salmo gairdneri* Richardson infected with *Cryptobia salmositica* (Sarcomastigophora: Kinetoplastida) and its relationship to the anaemia in cryptobiosis. *Journal of Fish Diseases*. **12(4)**: 395-397.

Thomson, A. 1994. The Cytokine handbook, 2nd edition. London: Academic Press.

Thoney, D.A. and Burreson, E.M. 1988. Lack of specific antibody response in *Leiostomus xanthurus* (Pisces: Sciaenidae) to parasitic copepods and monogeneans. *Journal of Parasitology*. **74(1)**:191-194.

Thuvander, A. 1989. Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. *Journal of Fish Biology*. **35(4)**: 521-529.

Tirard, C. and Raibaut, A. 1989. Quelques aspects de l'ecologie de *Lernaeocera lusci* (Basset-Smith, 1986), copepode parasite de poisons merluciidae et gadidae. *Bulletin d'Ecologie*. **20(4)**: 289-294.

Tirard, C., Berrebi, P., Raibaut, A., and Renaud, F. 1993. Biodiversity and biogeography in heterospecific teleostean (Gadidae) - copepod (*Lernaeocera*) associations. *Canadian Journal of Zoology*. **71**, 1639-1645.

Titus, R.G., Bishops, J.V., Mejia, J.S. 2006. The immunomodulatory factors of arthropod saliva and the potential for these factors to serve as vaccine targets to prevent pathogen transmission. *Parasite Immunology*. **28(4)**: 131-141.

Tort, L., Sunyer, J.O., Gomez, E., Molinero, A. 1996. Crowding stress induces changes in serum haemolytic and agglutinating activity in the gilthead sea bream *Sparus aurata*. *Veterinary Immunology and Immunopathology*. **51(1-2)**: 179-188.

Tort, J., Brindley, P.J., Knox, D., Wolfe, K.H., Dalton, J.P. 1999. Proteinases and associated genes of parasitic helminths. *Advances in Parasitology*. **43**: 161-266.

Tyson, K., Elkins, C., Patterson, H., Fikrig, E., de Silva, A. 2007. Biochemical and functional characterisation of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Molecular Biology*. **16(4)**: 469-479.

Urioste, S., Hall, L.E., Telford III, S.R., and Titus, R. 1994. Saliva of the Lyme disease vector, *Ixodes dammini*, blocks cell activation by a non-prostaglandin E2-dependent mechanism. *Journal of Experimental Medicine*. **180**: 1077-1085.

Vallejo, A.N., Miller, N.W., Clem, L.W. 1992. Antigen processing and presentation in teleost immune responses. *Annual Review of Fish Diseases*. **2**: 73-89.

Van Banning, P. 1974. Two remarkable infestations by *Lernaeocera spp.* (Copepoda parasitica). *Journal du Conseil*. **35(2)**: 205-206.

Van Damme, P.A. and Hamerlynck, O. 1992. The infection dynamics and dispersion pattern of *Lernaeocera branchialis* L. on 0+ whiting (*Merlangius merlangus* L.) in the Oosterschelde (SW Netherlands). *Journal of Fish Biology*. **41**: 265-275.

Van Damme, P.A. and Ollevier, F. 1995. Morphological and morphometric study of crustacean parasites within the genus *Lernaeocera*. *International Journal for Parasitology* **25**: 1401-1411

Van Damme, P.A. and Ollevier, F. 1996. The population dynamics of *Lernaeocera lusci* and *L. branchialis* on intermediate hosts. *Helgoland Marine Research*. **50(2)**: 177-190.

Van Damme, P.A., Ollevier, F., and Hamerlynck, O. 1994. Pathogenicity of *Lernaeocera lusci* and *L. branchialis* in bib and whiting in the North Sea. *Diseases of Aquatic Organisms*. **19**: 61-65.

Van Damme, P. A., Geets, A. and Hamerlynck, O. 1997. The suprapopulation dynamics of *Lernaeocera branchialis* and *L. lusci* in the Oosterschelde: seasonal abundance on three definitive host species. *ICES Journal of Marine Science* **54**: 24-31.

Van den Broek, W.L.F. 1978. The effects of *Lernaeocera branchialis* on the *Merlangius merlangus* population in the Medway estuary. *Journal of Fish Biology* **13**, 709-715

Van den Broek, W.L.F. 1979. Infection of estuarine populations by *Lernaeocera branchialis* and its effect on whiting *Merlangius merlangus*. *New Zealand Journal of Zoology* **6**: 646-647.

Von Vaupel Klein, J.C. and Koomen, P. 1994. The possible origin of mucus jets used for immobilizing prey in species of *Euchirella* (Copepoda, Calanoida, Aetideidae). I. Theoretical considerations in relation to swimming and feeding behaviour. *Crustaceana*. **66(2)**: 184-204.

Wagner, G.N., Fast, M.D., Johnson, S.C. 2008. Physiology and immunology of *Lepeophtheirus salmonis* infections of salmonids. *Trends in Parasitology*. **24(4)**: 176-183.

Wang, H.. and Nuttall, P.A. 1995. Immunoglobulin-G binding proteins in the ixodid ticks, *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus. Parasitology*. **111(2)**: 161-165.

Wang, R., Neumann, N.F., Shen, Q., Belosevic, M. 1995. Establishment and characterisation of a macrophage cell line from the goldfish. *Fish and Shellfish Immunology*. **5(5)**: 329-346.

Wang, H., Henbest, P.J., Nuttall, P.A. 1999. Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by re-programming of salivary gland protein expression. *Parasitology*. **119**: 143-149.

Wehnert, S.D. and Woo, P.T. 1980. *In vivo* and *in vitro* studies on the host specificity of *Trypanoplasma salmonositica*. *Journal of Wildlife Diseases.* **16(2)**: 183-187.

Wells, P.R. and Cone, D.K. 1990. Experimental studies on the effect of *Gyrodactylus colemanensis* and *G. salmonis* (Monogenea) on density of mucous cells in the epidermis of fry of *Oncorhynchus mykiss. Journal of Fish Biology.* **37(4)**: 599-603.

Wester, J., Sixma, J.J., Geuze, J.J., Heijnen, H.F.G. 1979. Morphology of the hemostatic plug in human skin wounds. Trasformation of the plug. *Lab. Invest.* **41**: 182.

Whitfield, P.J., Pilcher, M.W., Grant, H.J., and Riley, J. 1988 Experimental studies on the development of *Lernaeocera branchialis* (Copepoda: Pennellidae): population processes from egg production to maturation on the flatfish host. *Hydrobiologia*. **167**: 579-586.

Whittington, I.D. and Cribb, B.W. 1998. Glands associated with the anterior adhesive areas of the monogeneans, *Entobdella sp*. and *Entobdella australis* (Capsalidae) from the skin of *Himantura fai* and *Taeniura lymma* (Dasyatidae). *International Journal for Parasitology*. **28**: 653-665.

Whyte, S.K., Chappell, L.H., Secombes, C.J. 1989. Cytotoxic reactions of rainbow trout, *Salmo gairdneri* Richardson, macrophages for larvae of the eye fluke *Diplostomum spathaceum* (Digenea). *Journal of Fish Biology*. **35(3)**: 333-345.

Widholm, Lundback, A., Daggfeldt, A., Magnadottir, B., Warr, G.W. Pilstrom, L. 1999. Light chain variable region diversity in Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology*. **23(3)**: 231-240.

Wiegertjes, G.F., Yano, T., van Muiswinkel, W.B. 1993. Estimation of the genetic variation in complement activity in common carp (*Cyprinus carpio* L.). *Veterinary Immunology and Immunopathology*. **37(3-4)**: 309-19.

Wikel, S.K. 1996. Host immunity to ticks. *Annual Review of Entomology*. **41**: 1-22.

Wikel, S.K., Ramachandra, R.N., Bergman, D.K. 1994. Tick-induced modulation of the host immune response. *International Journal of Parasitology*. **24(1)**: 59-66.

Williams, K.I., Higgs, G.A. 1988. Eicosainoids and inflammation. *Journal of Pathology*. **156(2)**: 101-110.

Williamson, A.L., Lustigman, S., Oksov, Y., Deumic, V., Plieskatt, J., Mendez, S., Zhan, B., Bottazzi, M.E., Hotez, P.J., Loukas, A.. 2006. *Ancylostoma caninum* MTP-1, an Astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. *Infection and Immunity*. **74(2)**: 961-967.

Wilson, C.B. 1917. North American parasitic copepods belonging to Lernaeidae, with a revision of the entire family. *Proceedings of the United States National Museum* **53**: 1-150.

Wilson, M.,Bengten, E.,Miller, N. W.,Clem, W.,Du Pasquier, L., and Warr, G. W. 1997. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proceedings of the National Academy of Sciences of the United States of America*. **94**: 4593-4597.

Wolfson, M., McPhail, L.C., Nasrallah, V.N., Snyderman, R. 1985. Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: potential role in the activation of the respiratory burst enzyme. *The Journal of Immunology*. **135(3)**: 2057-2062.

Woo, P.T.K. and Shariff, M. 1990. *Lernea cyprinacea* L. (Copepoda: Caligidae) in *Helostoma temmincki* Cuvier & Valenciennes: the dynamics of resistance in recovered and naïve fish. *Journal of Fish Diseases*. **13(6)**: 485-493.

Xu, Y., Bruno, J.F., Luft, B.J. 2005. Identification of novel tick salivary gland proteins for vaccine development. *Biochemical and Biophysical Research Communications*. **326(4)**: 901-904.

Yano, T. 1992. Assays of hemolytic complement activity. In: *Techniques in Fish Immunology*. (eds. J.S. Stolen, T.C. Fletcher, D.P. Anderson, S.L. Kaatari, A.F. Rowley. SOS Publications, Fairhaven, NJ.

Zarkadis, I.K., Mastellos, D., and Lambris, J.D. 2001. Phylogenetic aspects of the complement system. *Developmental Comparative Immunology*. **25**: 745-762.

Zeni, C. and Zaffagnini, F. 1992. Labral glands of *Leptestheria dahalacensis* (Branchiopoda: Spinicaudata); an ultrastructural study. *Journal of Crustacean Biology*. **12(4)**: 661-676.

Zhao, X., Findly, R.C., Dickerson, H.W. 2008. Cutaneous antibody-secreting cells and B cells in a teleost fish. *Developmental and Comparative Immunology*. **32(5):** 500-508.

Zhu, K., Sauer, J.R., Bowman, A.S., and Dillwith, J.W. 1997. Identification and characterisation of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). *Journal of Parasitology*. **83**: 38-43.

Zipfel, P.F., Wurzner, R, Skerka, C. 2007. Complement evasion of pathogens: common strategies are shared by diverse organisms. *Molecular Immunology*. **44(16)**: 3850-3857.

Ziskowski, J., Mercaldo-Allen, R., Pereira, J.J., Kuropat, C., Goldberg, R. 2008. The effects of fin rot disease and sampling method on blood chemsitry and haematocrit measurements of winter flounder, *Pseudopleuronectes americanus* from New Haven Harbor (1987-1990). *Marine Pollution Bulletin*. **56(4)**: 740-750.

Zong, W.X. and Thompson, C.B. 2006. Necrotic death as cell fate. *Genes and Development*. **20**: 1-15

Zuo, X. and Woo, P.T.K. 1997a. *In vivo* neutralisation of *Cryptobia salmositica* metallo-protease by a-2-macroglobulin in the blood of rainbow trout, *Oncorhynchus mykiss* and in brook char, *Salvelinus fontinalis. Diseases of Aquatic Organisms*. **29**: 67-72.

Zuo, X. and Woo, P.T.K. 1997b. Natural antiproteases in rainbow trout, *Oncorhynchus mykiss* and brook char, *Salvelinus fontinalis*, in the *in vitro* neutralisation of fish alpha-2-macroglobulin by the metalloprotease of the pathogenic haemoflagellate, *Cryptobia salmositica. Parasitology*. **114**: 375-382.

Zou, J., Cunningham, C., Secombes, C.J. 1999. The rainbow trout *Oncorhynchus mykiss* interleukin-1B gene has a different organization to mammals and undergous incomplete splicing. *European Journal of Biochemistry*. **259(3)**: 901-908.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W., Chinchar, V.G. 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). *Developmental and Comparative Immunology*. **27(10)**: 845-858.

Appendix 1

Buffers

ELISA buffers

10x High salt wash buffer

Trisma base (Sigma)	0.2M
Sodium chloride (Sigma)	5M
Tween®20 (Sigma)	5ml

Dissolve in 400ml distilled water, pH to 7.7 with HCl and make up to 500ml. Dilute 1:10 before use.

10x Low salt wash buffer

Trisma base	0.2M
Sodium chloride	3.8M
Tween®20	0.5%

Dissolve in 400ml distilled water, pH to 7.3 with HCl and make up to 500ml. Dilute 1:10 before use.

Substrate buffer

Citric acid (Sigma)	0.055M
Sodium acetate (Sigma)	0.05M

Dissolve in 80ml distilled water, pH to 5.4 and make up to 100ml.

Fixatives

10% Neutral buffered saline (10% NBF)

Sodium dihydrogen phosphate (monohydrate; VWR)	0.029M
Disodium hydrogen phosphate (anhydrous; VWR)	0.046M

Formaldehyde (Sigma)	100ml
distilled water	make up to 1 litre

Davidsons alcohol / formaldehyde / acid fixative (Davidsons AFA fixative)

95% ethanol	330ml
Formaldehyde	220ml
Glacial acetic acid (VWR)	115ml
distilled water or filtered seawater	335ml

General buffers

Phosphate buffered saline, pH 7.4 (PBS)

NaH_2PO_4 (VWR)	0.007M
Na_2HPO_4 (VWR)	0.018M
Sodium chloride	0.150M

Dissolve in 400ml distilled water, pH to 7.4, make up to 500ml and autoclave.

Tris buffered saline (TBS), pH 7.6

Trisma base	10mM
Sodium chloride	0.5M

Dissolve in 400ml distilled water, pH to 7.2-7.6 and make up to 500ml.

In situ hybridisation buffers

Phosphate buffered saline (PBS), pH 7.4

Sodium chloride	0.14M
Potassium chloride (Sigma)	0.003M
Na_2HPO_4	0.008M

KH_2PO_4 (Sigma)	0.0015M

pH to 7.4. DEPC-treat overnight and autoclave twice.

Proteinase K buffer

Tris-HCl, pH 7.5 (RNAse-free)	50mM
EDTA, pH 8.0 (RNAse-free; Sigma)	5mM

Made up in nanopure water.

Hybridisation buffer

Deionised formamide RNAse-free (Sigma)	5ml
50% Dextran sulphate RNAse-free (Sigma)	2ml
Sheared salmon sperm DNA (RNAse-free; Sigma)	500µg
10X salt solution (RNAse-free)	1ml
500mM Tris-HCl, pH 7.5 (RNAse-free)	200µl
50x Dendhardt's solution	200µl

Make up to 10ml with DEPC-treated water, aliquot and store at -20°C.

50x Dendhardt's solution

Polyvinylpyrrolidone (PVP; molecular grade; Sigma)	1%
Ficoll (molecular grade; Sigma)	1%
Bovine serum albumin (molecular grade; Sigma)	1%
DEPC-treated water	5ml

Aliquot and store at -20°C.

10x salt solution

NaCl (molecular grade)	3M
Na_2HPO_4 (RNAse-free)	100mM

EDTA (molecular grade)	50mM
Tris-HCl, pH 7.5 (RNAse-free)	100mM

Make up to 500ml with nanopure water.

20x SSC

NaCl	3M
Sodium citrate (Sigma)	0.3M

Made up to 800ml with distilled water, pH to 7.4 and make up to 1L with distilled water.

RNAse buffer

Trisma base	10mM
EDTA	1mM
Sodium chloride	0.5M

Add 800ml distilled water, pH 7.5 and make up to 1L.

Tris-buffered saline (TBS)

Trisma base	0.05M
Sodium chloride	0.15M

Add 900ml distilled water, pH to 7.6 and make up to 1L with distilled water.

Blocking buffer for alkaline phosphatase (AP) detection

Normal goat serum (Sigma)	10%
Bovine serum albumin	0.2%

Make up to 5ml with Tris-buffered saline.

TNT wash buffer

Tris-HCl, pH 7.5	0.1M
Sodium chloride	0.15M
Tween®20	0.05%

TNB blocking buffer

Tris-HCl, pH 7.5	0.1M
Sodium chloride	0.15M
Blocking reagent (supplied in TSA kit by PerkinElmer)	0.5%

In situ hybridisation cRNA probe production

1.2% agarose gel

Agarose (molecular grade)	1.2%

Add 60ml sodium hydroxide – boric acid buffer, dissolve by heating in the microwave, allow to cool 5 min, add 5µl 1mg.ml^{-1} ethidium bromide, pour into gel mould and allow to set for atleast 40 min.

Dot blot detection buffer

Tris-HCl, pH 8.4	100mM

Dot blot blocking buffer

Skimmed milk powder	1%

Dissolved in dot blot wash buffer.

Dot blot wash buffer

Maleic acid, pH 7.5 (Sigma)	0.1M
Sodium chloride	0.15M

Dot blot RNA dilution buffer

20x SSC	3ml
Formaldehyde	2ml
DEPC-treated nanopure water	5ml

10x Sodium hydroxide – boric acid buffer

Sodium hydroxide	0.4%
Distilled water	400ml

Add Boric acid until the buffer reaches pH 8.5, make up to 500ml with distilled water and dilute 1:10 prior to use.

SDS-PAGE

SDS separating buffer

Trisma base	1.5M
SDS (Sigma)	0.4%

Dissolve in 400ml distilled water, pH to 8.8 and make up to 500ml.

SDS stacking buffer

Trisma base	0.5M
SDS	0.4%

Dissolve in 80ml distilled water, pH to 6.8 and make up to 100ml.

12% polyacrylamide separating gel

SDS separating buffer	5ml
Distilled water	6.9ml
30% acrylamide/bis acrylamide (Severn Biotech Ltd., Kidderminster, UK)	8ml

De-gas for 15 min

TEMED (Sigma)	10µl
Ammonium persulphate (100mg.ml^{-1} in distilled water; Sigma)	100µl

Set in gel caste for 1 h.

4% stacking gel

SDS stacking buffer	2.5ml
distilled water	6.1ml
30% acrylamide/bis acrylamide (Severn Biotech Ltd., Kidderminster, UK)	1.3ml

De-gas for 15 min

TEMED	10µl
Ammonium persulphate (100mg.ml^{-1} in distilled water)	50µl

Set in gel caste for on top of separating gel with well comb for 1 h.

5x SDS running buffer

Trisma base	0.124M
Glycine (Sigma)	0.96M
SDS	0.5%

Dissolve in 400ml distilled water, pH to 8.3 and make up to 500ml. Dilute 1:5 prior to use.

Stains

1% (w/v) alcian blue, pH 2.5

Alcian blue	1%
Glacial acetic acid	3ml

Make up to 100ml with distilled water and filter.

1% acid alcohol

Methylated spirits	1980ml
Hydrochloric acid	20ml

Mayer's Haematoxylin

Haematoxylin	0.1%
Sodium iodate	0.02%
Potassium alum	5%
Citric acid	0.1%
Chloral hydrate	5%
Distilled water	2L

Allow haematoxylin, potassium alum and sodium iodate to dissolve in distilled water overnight. Add chloral hydrate and citric acid and boil for 5 min.

Eosin

1% Eosin	240ml
Putt's Eosin	80ml

1% Eosin

Eosin yellowish	1%

Pre-dissolve in 600ml distilled water and then make up to 2L.

Putt's Eosin

Eosin yellowish	1%
Potassium dichromate	0.5%
Saturated aqueous picric acid	40ml
Absolute alcohol	40ml

Distilled water	320ml

Dissolve eosin and potassium dichromate in the ethanol, add the water and then the picric acid.

Scott's tap water substitute

Sodium bicarbonate	0.042M
Magnesium sulphate	0.166M
Tap water	1L

Dissolve by heating if necessary and add a few thymol crystals to preserve.

Method for sodium sulphate precipitation of serum proteins

Add 14% (w/v) sodium sulphate (Sigma) to 2ml serum at 25°C. Incubate in water bath at 25°C for 5 min followed by centrifugation at 13 000 xg for 15 min at room temperature. Remove and dispose of the supernatant, and wash the precipitate twice in 14% (w/v) sodium sulphate warmed to 25°C with centrifugation. Dissolve the precipitated proteins in 2ml PBS at 25°C, ready for use.

Add 14% (w/v) sodium sulphate (Sigma) to 2ml serum at 25°C. Incubate in water bath at 25°C for 5 min followed by centrifugation at 13 000 xg for 15 min at room temperature. Remove and dispose of the supernatant, and wash the precipitate twice in 14% (w/v) sodium sulphate warmed to 25°C with centrifugation. Dissolve the precipitated proteins in 2ml PBS at 25°C, ready for use.