Thesis
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Piscirickettsia salmonis: characterisation, infection and immune response in salmonid fish

Thesis submitted for the degree of
Doctor of Philosophy

by

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June 2005

05/06

Dedication

This thesis is dedicated to the memory of

Denis Brereton,

the first person to call me Doc.

Declaration

I hereby declare that the work and results presented in this thesis were carried out by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not previously been submitted for any other degree or qualification. All information from other sources has been acknowledged.

Úna McCarthy

Acknowledgements

An enormous thank you to my supervisors: Dr. Kimberley Thompson for her kindness, guidance, encouragement and, most of all, her humanity, for which I will be eternally grateful (bless you); Dr. Tony Ellis for very many useful discussions about results, life and the universe; Prof. Alexandra Adams for arranging this project and seeming never to be phased by any 'catastrophes'.

Prof. Patrick Smith (Aquaculture Vaccines Ltd.– now Schering Plough Aquaculture) very generously provided the industrial sponsorship for this project and was encouraging and supportive throughout the entire project. I wish also to thank the University of Stirling for providing the other half of my funding, through a CASE studentship.

Fiona Muir and Jacqueline Ireland adopted both *Piscirickettsia salmonis* and me and thoroughly, efficiently and diligently introduced us to the ways of the Virology Laboratory. Along the way we encountered big green stripes and six nationalities with hilarious consequences – thank you so much for a fabulous three years!

Of course, no fish, no project! Thank you Niall for always managing to find fish, sometimes at very short notice. Hope all the t's are crossed and the tanks are clean?

Mr. Linton Brown deserves a medal for his patience – without his hard work I would never had such good and plentiful grids for TEM. It was also due to his observation that the 'typo' in Section 3.2.7 was detected almost at the outset.

Dr. James Bron sacrificed lunches and evenings to provide me with the confocal microscope service and, together with Dr. Andy Shinn, was always offering assistance and ideas which are much appreciated.

Drs. William Starkey, John Taggart and Michael Leaver were an invaluable source of help and advice – way beyond the call of duty – and I am very grateful to them.

Prof. Hugh Ferguson provided expert analysis of histological sections and I am grateful that he took time to discuss and demonstrate these interpretations for me.

The technical staff in the Institute were more than helpful and often went out of their way to be of assistance. In particular, I would like to thank Stuart Miller, Cathryn Dickson, Gillian Dreczkowski, Maureen Menzies and Debbie Faichney, Steve Powell, Ann Gilmour, Hilary McEwan and Karen Snedden. Of course, cannae forget that source of all chemicals, cars and codes – the end of F corridor! Charlie, Jane, Betty, Brian and Iain – does a body good to come in past you lot in the morning!

To Dr. Janina Zuleica de Garcia e Costa – go raibh mile, mile maith agat agus go n-eiri an t'ádh leat i rith do shaol. I'd never have survived without the Crush Hall coffees, the dancing in H25, the zebra-crossing conferences and the Portugese words of wisdom.

Thank you to Rachel Flockton for being a fantastic flatmate, for the secrets of beetroot and chickpeas and for arranging things so I got to run away to sea – *finally*!!

My parents must be relieved to know that I might now decide I've had enough Third Level education and may finally get to a stage where I give *them* financial support instead – in my non-slip sailing shoes! Sarah, good luck with yours and, officially (!!!) thank you for the 'This is your life'.

My fellow students and my flatmates – I was always glad to be coming back to Stirling because you were all here. It always felt like coming home. Thank you.

Abstract

The pathogen *Piscirickettsia salmonis*, has been isolated from all species of salmonid and has been found in Chile, Canada, Ireland, Norway and Scotland. Rickettsia-like organisms from European sea bass (*Dicentrarchus labrax*) were found to share common antigens with the *P. salmonis* type-strain, LF-89 using the indirect fluorescent antibody test (IFAT) and immunohistochemistry (IHC). In addition, the DNA sequences of the 16S rDNA and 16S-23S internal transcribed spacer region (ITS) were compared with those published for *P. salmonis* strains and showed that the sea bass piscirickettsia-like organism (SBPLO) was another strain of *P. salmonis*, closely related to the salmonid pathogens.

The ability of *P. salmonis* to survive and replicate within head kidney (HK) macrophages of rainbow trout infected *in vitro* was demonstrated using transmission electron microscopy (TEM) at various times post-infection (p.i.). However, macrophages derived from fish vaccinated against *P. salmonis* appeared to clear *in vitro* infection more rapidly than macrophages from naïve fish.

Polymerisation of filamentous actin within the cytoplasm of the host cell is used by some mammalian rickettsiae to achieve intercellular spread by actin-based motility (ABM). Both TEM and confocal microscopy were used to investigate possible actin tail formation by *P. salmonis*. No evidence of tail formation was found.

Respiratory burst (RB) by *P. salmonis* was measured following exposure of rainbow trout HK macrophages to the organisms *in vitro*. Because of background stimulation of the RB by growth media and debris from the CHSE-214 cells used to culture *P. salmonis*, it was not possible to detect any effect of the pathogen on the burst.

Schering Plough Aquaculture has developed a recombinant vaccine against *P. salmonis*. The ability of the vaccine to elicit a memory response against *P. salmonis* was investigated by measuring three different immune responses:

a) the expression of iNOS was measured by reverse transcription polymerase chain reaction (RT-PCR) to detect mRNA levels or by the Greiss reaction to quantify the end-products of nitric oxide metabolism in the serum. Increased iNOS expression was not detected in rainbow trout kidney or serum following vaccination/challenge with *P. salmonis*. However,

iNOS expression was detected in gill tissue from naïve trout which suggests that expression may be constitutive in this tissue.

b) the production of macrophage activating factor (MAF) by lymphocytes from vaccinated trout, following stimulation *in vitro* with *P. salmonis*, was measured by the ability of supernatants from these cells to prime elevated RB in naïve macrophages. No difference in priming ability between supernatants from vaccinated and non-vaccinated fish was detected. However, macrophages among the immune leukocytes used to produce the MAF supernatants did exhibit elevated RB compared with macrophages from non-immune fish, suggesting that vaccination had produced a population of lymphocytes capable of priming activation of macrophages.

c) by screening individual sera concurrently against the rickettsial and CHSE antigen preparations, the antibody response to *P. salmonis* could be detected specifically and was found to increase significantly in immunised fish by 6 weeks post-vaccination. Specificity of the response was demonstrated by screening the sera against *Aeromonas salmonicida.*

List of abbreviations

2ME	: 2 mercaptoethanol
Ab	: antibody
ABM	: actin-based motility
ANOVA	: analysis of variance
APC	: antigen presenting cell
ATCC	: American type culture collection
bp	: base pair
BF-2	: bluegill fry (cell line)
BSA	: bovine serum albumin
CHH-1	: Chum salmon heart (cell line)
CHSE-214	: Chinook salmon embryo (cell line)
CSE-119	: Coho salmon embryo (cell line)
CSF	: colony stimulating factor
CMI	: cell-mediated immunity
Con A	: concanavalin A
CPE	: cytopathic effect
CTL	: cytotoxic T-lymphocyte
d	: day
DAB	: 3,3'-diaminobenzidine tetrahydrochloride
dH_2O	: distilled water
DMSO	: dimethylsulphoxide
DNA	: deoxyribonucleic acid
ELISA	: enzyme-linked immunosorbent assay
EMEM	: minimal essential medium with Earle's salts
eNOS	: endothelial nitric oxide synthase
ECP	: extracellular product
EPC	: epithelioma papulosum cyprini (cell line)

FAD	: flavine adenine dinucleotide
FBS	: foetal bovine serum
FCA	: Freund's complete adjuvant
FHM	: fathead minnow (cell line)
FIA	: Freund's incomplete adjuvant
FITC	: fluorescein isothiocyanate
GAPDH	: glyceraldehyde-3-phosphate dehydrogenase
H_2O_2	: hydrogen peroxide
HK	: head kidney
HRP	: horseradish peroxidase
HSWB	: high salt wash buffer
i.p.	: intraperitoneal
IFAT	: indirect fluorescent antibody test
Ig	: immunoglobulin
IHC	: immunohistochemistry
IFN	: interferon
IL	: interleukin
iNOS	: inducible nitric oxide synthase
IROMP	: iron-regulated outer membrane protein
ITS	: internal transcribed spacer
kDa	: kilodalton
KOH	: potassium hydroxide
l	: litre
L-15	: Leibovitz-15
LPS	: lipopolysaccharide
LSWB	: low salt wash buffer
M	: molar
mAb	: monoclonal antibody
MAF	: macrophage activating factor
MEM	: minimal essential medium
MHC	: major histocompatability complex

μl	: microlitre
μm	: micrometer
mM	: millimolar
min	: minute
MLR	: mixed leukocyte reaction
MPO	: myeloperoxidase
MOI	: multiplicity of infection
mRNA	: messenger RNA
MW	: molecular weight
NADPH	: nicotinamide adenine dinucleotide phosphate
NBF	: neutral buffered formalin
NBT	: nitroblue tetrazolium
NEAA	: non-essential amino acids
N^G-MMLA	: N^G-monomethyl-L-arginine
Nm	: nanometer
nNOS	: neuronal nitric oxide synthase
NO	: nitric oxide
O_2^-	: superoxide anion
OsO_4	: osmium tetroxide
pAb	: polyclonal antibody
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PMA	: phorbol myristate acetate
PRF-HBSS	: phenol red-free Hank's balanced salt solution
RB	: respiratory burst
RLO	: rickettsia-like organism
RNA	: ribonucleic acid
RNI	: reactive nitrogen intermediate
ROI	: reactive oxygen intermediate
RT	: room temperature
RTG-2	: rainbow trout gonad (cell line)

rDNA	: DNA sequence coding for rRNA
rRNA	: ribosomal RNA
RT	: reverse transcription
RT-PCR	: reverse-transcription PCR
SBPLO	: sea bass piscirickettsia-like organism
SDS-PAGE	: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	: standard deviation
SEM	: standard error of the mean
sEMEM	: MEM supplemented with FBS, NEAA and L-glutamine
SFG	: spotted fever group
SOD	: superoxide dismutase
SRS	: salmonid rickettsial septicaemia
Sp.	: species
Spp.	: species (plural)
Subsp.	: subspecies
T-25	: 25 cm^2 tissue culture flask
T-75	: 75 cm^2 tissue culture flask
TBS	: Tris buffered saline
$TCID_{50}$	: 50% tissue culture infective dose
TcR	: T-cell receptor
TE	: Tris-EDTA buffer
TEM	: transmission electron microscopy
TG	: typhus group
TGF	: transforming growth factor
TMB	: 3'3'5'5'- tetramethyl-benzidine dihydrochloride
TNF	: tumour necrosis factor
v/v	: volume per volume
w/v	: weight per volume

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Chapter 1 - Introduction

1.1 Historical background

1.1.1 First report of the pathogen

Between April and December, 1989, a disease outbreak caused significant mortalities in coho salmon, *Oncorhynchus kisutch*, in seawater cages in southern Chile. Initial losses occurred in first year fish and followed a period of fluctuating water temperatures coinciding with an algal bloom in the March of that year (Branson and Diaz-Munoz, 1991). Affected fish were from different stocks and freshwater sources (Fryer *et al.* 1990; Cvitanich *et al.* 1991). A second peak of mortalities occurred in late 1989, this time in harvest-size fish. External signs were similar to those in first-year fish but few of the internal gross characteristics were present. Seawater temperatures were 12.5°C during the first outbreak and 18°C during the second outbreak. After these initial outbreaks, epizootics occurred predictably at seawater sites in Spring and Autumn when water temperatures were 10 –16°C (Lannan and Fryer, 1994). Outbreaks commonly occurred 6-12 weeks following transfer of fish to sea (Lannan and Fryer, 1993), but horizontal transmission in seawater appeared to occur quite readily, as new stocks became infected as early as two weeks post-transfer to an infected sea farm and, often, in the absence of any apparent vector (Bravo, 1994). As the disease represented a significant loss to the Chilean fish farming industry, intensive efforts were made to isolate the organism responsible and develop diagnostic methods (Fryer *et al.* 1990; Cvitanich *et al.* 1991).

1.1.2 Identification of the pathogen

Several bacterial pathogens were suspected, but attempts to culture the organism in bacteriological media at 15°C were unsuccessful. Kidney tissue from diseased fish was also inoculated onto an established chinook (*O. tshawytscha*) salmon cell line, CHSE-214 (ATCC CRL 1681) (Lannan *et al.* 1984), which is used routinely in the diagnosis of salmonid viral diseases (Fryer *et al.* 1990; Cvitanich *et al.* 1991). On development of a cytopathic effect (CPE) in CHSE-214 cultures incubated at 15°C, spent tissue culture medium was aliquoted onto fresh monolayers and incubated at 4, 10, 15, 18 and 21°C. In addition, supernatants were inoculated onto CHSE-214 cells in MEM-10 supplemented with antibiotic and, following incubation at 15°C for 14 days, spent medium from these flasks was inoculated into antibiotic-free cell cultures (Fryer *et al.* 1990). Culture supernatants were also inoculated onto a number of other fish cell lines. A CPE was observed in antibiotic–free CHSE-214 cultures incubated at 10-21°C, with development of CPE retarded at temperatures below 15°C and above 18°C (Fryer *et al.* 1990). The organism was cytopathic in all of the salmonid cell lines tested but no CPE was observed in BB (ATCC CCL 59), from brown bullhead (*Ictalurus nebulosus*) or BF-2 (ATCC CCL 91), from bluegill (*Lepomis macrochirus*), cultures. As no growth was observed on bacteriological media and, with the exception of penicillin, replication *in vitro* was inhibited in cell cultures containing antibiotics, the organism was considered to be an obligate intracellular parasite (Fryer *et al.* 1990). The infectivity of the organism was determined by an end-point dilution assay (50% tissue culture infective dose ($TCID_{50}$)) in 96-well plates or by plaque assay in 24-well plates. The method of Reed and Muench (1938) was used to calculate dilution endpoints (Fryer *et al.* 1990; Lannan and Fryer, 1993).

Examination of Giemsa-stained smears revealed large numbers of darkly stained micro-organisms, present within cytoplasmic inclusions in infected cells. The isolated organism was Gram-negative and stained positively with a modification of the Gimenez stain developed for *Rickettsia tsutsugamushi*, a spotted fever group rickettsia (Fryer *et al.* 1990; Cvitanich *et al.* 1991). Transmission electron microscopy (TEM) showed individual or paired micro-organisms enclosed within membrane-bound vacuoles. Each organism was bound by a closely-apposed inner membrane and an undulating outer membrane with ribosome-like structures concentrated near the plasma membrane. Deoxyribonucleic acid-like material was localised in the central region and electron-lucent spheroid structures were present in many of the organisms. Some organisms appeared to be undergoing binary fission (Fryer *et al.* 1990) (Fig. 1.1). Examination of infected cells by scanning electron microscopy (SEM) after a 24h incubation revealed irregular coccoid organisms, approximately 1 μm in diameter, attached to the exterior surfaces of the host cells. Following incubation for 8 days, pleomorphic organisms with highly folded outer membranes were being released from ruptured cells into the intercellular spaces (Fryer *et al.* 1990).

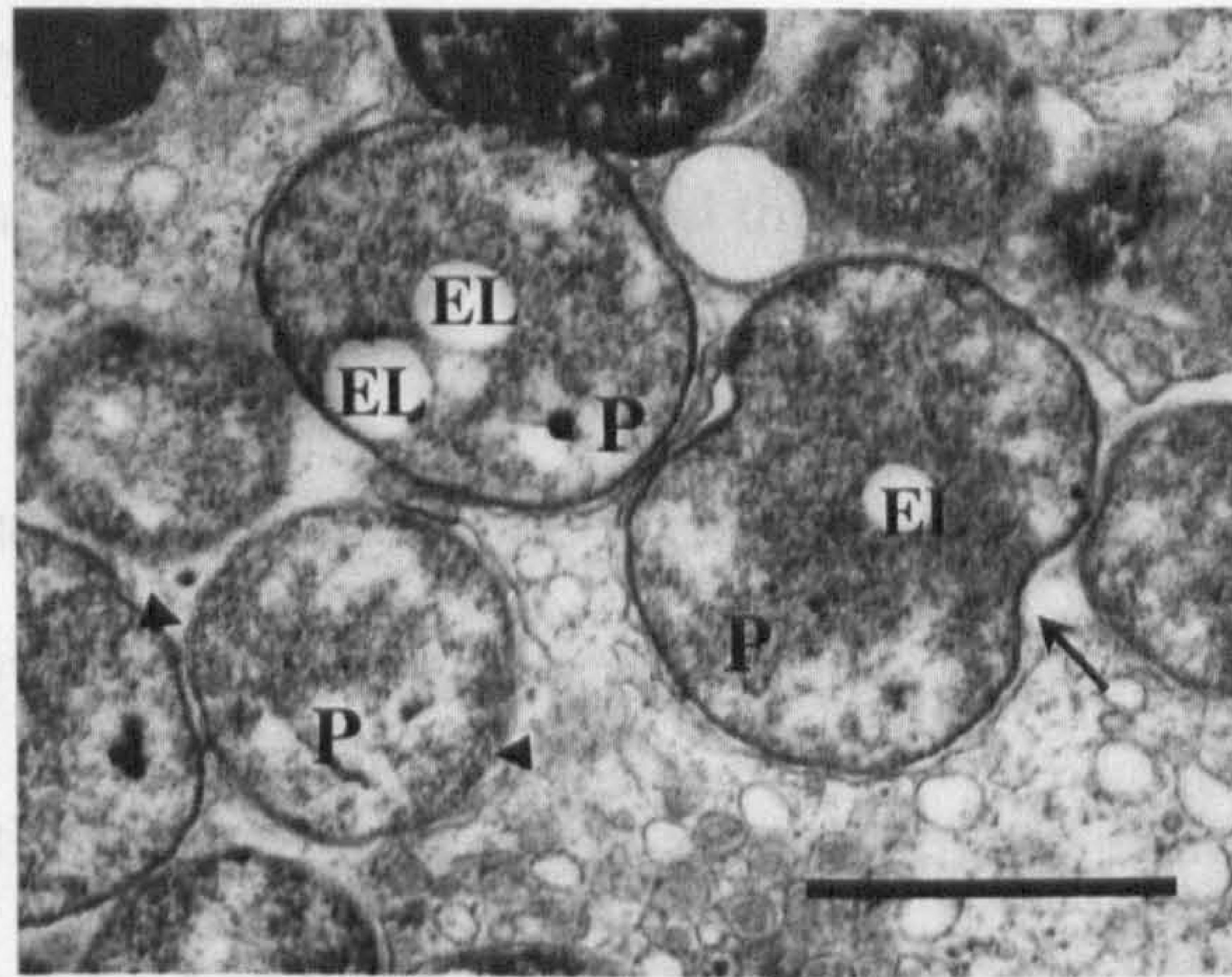


Fig. 1.1 Transmission electron micrograph showing a number of *P. salmonis* organisms (P) inside a rainbow trout head kidney macrophage, infected *in vitro*.The closely apposed inner membrane and undulating outer membrane are indicated by arrowheads. Electron-lucent vacuoles are labelled EL. One organism is in the process of dividing by binary fission (solid arrow). The scale bar represents 1.0 μm.

1.1.3 Classification of the organism

Based on similarities with certain rickettsial species, namely, replication within cytoplasmic inclusions in host cells, a rippled cell wall and electron-lucent bodies, Fryer *et al.* (1990) suggested that the organism be placed within the order *Rickettsiales*, tribe Ehrlichieae. Subsequently, Cvitanich *et al.* (1991) identified rickettsia-like organisms (RLOs) in kidney, spleen, liver, muscle, skin, heart, blood, brain, testes, ovaries and ovarian fluid, intestine, visceral fat and gills of coho salmon, Atlantic salmon (*Salmo salar*), chinook salmon (*O.*

tshawytscha) and rainbow trout from farms experiencing disease outbreaks. Based on the tentative identification of the agent as a rickettsia and observation of the disease in salmonid species other than coho, these authors proposed the name Salmonid Rickettsial Septicaemia (SRS) for the disease.

The RLO isolate which has been designated as the type strain, LF-89, was cultured by Fryer *et al.* (1992) from a moribund 2 year old coho salmon collected from a seawater pen during an epizootic. Although the organism is morphologically similar to organisms in the tribe Ehrlichieae of the family Rickettsiaceae and has the most common feature of this group, i.e. replication within membrane-bound cytoplasmic inclusions in host cells, it does not match any established genera in terms of host specificity and serological characteristics (Fryer *et al.* 1990). Unlike previously described Ehrlichieae, this organism is a pathogen of poikilotherms, replicating optimally in fish cell cultures at temperatures between 15°C and 18°C. Furthermore, it shares few antigens in common with other fish pathogens as shown by the absence of reaction in indirect immunofluorescence tests (Lannan *et al.* 1991).

As phenotype alone was insufficient to determine specific placement, 16S ribosomal RNA (rRNA) from LF-89 and other Gram negative bacteria was compared to determine the phylogenetic position of the agent (Mauel *et al.* 1996). From analysis of rRNA sequences, members of the genus *Rickettsia* have been placed in a monophyletic cluster within the alpha subdivision of the class Proteobacteria (Roux *et al.* 1997). In contrast, the 16S rDNA sequence of LF-89 conformed to the secondary structural models for the gamma subdivision of the Proteobacteria (Fryer *et al.* 1992). However, the DNA sequence showed no specific relationship to any of the bacterial 16S rRNA sequences examined and, although more closely related to *Wolbachia persica* and *Coxiella burnetti*, sequence comparison

indicated that LF-89 was not a specific relative of either of these intracellular bacteria. Therefore, Fryer *et al.* (1992) proposed that this unique 16S rRNA sequence, together with its growth temperature requirements, host range and serological characteristics justified the classification of this pathogen as a new genus and species *Piscirickettsia salmonis* gen. nov., sp. nov.

The species *Piscirickettsia salmonis* gen. nov., sp. nov. is described as pleomorphic, predominantly coccoid, ca. 0.5 to 1.5 μm in diameter, but also occurring as rings or pairs of curved rods. Gram negative and non-motile, the organism is a pathogen of salmonid fishes and replicates by binary fission within membrane-bound cytoplasmic vacuoles or inclusions in cells of susceptible fish hosts or in fish cell lines including CHSE-214, CHH-1, CSE-119 and RTG-2 from salmonid fishes and EPC and FHM from non-salmonid fishes. Replication *in vitro* is optimal between 15°C and 18°C, is greatly retarded at temperatures above 20°C and below 10°C, and does not occur at or above 25°C. *Piscirickettsia salmonis* produces CPE in the form of clusters of rounded cells and produces a titre of 10^6 to 10^7 $TCID_{50}.ml^{-1}$ in cultured fish cells. The titre is reduced by >99% by one freeze-thaw cycle at -70°C, but the presence of 10 % dimethyl sulphoxide (DMSO) has a cryopreservative effect. After detection in cell culture or by acridine orange stain, confirmatory identification of the organism is made by serological methods, e.g., fluorescent-antibody staining. The type strain LF-89 was deposited with the American Type Culture Collection as strain ATCC(R) VR 1361. Lannan and Fryer (1993) proposed the name, piscirickettsiosis, for the disease caused by *P. salmonis*.

1.1.4 Pathology

The clinical signs of the disease are variable and, in many cases, affected fish exhibit no external symptoms. The most consistent signs are pale gills, swollen kidneys and enlarged spleens. The most characteristic lesions, seen in some fish, are pale, subcapsular nodules, or ring-shaped foci, in the liver. Petechial haemorrhaging on stomach, intestines, pyloric caecae, swim bladder and visceral fat was observed frequently in coho salmon. Haematocrits were reduced to 4 – 34% in moribund fish. Large numbers of macrophages were found in peripheral blood smears with most containing cell debris or organisms within cytoplasmic vacuoles (Branson and Diaz-Munoz, 1991; Cvitanich *et al.* 1991).

In coho salmon, pathological changes were reported in kidney, liver, spleen, heart, brain, intestine, ovary and gill (Cvitanich *et al.* 1991). Invasion by inflammatory cells and diffuse chronic inflammatory tissue were found in kidneys and spleens, with necrotic cells primarily associated with vessels. Intravascular coagulation and fibrin thrombi in large vessels were observed in kidney and liver. Severe focal or diffuse necrotic lesions were found in the liver, with oedema, invasion by inflammatory cells and some fibrosis, and RLOs were observed within the cytoplasm of degenerating hepatocytes as well as within macrophages (Cvitanich *et al.* 1991). Pericarditis and endocarditis were evident and intravascular coagulation was often seen in blood within the heart. Chronic inflammatory lesions were common in the lamina propria of the large intestine, usually accompanied by severe necrosis and sloughing of the mucosal epithelium into the lumen. In the gills, epithelial hyperplasia and lamellar fusion were occasionally accompanied by necrotic areas and thrombi in lamellar capillaries (Branson and Diaz-Munoz, 1991; Cvitanich *et al.* 1991).

Because of the systemic nature of the disease, it was common to find infected cells in most tissues and mild inflammatory and thrombotic lesions were also seen in brain, pancreas, ovaries and adipose tissue (Cvitanich *et al.* 1991). The pathological lesions, particularly the vascular lesions with diffuse intravascular coagulation and fibrin thrombi, as well as perivascular inflammation, are similar to those described for rickettsial diseases of other animals (Feng *et al.* 1994).

1.1.5 Diagnosis

Moribund fish are lethargic, anaemic and dark in colour, the gills are pale and haematocrits low (<25%). While swollen kidneys and enlarged spleens are consistently observed, grey, mottled lesions in the liver, although diagnostic, do not often occur. There is extensive necrosis of haemopoietic tissues and the RLOs can be observed microscopically within cytoplasmic vacuoles in histological sections and in imprints and smears from the kidney and other organs (Lannan and Fryer, 1993). *Piscirickettsia salmonis* produces a characteristic CPE in CHSE-214 cell monolayers (Fig. 1.2), and confirmatory diagnosis is achieved by indirect fluorescent antibody test (IFAT) or polymerase chain reaction (PCR) assay of positive cultures, according to the methods set out in the OIE regulations (OIE, 2003). The PCR assays can also be conducted directly on tissues and thus PCR assays on tissues, along with the observation of suspect organisms within macrophages or hepatocytes, are also suitable methods for confirmatory diagnosis. Alternatively, *P. salmonis* can be detected in Giemsa-stained tissue smears, followed by IFAT for positive identification (Lannan and Fryer, 1991). An enzyme-linked immunosorbent assay (ELISA) for detecting *P. salmonis* is commercially available (Birrell *et al.* 2003).

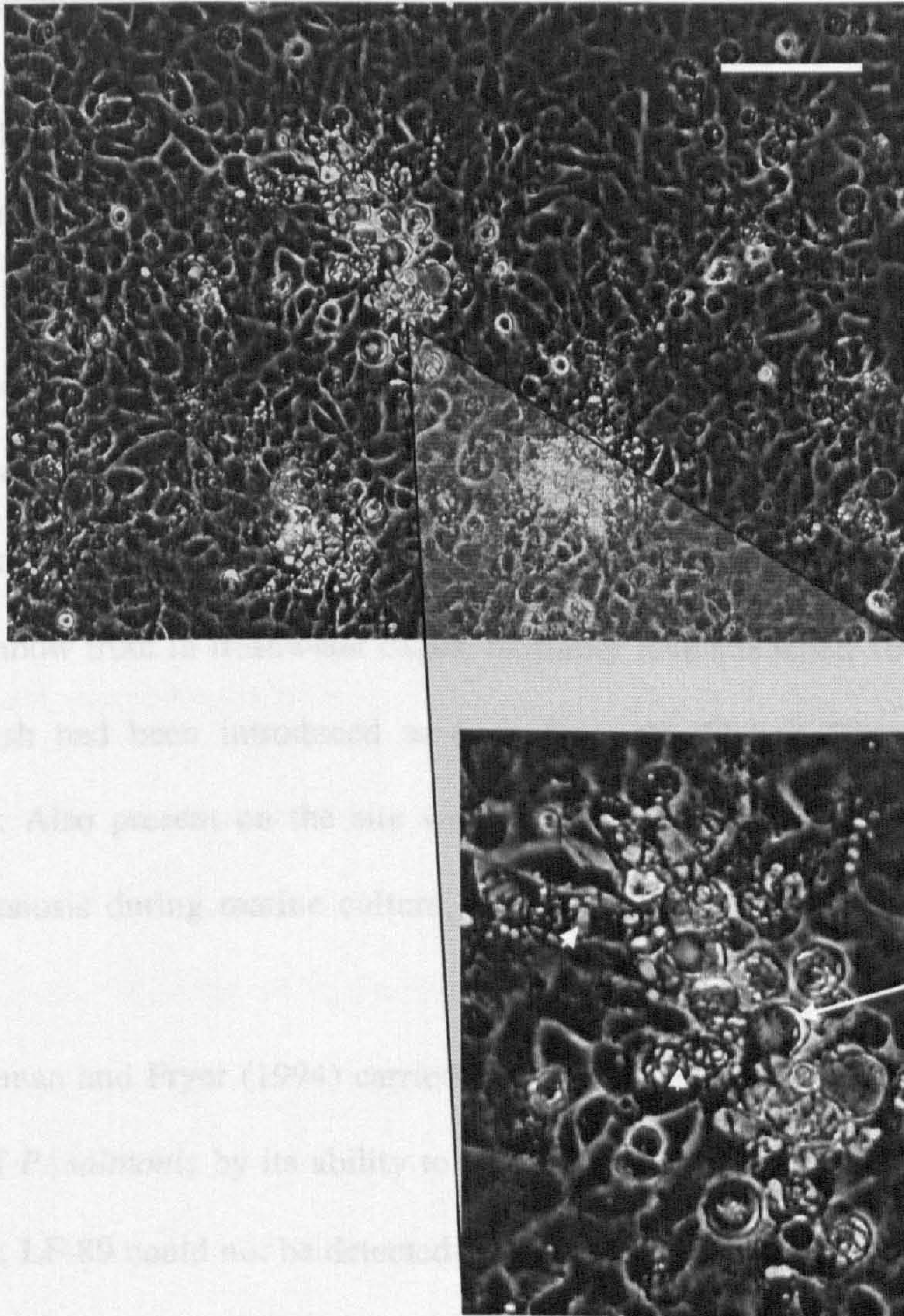


Fig. 1.2. Foci of *P. salmonis* infection in CHSE-214 cell monolayers (main picture). In the inset, characteristic CPE caused by *P. salmonis* on these host cells is more clearly visible. The initial appearance of irregularly-shaped vacuoles (arrowheads) is followed by the development of 'crescent-moon' cells (solid arrow), in which host-cell cytoplasm is displaced by a large vacuole containing *P. salmonis* organisms. The scale bar represents 100 μm.

1.1.6 Transmission of the disease

The pathogen *P. salmonis* has now been detected in all species of salmonid reared in Chile: coho, chinook, Atlantic and Sakura salmon (*O. masou*) and rainbow trout. The disease was thought to be confined to fish during their seawater phase until the etiological agent, *P. salmonis*, was isolated in September 1993, from juvenile coho salmon, Atlantic salmon and rainbow trout reared in freshwater (Gaggero *et al.* 1995). These fish were offspring of broodstock which had survived a saltwater *P. salmonis* outbreak. In another outbreak among rainbow trout in freshwater cages, mortality levels reached 10% (Bravo, 1994). The affected fish had been introduced as eggs from the United States and reared only in freshwater. Also present on the site were offspring of coho salmon which had survived piscirickettsiosis during marine culture, but these tested negative by fluorescent antibody test.

Lannan and Fryer (1994) carried out a series of experiments to assess extracellular survival of *P. salmonis* by its ability to infect CHSE-214 cells. Infectivity of semipurified type strain, LF-89 could not be detected at any time in freshwater but was maintained more than 14 days in seawater (3.2% salinity) at 5, 10 and 15°C. The extended survival of the rickettsial agent in seawater suggested that direct transmission was a possibility and this would seem to be borne out by findings from experimental (Cvitanich *et al.* 1991; Almendras *et al.* 1997) and natural situations (Bravo, 1994). Rapid inactivation of the rickettsial agent in freshwater may explain why the disease is rarely observed at freshwater sites. However, the infectious agent has been observed in gonadal tissues, seminal, ovarian and coelomic fluids, and vertical transmission has been demonstrated (Cvitanich *et al.*

1991; Larenas *et al.* 2003). Furthermore, experimental transmission to co-habitant fish has been achieved in freshwater (Cvitanich *et al.* 1991). These findings suggest that the disease agent may have been introduced to the freshwater sites by vertical transmission from infected broodstock and is capable of survival and transmission to adjacent fish. The ability to be transmitted in this manner has serious implications for the control of spread and infection by *P. salmonis*, and Larenas *et al.* (2003) recommend the screening of seminal and ovarian fluids, in addition to kidney tissue, to ensure the negative carrier status of broodstock.

1.1.7 *P. salmonis* outside Chile

Norway: In 1988, mortalities were observed in seawater-farmed Atlantic salmon in Norway (Olsen *et al.* 1997) and, on examination of tissues, intracellular, intravacuolar bacteria-like inclusions were found. In 1992, an RLO was isolated from diseased fish. The RLO was diagnosed in 51 Atlantic salmon farms during 1988 to 1992, with most of the outbreaks occurring in 1988 and the majority of cases occurring in Autumn (August to December). Except for one case in adult fish, all incidents occurred in post-smolts. The geographical distribution of the affected farms in 1988 coincided with the geographical spread of a large algal bloom and the disease was frequently recorded after algal blooms, with fish observed feeding on zooplankton. Skin lesions were sometimes observed and, in a few fish, skin lesions were the only gross findings. The liver was the organ most consistently affected. The RLO appeared morphologically similar to *P. salmonis* and to an RLO causing disease in Ireland, and serological tests using antibodies against the type strain, LF-89, suggested that the Norwegian isolate was indeed *P. salmonis*. Subsequently, the 16S rDNA, 23S

rDNA and internal transcribed spacer (ITS) regions were sequenced and comparison of 16S rDNA and ITS sequences showed 99.4% and 97.8% similarity between the Norwegian isolate (NOR-92) and the type strain, indicating that they were the same species (Mauel *et al.* 1999).

Ireland: In 1991, low level mortalities occurred among post-smolt Atlantic salmon on a marine farm in Ireland, at water temperatures of 14-16°C (Rodger and Drinan, 1993). Affected fish had pale gills, congestion of the pyloric caecae and intestine, splenomegaly, swollen kidneys and yellow mottling of the liver which had the appearance of ring formations in some fish. Histopathological examination revealed extensive necrosis of haemopoietic tissue in the kidney and spleen and of liver hepatocytes. In all three organs, numerous enlarged, vacuolated cells contained basophilic granules which stained Gram negative and Giemsa positive. The vacuolated cells included hepatocytes and macrophages in the liver and macrophages in the kidney and spleen. Necrosis in the liver was frequently perivascular with inflammation and fibrosis, and thrombi were present in some blood vessels. Electron microscopy revealed coccoid organisms (0.7 – 1.3 μm) containing electron dense areas and electron lucent vacuoles with undulating outer cell membranes. These were enclosed in membrane-bound cytoplasmic vacuoles. Subsequently, in 1995 and 1996, RLO-associated disease was found on four Atlantic salmon marine farms, occurring at a time of declining water temperatures (16°C – 12°C) during the Autumn period (Palmer *et al.* 1996). Three of the cases were in post-smolts while one involved adjacent stocks of post-smolts and one-sea-winter fish. The pathology, CPE and antibiotic sensitivity were consistent with those reported for *P. salmonis*, and smears of fish tissues and infected cell cultures were IFAT positive using *P. salmonis* antiserum.

Canada: In November, 1991, a rickettsial agent, morphologically identical to the *P. salmonis* type strain, LF-89, was isolated from marine-farmed Atlantic salmon in British Columbia, Canada (Brocklebank *et al.* 1992). This agent had been observed sporadically since 1970 in salmonids held in seawater in the area. However, although it produced pathology in infected fish similar to that observed with LF-89, mortality was low at 0.06% per day. Pathological changes included dermal ulcers, adhesions and petechiae in the peritoneal cavity, and fibrosis, necrosis and inflammation of spleen, liver and kidney. Basophilic inclusions in the cytoplasm of macrophages stained Gram negative, periodic acid-Schiff negative and Giemsa positive. The agent reacted in a fluorescent antibody test with polyclonal antiserum against LF-89 (Lannan and Fryer, 1993) and subsequent comparison of 16S rDNA sequences revealed 99.4% similarity between this isolate, ATL-4-91, and LF-89 (Mauel *et al.* 1999).

Scotland: The presence of an RLO in the brain of marine farmed Atlantic salmon was reported by Grant *et al.* (1996) but this organism was negative in a latex agglutination test for *P. salmonis*. Subsequently, Birrell *et al.* (2003) identified *P. salmonis* in the kidney of seawater farmed Atlantic salmon by ELISA. The organism produced characteristic CPE in CHSE-214 monolayers, and culture supernatants gave positive results in PCR amplifications using *P. salmonis*-specific primers. Histological examination revealed focal necrosis and inflammation in haematopoietic tissues, with perivascular inflammation and infiltration of granulocytes in the liver. The presence of intracellular bacteria and hypertrophic macrophages was also reported. Reid *et al.* (2004) have conducted phylogenetic analyses on Scottish isolates of *P. salmonis* and found them to cluster with Norwegian and Canadian Atlantic salmon isolates.

The pathology found in the Norwegian disease shares similarities with the Chilean cases in that tissue necrosis, vascular damage and an affinity for reticuloendothelial cells, other fixed phagocytic cells and macrophages are characteristic findings. However, multifocal gill hyperplasia and inflammation and necrosis in the gut, which are commonly found in Chilean coho salmon, were not observed in the Norwegian or Irish disease in Atlantic salmon. Similarly, while extensive thrombosis was seen in salmonids in British Colombia, thrombosis was only observed occasionally in the Norwegian and Irish cases (Olsen *et al.* 1997). On the other hand, hyperplasia was reported in the gills of Scottish salmon and there were mild thrombotic lesions in the pancreas (Birrell *et al.* 2003).

The variety in the expression and severity of the RLO infections may be due to modulations by the host species and its immune status, dose dependence and the presence or absence of concurrent disease, but it will also be crucial to determine if intrinsic differences exist between the different isolates which affect their virulence (Fryer and Mauel, 1997).

1.1.8 Antigenic characterisation

Although organisms identified as *P. salmonis* have been isolated from disease outbreaks in Canada, Norway, Ireland and Scotland, these incidents resulted in minor losses when compared with outbreaks in Chile (Brocklebank *et al.* 1992; Fryer *et al.* 1992; Rodger and Drinan, 1993; Grant *et al.* 1996; Palmer *et al.* 1996; Olsen *et al.* 1997; Birrell *et al.* 2003; Reid and Birkbeck, 2003). The reasons for the greater severity of the disease in Chile are unknown but it is thought that environmental factors, greater host susceptibility or more virulent strains of *P. salmonis* may be responsible (Olsen *et al.* 1997). Antigenic characterisation of isolates from different geographical locations by electrophoresis and

immunoblotting may help to identify differences between strains which are responsible for increased virulence. In addition, identification of shared or immunodominant antigens will be important in designing effective vaccines.

Mammalian rickettsiae are obligate intracellular parasites that cannot be cultivated in cell-free media and are generally unstable when separated from host components (Tamura *et al.* 1982). With regard to the antigens of rickettsiae, most of what is known was initially derived using antisera and monoclonal antibodies (mAbs) to whole organisms. Immunoblotting of *R. conorii* and *R. rickettsii*, spotted fever group (SFG) rickettsiae, using immune sera and mAbs, revealed a range of high molecular weight proteins, 100-200 kDa, and a ladder-like pattern of lipopolysaccharide (LPS) (Schuenke and Walker, 1994). Typhus group (TG) rickettsiae, meanwhile, possess group-specific antigens which are sensitive to sodium metaperiodate, resistant to trypsin, stable in 0.2 M sodium hydroxide and include LPS. The species-specific antigens of *R. typhi* and *R. prowazekii* (TG rickettsiae), on the other hand, are destroyed by incubation at 56°C for 45 min, suggesting that they are proteins (Amano *et al.* 1998).

Rickettsial LPS forms a pattern of ladder-like bands following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver-staining (Amano *et al.* 1998). LPS is found exposed on the outer membrane of Gram negative bacteria. It is attached to the outer membrane by Lipid A which, in turn, is connected to a core polysaccharide unit consisting of 2-keto-3-deoxyoctonic acid (KDO), heptose and other sugars. The outermost section is referred to as the O-antigen and is composed of repeating sugar units which may vary in the number and type of repeats. This variation can be found between species and even between individual isolates and, as the O-antigen is capable of

stimulating a humoral response, the variation may be used to analyse strain differences by immunoblotting.[a]

Because of the difficulties in producing sufficient purified antigen from cultured mammalian rickettsiae, analysis of proteins and antigens is being conducted at the molecular level through the use of DNA sequencing and PCR amplification (Schuenke and Walker, 1994; Díaz-Montero *et al.* 2001). There have been attempts to clone and express immunogenic antigens in *Escherichia coli*, in order to determine which proteins are important in mammalian immunity to rickettsial infections. For example, Díaz-Montero *et al.* (2001) achieved protection in mice against challenge with *R. conorii* following immunisation with plasmid constructs expressing fragments of two major outer membrane proteins and with the corresponding recombinant proteins. Another *R. conorii* gene, coding for a 120 kDa antigen, was cloned and sequenced (Schuenke and Walker, 1994) and expressed as a β-galactosidase fusion protein in *E. coli*. The finding that this 120 kDa protein was recognised by convalescent serum and was found to react with T-lymphocytes would suggest that the protein plays a role in protective immunity. Furthermore, the presence of this antigen in a number of different strains indicated that it might be useful as a vaccine candidate.

[a] (http://smms.ilc.uq.edu.au/resources/MICR2008_lecture11.pdf,
http://www.bact.wisc.edu/MicroTextbook/BacterialStructure/CellWall.html)
accessed 5/3/02 via yahoo.com search structure of lipopolysaccharide)

The ability of antibodies against heat-sensitive epitopes to protect against challenge has been demonstrated (Anacker *et al.* 1987; Li *et al.* 1988), which suggests that these epitopes may have a function in attachment to or penetration of host cellular membranes. Since mAbs against some surface components, such as LPS-like antigens, do not confer protection against infection, it would appear that a mechanism other than opsonisation may be involved in the protective effect (Anacker *et al.* 1987). The protective mAbs may exert their effect by blocking attachment of the rickettsiae to the host. Thus, not only is the identification of heat-labile epitopes important in the search for protective antigens but further investigation of the structure of the native epitope may help to elucidate the mechanism of attachment and entry to the host. Therefore, it will be important to look at the immunoreactivity of non-denatured proteins, and examination of immunoblots using non-heat denatured antigen preparations may give more useful information about the host immune response to the native organism.

While *P. salmonis* is not related to mammalian rickettsiae, many of the same techniques may be applied in the search for important immunogenic antigens. Following Percoll density gradient centrifugation to obtain purified *P. salmonis* from cell cultures, Kuzyk *et al.* (1996) used western blotting to analyse type-strain, LF-89, antigens. Rabbit anti-*P. salmonis* serum identified six immunoreactive antigens of relative molecular weights 65, 60, 54, 16 and ~11 kDa. Digestion with proteinase K (PK) was used to determine if any of these antigens were carbohydrate; this digestion destroyed all but the 16 and 11 kDa antigens.

Silver-staining of PK-digested *P. salmonis* was carried out to ascertain if the organism had an LPS-like component. This revealed a faint ladder-like banding pattern of

carbohydrates ranging from 16-35 kDa, with a discrete band seen at 20 kDa and a strongly staining major band at ~11 kDa. The 11 kDa band was considered to be the lipo-oligosaccharide (LOS) component of LPS. The ladder-like banding pattern was thought to be core region LOS attached to a diminishing number of polysaccharide O-antigen repeat units, while the 16 kDa band was thought to represent core region LOS with a single unit of O-antigen.

Immunoblot analysis was used to investigate possible antigenic variation in isolates experimentally passaged through different salmonid species. Analysis of four isolates by western blot, using convalescent rainbow trout serum, revealed minor immunoreactive bands between 10 and 70 kDa. A PK-resistant band at ~11 kDa was also detected using salmonid serum but was more weakly detected than by rabbit antiserum. No difference was observed in the banding patterns of the four isolates.

Barnes *et al.* (1998) used diatrizoate meglumine and diatrizoate sodium (DMDS or Hypaque) density gradient centrifugation to purify *P. salmonis* (LF-89) from cell cultures. Immunoblots using polyclonal anti-*P. salmonis* rabbit serum detected antigens of molecular weight 108, 95, 64, 60, 56, 40, 36, 32 and 20 kDa. Three of these antigens - 64, 60, 56 – were of similar molecular weight to three of the six antigens identified by Kuzyk *et al.* (1996). It was suggested that the reason for different antigens being identified in the two studies was a result of different separation methods used i.e. Hypaque v Percoll and because of recognition of different epitopes by the two different preparations of rabbit antiserum.

Jones *et al.* (1998) examined four strains of piscirickettsia using rabbit antiserum generated against LF-89 – two isolates from Chile, CR288 and CR1010, strain ECR0811 isolated from Atlantic salmon in Eastern Canada, and the type-strain LF-89. Following PK-

digestion, SDS-PAGE and immunoblotting, a range of PK-resistant bands was observed extending from 20 kDa to greater than 95 kDa. However, in 1999, Kuzyk *et al.* (1999) reported a PK-resistant, ladder-like pattern on immunoblots of *P. salmonis* grown in CHSE-214 and EPC cell lines and probed with anti-*P. salmonis* rabbit serum prepared against Percoll-purified *P. salmonis*. The pattern, which was like that reported by Jones *et al.* (1998), was observed also in uninfected cell lines and, on further investigation, was found to be similar to that of two different mycoplasma antigens. Mycoplasma-specific products were amplified subsequently by PCR from contaminated cell line samples (Kuzyk *et al.* 1999). This finding highlights the potential for misdiagnosis of *P. salmonis* antigens and underlines the need for adequate controls and precautions when propagating and examining isolates from cell culture.

Polyclonal antiserum to *P. salmonis* from different laboratories is likely to recognise different epitopes or to cross-react with epitopes from cells used to culture the organism (Barnes *et al.* 1998; Jones *et al.* 1998) thus leading to inconsistent results between laboratories. Monoclonal antibodies, on the other hand, allow specific detection of a particular antigen and could be used to standardise the findings of different groups. Jamett *et al.* (2001) generated mAbs to the type-strain LF-89 and eight other isolates from Chile. Using a panel of these mAbs, a number of bands ranging from approximately 10 kDa to 105 kDa were identified. Different mAbs recognised different epitopes with some recognising a ladder-like pattern similar to the LPS reported in *R. typhi* and *R. prowasekii*. A group of six antibodies was selected for specificity by ELISA and checked for absence of cross-reactivity with other fish pathogens. The selected antibodies were able to detect all *P. salmonis* isolates tested by indirect immunofluorescence assay. In addition, four of the

antibodies were capable of detecting *P. salmonis* by IFAT on kidney and brain smears. These workers suggested that the specificity of mAbs and the fact that they can be produced on a large scale would allow optimisation of the ELISA assay to achieve the most sensitive and reproducible detection of *P. salmonis* (Jamett *et al.* 2001; Aguayo *et al.* 2002).

The use of mAbs to identify important *P. salmonis* antigens and analysis of these antigens at the molecular level are essential to improve our understanding of how this organism interacts with the host. As it has been shown that the humoral response in the natural host species is very different to the response in the rabbit, further work is needed to identify those antigens which are recognised by salmonids and which may be capable of stimulating protection (Kuzyk *et al.* 1996). Recombinant DNA technology has proven useful in the production of immunogenic antigens from terrestrial rickettsiae and, already, the first vaccine based on a recombinant protein of *P. salmonis* has been shown to confer protection against challenge (Kuzyk *et al.* 2001a); (Kuzyk *et al.* 2001b).

1.1.9 Challenge tests

Several challenge tests for *P. salmonis* have been reported in the literature, from initial challenges to confirm Koch's postulates to experiments designed to examine possible entry routes for the pathogen (Cvitanich *et al.* 1991; Garcés *et al.* 1991; Almendras *et al.* 1997).

Although SRS, or piscirickettsiosis, is a disease primarily affecting fish 6–12 weeks following transfer to saltwater, nearly all of the challenge tests have been carried out in freshwater aquaria and using juvenile fish (10 – 20g), presumably for convenience or for lack of appropriate seawater facilities. However, Cvitanich *et al.* (1991) reported earlier onset of disease in saltwater compared with freshwater (7-9 days versus 10-11 days).

Furthermore, *P. salmonis* has been shown to survive longer than 14 days in seawater whereas it is inactivated rapidly in freshwater (Lannan and Fryer, 1994). While incidences of SRS have been reported from freshwater sites (Bravo, 1994; Gaggero *et al.* 1995), these are the exception and, if a challenge is designed to yield results relevant to the natural disease, it may be more meaningful to conduct the challenge in seawater.

One dilemma when administering challenge tests is deciding on the route by which the challenge dose is to be delivered. Again, the difficulty lies in whether or not to imitate the natural mode of infection; in some cases, it may be more important to ensure that a reproducible, defined dose is administered. Almendras *et al.* (1997) administered the challenge dose by three routes: intraperitoneal injection (IP), oral intubation (PO) and dispensing the inoculum over the gill surface (GS). In addition, uninoculated fish were placed in the same tank or in a downstream tank to act as contact and non-contact co-habitants, respectively. The PO group showed a pattern of mortality that was significantly delayed compared with the IP and GS routes, although there were significant differences between replicates in this group. However, when the invasion of the tissues by the organism was examined, two different patterns of infection were observed in directly challenged fish. In the IP challenged group a capsular (serosal) pattern of *P. salmonis* infection was observed in the liver and spleen. This infection progressed to the leucocytes and other parenchymal cells of the spleen, liver and kidney. In contrast, a haematogenous pattern of infection was observed in PO and GS challenged fish, where the organism invaded the organs from the blood vessels into the surrounding tissues, and no evidence of capsular infection by *P. salmonis* was observed in these groups. Leukocytes carrying *P. salmonis* in their cytoplasm were frequently observed within hepatic sinusoids and blood vessels. This

pattern of infection in gill and orally infected fish resembles that observed in natural outbreaks of SRS. A haematogenous infection was also observed in co-habitant fish, which further supports the idea that this pattern of infection is more representative of the natural disease.

However, using cohabitation as a means of challenging fish with *P. salmonis* has not proven reliable. While both Almendras *et al.* (1997) and Cvitanich *et al.* (1991) succeeded in reproducing the disease in target fish, Garcés *et al.* (1991) found no evidence for horizontal transmission to contact co-habitants, despite 100% mortality in challenged fish and, presumably, a significant shedding of infectious agent into the holding tank. Several factors may have contributed to these contradictory results. In the study by Garcés *et al.* (1991), fish were held in aquaria with a flow rate of 6L min^{-1}, compared with a flow rate of 2L min^{-1} used by Almendras *et al.* (1997) or in a recirculating system used by Cvitanich *et al.* (1991). The use of freshwater together with a higher flow rate may have combined to inactivate or rapidly flush infectious agent from the aquarium. Also, sham-vaccination (Cvitanich *et al.* 1991) and an underlying infection with *Aeromonas salmonicida* (Almendras *et al.* 1997) could have imposed an additional stress on co-habitant fish which may have rendered them more susceptible to infection with *P. salmonis*.

Smith *et al.* (1999) examined a number of possible routes of entry by *P. salmonis*. In addition to subcutaneous injection, this study assessed the efficiency of entry via the skin and gills, using pathogen-impregnated paper patches, and via oral and anal intubation. In contrast to Almendras *et al.* (1997), these workers observed only low level mortality (2%) in fish infected by the gastric route but suggested that, in fasting fish, *P. salmonis* may be more susceptible to acid degradation in the stomach. Both oral and anal routes were less

efficient than the percutaneous routes for the development of SRS. The skin-patch method used by Smith *et al.* (1999) proved an effective way of administering a *P. salmonis* challenge and produced considerable cumulative mortality (52%). The ability of *P. salmonis* to penetrate intact skin and invade underlying tissues suggests that this route of entry may be of significance in the natural disease. Likewise, the gills have been shown to be a route of entry (Almendras *et al.* 1997; Smith *et al.* 1999) and it appears that *P. salmonis* may be able to reach the gill capillaries rapidly and, from there, disseminate through the body.

Smith's group found that the most efficient method of administering a *P. salmonis* challenge in terms of mortality was via subcutaneous or intraperitoneal (i.p.) injection. It is possible that the subcutaneous route may mimic a natural challenge where the skin has been damaged or where arthropod ectoparasites have inflicted skin wounds. In terrestrial infections, rickettsiae are transmitted via an arthropod vector (Hickman *et al.* 1993; Walker *et al.* 2000) and, in the case of SRS, *P. salmonis* has been detected in *Ceratothoa gaudichaudi* which is parasitic on farmed salmon in Chile (Garcés *et al.* 1994). While i.p. injection is unlikely to represent the natural route of entry, and may produce a pathology which does not truly mirror that found in nature (see above), it is frequently the most convenient method available for administering a challenge. It allows a known dose to be delivered and variation in dose rate between individual fish can be minimised.

The dose of infectious agent given in the challenge tests varies considerably between groups. Cvitanich *et al.* (1991) made dilutions of infectious material which were used to inoculate cell culture monolayers in 24-well plates. Discrete foci of CPE were counted to obtain the number of infectious rickettsial units (IRUs), and all challenge doses

were reported as IRUs. In all other reports, infectivity titres are calculated by endpoint dilution assay ($TCID_{50}$) on cell monolayers. While Smith *et al.* (1997) produced a cumulative mortality of 60% in coho salmon receiving 50 $TCID_{50}$, Kuzyk *et al.* (1999) obtained only 66.7% mortality in coho challenged with $10^{3.6}$ $TCID_{50}$. In another part of their study, Kuzyk's group produced 58% mortality in 15 g coho by administering $10^{5.0}$ $TCID_{50}$ while Garcés *et al.* (1991) produced 100% mortality in 10 g coho using a dose of $10^{5.3}$ $TCID_{50}$.

It is apparent that the isolate used has a significant effect on the level and rate of onset of mortality, indicating different levels of virulence. Therefore, the strength of the challenge is not always a reflection of the dose administered. For example, using strain LF-89, House *et al.* (1999) produced 57 % mortality in coho receiving 10^3 $TCID_{50}$ but only 32 % mortality in fish receiving 10^3 $TCID_{50}$ of strain ATL-4-91. Cumulative mortality of 76 % was produced using 10^4 $TCID_{50}$ of strain ATL-4-91 while Smith *et al.* (1999) produced 98 % mortality in fish receiving 10^4 $TCID_{50}$ of strain SLGO-95. However, as shown above, even when using the same isolate there is still considerable variability in the level of mortality produced. For instance, while 10^3 $TCID_{50}$ of LF-89 produced 57 % mortality in the challenge by House *et al.* (1999), in the test by Garcés *et al.* (1991) a dose of $10^{3.3}$ $TCID_{50}$ resulted in a mortality of 88 % in coho of similar size and at a similar water temperature.

The virulence of the test organism will affect its ability to reproduce the natural disease. The number of passages the organism has undergone *in vitro* and the type of cell in which it is propagated may serve to alter the expression of virulence factors, with a resultant effect on the infectivity (Lopez-Doriga *et al.* 2001). House *et al.* (1999) used organisms

which were all of the same passage number (passage 9) when comparing the virulence of different isolates. Both Jones *et al.* (1998) and Smith *et al.* (1999) also stated the passage number of the isolate used in their studies. However, with the exception of Birkbeck *et al.* (2004b), other authors have not reported the passage number of the isolates used and, to date, there has been no examination of the effect of passage *in vitro* on the expression of virulence factors by *P. salmonis*.

Fish of different sizes and species will exhibit different susceptibilities to the challenge organism. For example, Smith *et al.* (1996) found that while pathological effects in rainbow trout were more severe than in coho salmon, cumulative mortalities were lower in trout and they appeared to be more efficient at clearing the *P. salmonis* organism from tissues. Jones *et al.* (1998) reported that the virulence of isolate ECR0811, from Eastern Canada, varied between fish species such that a dose of $10^{2.875}$ $TCID_{50}$ produced 100 % mortality in Atlantic salmon (10 g), 62 % in coho (10 g), 22.5% in rainbow trout (25 g) and no mortality during the observation period in common carp, *Cyprinus carpio*, (5 g).

The ability to induce experimental infection is an important tool in testing vaccine efficiency. In an attempt to standardise challenge tests for evaluation of vaccines, it has been recommended that mortality in the control group should reach 60% while non-specific mortality should be less than 10 % (Nordmo, 1997). However, it is obvious from the studies above that several factors may influence the outcome of any challenge test. The challenge dose, virulence of the challenge strain and the susceptibility of the fish species will all affect the severity of the challenge. In addition, stress factors such as recent handling or underlying infections will increase the chance of fish succumbing to challenge. Therefore, it may be difficult to achieve reproducibility between repeated challenge tests using *P.*

salmonis. Furthermore, while the aim of the challenge test is to reproduce the natural disease as closely as possible, it may not be feasible for routine vaccine testing, in terms of cost or facilities, to carry out seawater challenges on fish of the correct age. Thus, for routine assessment of vaccine efficacy, it would be useful to develop a non-destructive test for some parameter which accurately indicates acquisition of protective immunity. Such a parameter might be the level of specific anti-*P. salmonis* antibodies in the serum of vaccinated fish, which could be sampled non-destructively. The development of such a test is discussed in Chapter 7.

1.2 The teleostean immune system

Teleosts are among the most primitive groups of vertebrates to exhibit both innate and acquired immune systems (Laing *et al.* 1999). As research progresses, the teleostean and mammalian immune systems have been found to have a number of similarities, at least on a functional level, and in teleosts, as in mammals, the inter-dependence of innate and acquired immunity is increasingly being underlined. While the innate immune system may be regarded as a 'constitutive' defence, in many cases the increased presence or expression of components can be induced following exposure to pathogens or inflammatory insult and by interactions with elements of the acquired immune system. However, a feature of the innate defences is that they act in a non-specific manner while the acquired, or adaptive, immune responses respond specifically to a particular pathogen or stimulus and produce a memory component capable of improved responses on subsequent exposure to the same stimulus. It is this adaptive response and the production of an effective memory component which are the targets of vaccination programmes.

Although the innate and acquired immune systems are inter-connected, for the sake of clarity they will be discussed separately in this review.

1.2.1 The innate immune system

The innate immune system is comprised of physical barriers, such as skin and mucus, non-specific humoral defences and phagocytic cells. Non-specific cytotoxic cells, which are considered to correspond to natural killer (NK) cells in mammals, have also been found in teleosts (Moody *et al.* 1985; Faisal *et al.* 1989; Greenlee *et al.* 1991). Mucus secreted onto the skin, gills and intestine contains antimicrobial compounds such as lysozyme, trypsin, antibodies (Abs), agglutinins and complement factors. Plasma defences include these compounds in addition to acute phase proteins (serum amyloid A, C-reactive protein), transferrins, protease inhibitors such as α_2-macroglobulin, cytokines, interferons and eicosanoids (Secombes and Fletcher, 1992; Yano, 1996; Ellis, 2001).

Teleosts possess a well-developed complement system (Boshra *et al.* 2004) which plays a significant role in non-specific defence (Olivier *et al.* 1986; Rose and Levine, 1992; Rao *et al.* 2001; Holland and Lambris, 2002). The complement cascade can be initiated by the classical Ab-mediated, mannan-binding lectin or the alternative pathways, forming the terminal complement complex in the extracellular fluid or the membrane attack complex (MAC) on the target cell. The assembly of the MAC involves aggregation of the lytic complement components (C5b-C9) which results in the formation of a pore-like hydrophilic channel, causing disruption of the membrane and death of the target cell (Yano, 1996; Kaattari and Piganelli, 1997; Zarkadis *et al.* 2005). The complement system also contributes significantly to opsonisation of foreign particles for uptake by phagocytes (C3b

complement component and its receptor CR3), to chemotaxis of leukocytes (C5a) and to activation of the respiratory burst in phagocytes (C3a & C5a), and components from all of these pathways have been identified in fish (Johnson and Smith, 1984; Rose and Levine, 1992; Lamas and Ellis, 1994a; Lamas and Ellis, 1994b; Yano, 1996; Boshra *et al.* 2004).

One of the defences found early in phylogenetic development, is the phagocytic cells. Their ability to engulf particles is critical, from their role as defence cells in invertebrates (Novoa *et al.* 1996; Seljelid and Eskeland, 1993; Dalmo *et al.* 1997) to their position as central participants in the innate and adaptive immune responses of higher vertebrates (Paulnock, 1994). Although our understanding of the functions and capabilities of phagocytes is largely obtained from studies of the mammalian immune system, increasingly, similarities between the mammalian and teleostean systems are being discovered (Enane *et al.* 1993; Brubacher *et al.* 2000; Dixon and Stet, 2001). While cells such as fibroblasts, epithelial and endothelial cells possess a degree of phagocytic capability, (Dalmo *et al.* 1997; Neumann *et al.* 2001) in teleosts, as in mammals, it is the neutrophil and the mononuclear phagocyte – the 'professional phagocytes' – which are principally responsible for preventing the multiplication and spread of pathogens (Secombes and Fletcher, 1992). As the possible involvement of macrophages in the immune response to *P. salmonis* forms a substantial part of this thesis, phagocytes (both neutrophils and monocyte/ macrophages) are discussed in more detail in Section 1.2.3.

1.2.2 The adaptive immune system

1.2.2.1 Humoral immunity

The adaptive or specific immune system of fish, as in mammals, is comprised of humoral, or antibody (Ab)-mediated, defences and cellular defences or cell-mediated immunity (CMI). Although the immune response of fish may differ in specifics from that of mammals, the humoral response does exhibit similarities in the basic structure of immunoglobulin (Ig), the cellular requirements for the induction of Abs and the role played by these Abs in activities such as neutralisation, complement fixation and opsonisation. In the teleost, two classes of Ig have been described, IgM and a recently discovered IgD homologue (Kaattari, 1994; Watts *et al.* 2001). The IgM molecule exists primarily as a tetramer, composed of four monomeric subunits, each containing two heavy (H) protein chains (70-81 kDa) and two light (L) chains (22-32 kDa). Individual H and L chains form pairs which are held together by disulphide bonds. The J-chain, present in mammalian IgM, is not seen in fish Ig. It is hypothesised that heterogeneity in disulphide cross-linking may be a means of providing structural diversity in fish Abs.

Fish Ab molecules possess relatively low intrinsic affinity compared with mammalian monomeric Abs and the antigen-binding site demonstrates limited heterogeneity. However, it has been shown that a limited degree of affinity maturation takes place and, given its multimeric format, the functional or overall affinity of fish IgM may equal or exceed that observed in mammalian IgG (Kaattari, 1994; Kaattari *et al.* 2002).

The induction of Ab responses to T-independent antigens (polysaccharides) requires the assistance of an accessory cell such as the monocyte or macrophage. The role of the macrophage appears to be the provision of interleukin 1 (IL-1) (Secombes and Fletcher,

1992). In trout, the IL-1 factor is crucial to the differentiation of the B-lymphocyte into an Ab-secreting plasma cell (Secombes *et al.* 1999). Protein antigens (T-dependent), meanwhile, require that the antigen be processed by the accessory cell and presented on the cell surface to a T-lymphocyte which triggers expression of other interleukins to stimulate B-cell differentiation (Secombes and Fletcher, 1992; Vallejo *et al.* 1992).

Fish IgM is an effective agglutinator and is also capable of activating complement via the classical pathway (Verho *et al.* 2005). In addition, antibody has an important role in opsonising pathogens which facilitates their uptake by macrophages via Fc receptors (Barnes *et al.* 2002; Barnes and Ellis, 2004). In many cases, this uptake results in more effective killing of the pathogen by the macrophage although, for a pathogen such as *R. salmoninarum*, this increased entry to the phagocyte is of benefit and promotes survival within the host (Gambrill and Wisseman, 1973; Griffin, 1983; Bandin *et al.* 1993; Bandin *et al.* 1995; Chen *et al.* 1998).

1.2.2.2 Cell-mediated immunity

Cytokines.

As in mammals, adaptive and innate immunity in teleosts operate in an integrated manner (Fig 1.3) (Köllner *et al.* 2002). To achieve coordination between the various facets of the immune system requires complex control mechanisms, effected to a large extent by the cytokine network. Cytokines have been classified as interferons (IFNs), interleukins (ILs), colony stimulating factors (CSFs), tumour necrosis factors (TNFs) or growth factors (GFs). They are polypeptides or glycoproteins of less than 30 kDa which act as signalling molecules within the immune system. Acting on target cells via high affinity specific receptors, they effect paracrine or autocrine signalling and can produce different responses

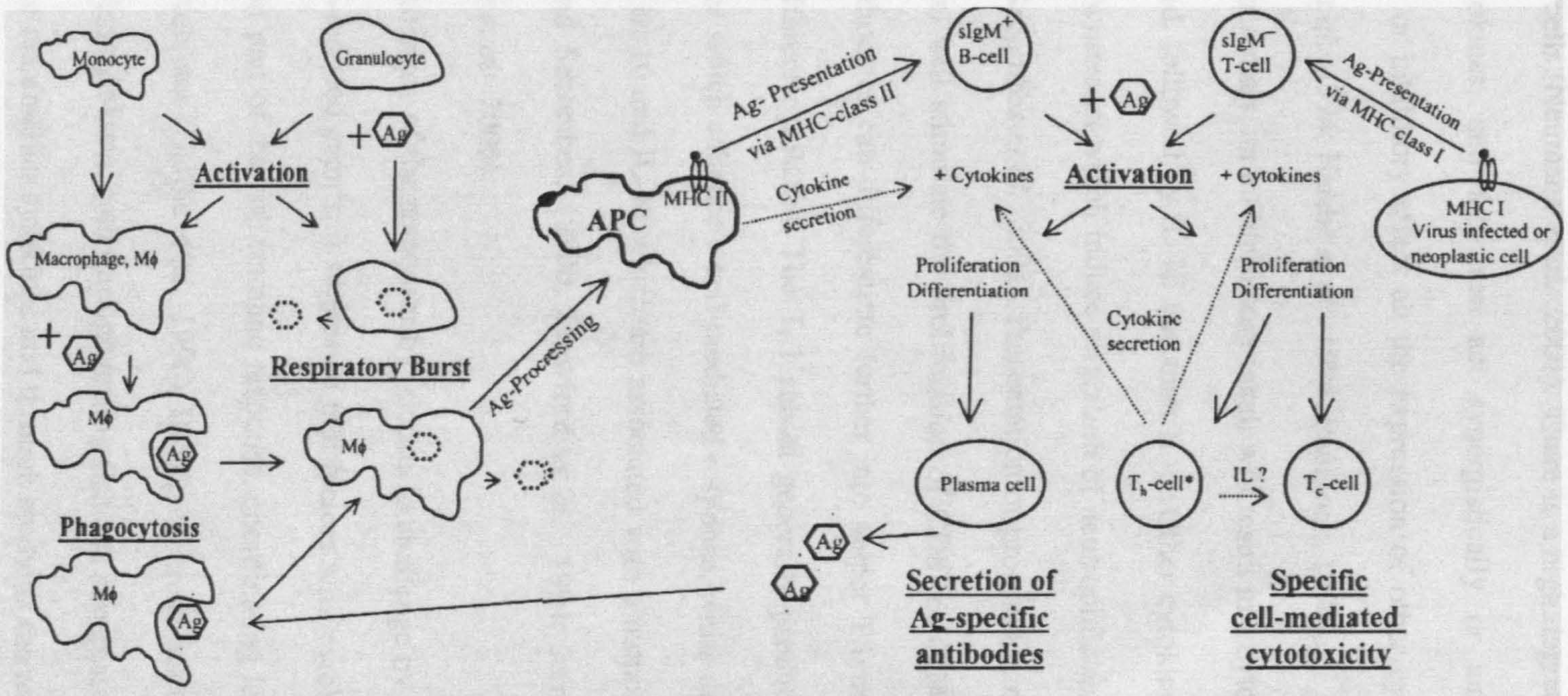


Fig. 1.3. Schematic overview of known immune functions of bony fish. For detailed information see text. Dotted arrows indicate functions which are predicted from proliferation assays only. *Abbreviations* :Ag, antigen; APC, antigen presenting cell; MHC, major histocompatibility complex; M, macrophage; sIgM, surface immunoglobulin M; Th-cell, helper T lymphocytes; Tc-cell, cytotoxic T-lymphocytes (*Both are predicted from functional assays only, not shown as specific cell population). Adapted from Köllner *et al.* (2002).

in different targets (Hardie *et al.* 1994): they may stimulate proliferation or gene expression in immune cells (Neumann *et al.* 2001). There is a high degree of redundancy and overlap among cytokines, and they can act synergistically or antagonistically and cause a stimulatory or inhibitory effect on the expression of other cytokines (Nacy *et al.* 1981; Crawford *et al.* 1994; Hardie *et al.* 1994; Paulnock, 1994).

In mammals, an inflammatory insult will result in a cytokine cascade whereby TNF-α is released, followed by IL-1β and then IL-6. Other cytokines, the chemokines, are then released downstream which induce migration of neutrophils and macrophages to the site of infection (Secombes *et al.* 2001). Presentation of processed antigens by antigen presenting cells (APCs) will stimulate the proliferation of $CD8^+$ or $CD4^+$ T-lymphocytes. Activated $CD4^+$ lymphocytes can differentiate further into helper T-lymphocyte (T_H) subsets which produce different cytokines. The T_H1 subset generally produces IL-2, TNF-α and IFN-γ, lymphokines which enhance a cell-mediated response, while the T_H2 subset produces IL-4, IL-5, IL-6, IL-10 and IL-13 which are associated with a humoral or Ab-mediated response (Graham and Secombes, 1990b; Crawford *et al.* 1994; Jerrells, 1997; Sinigaglia *et al.* 1999; Esser *et al.* 2003).

Since many of the responses by teleosts to challenge by pathogens *in vivo* are typical of cytokine-induced events, it suggested that factors with cytokine bioactivity must also be an important part of the fish immune response, coordinating leucocyte responses of fish as in other vertebrates (Hardie *et al.* 1996). Biological cross-reactivity has been utilized in the search for fish cytokines both through testing fish cell supernatants using biological assays designed for mammalian cytokines and through studying the reactivity of fish leucocytes to purified mammalian cytokines. However, while the latter approach proved useful,

Secombes *et al.* (2001) emphasised that care should be taken when interpreting results from assays designed for mammalian cytokines where non-cytokine factors in the fish supernatants may influence the outcome.

The pleiotropic cytokine, IL-1, plays a central role in mediating immune responses such as thymocyte proliferation, B-cell growth and differentiation, IL-2 and IL-6 production (Subramaniam *et al.* 2002). In mammals, bacteria and LPS stimulate macrophages to secrete IL-1 which sequentially stimulates the release of eicosanoids, which have pro-inflammatory and chemotactic activity. In fish, a similar process is apparent as LPS has been shown to induce IL-1 production by trout leucocytes, and the production of eicosanoids with leucocyte chemotactic activity by a variety of leucocytes has been reported (Ellis, 2001). It was shown that supernatants from LPS-stimulated monocytes of channel catfish (*Ictalurus punctatus*) can substitute for intact monocytes, allowing macrophage-depleted lymphocytes to undergo mitogen-induced proliferation and antigen-induced Ab production. This factor from fish cross-reacts with mammalian cells, which respond in a manner characteristic of IL-1 stimulation, namely, proliferation and IL-2 secretion by T-cell lines. Furthermore, the activity can be inhibited by antisera to human IL-1α and IL-1β, and cDNA probes to mammalian IL-1α hybridise with mRNA from catfish monocytes. Interleukin-1 exerts its effects via its receptor but, while the interleukin and its receptor have been identified or cloned in fish, knowledge of its role in the immune response of fish is limited (Secombes *et al.* 1999; Subramaniam *et al.* 2002).

Type I interferons are pH-resistant cytokines which are produced by many cell types in response to a viral infection. The Type I interferons (IFN-α and IFN-β) provide a rapid and powerful non-specific, antiviral defense mechanism. In humans, Type I IFNs cause

cells to synthesize a number of enzymes that collectively interfere with the replication of viral RNA or DNA, for example, proteins inhibitory to the translation of viral mRNA such as Mx proteins. In addition, there is evidence that these IFNs can promote the development of IFN-γ-producing T_H1 cells (Sinigaglia *et al.* 1999). Type 1 IFN-like activity has been demonstrated in fish but, while the protein has not been isolated, the genes encoding the Mx protein have been cloned from several species of fish (Graham and Secombes, 1990b; Leong *et al.* 1998; Sinigaglia *et al.* 1999; Ellis, 2001). The unrelated IFN-γ (Type II IFN) is induced primarily in T-lymphocytes following antigenic/mitogenic stimulation. The principal effector cytokine of T_H1 cells is IFN-γ. In mammals, IFN-γ activates monocytes and macrophages and promotes switching of B-lymphocytes to isotypes such as IgG2a which fix complement and promote phagocytosis by macrophages. Interferon-γ has been shown to be a potent macrophage activating factor (MAF), enhancing macrophage antimicrobial activity, and to induce MHC class II antigens.

Since purified or recombinant fish cytokines have not been available, studies addressing the regulation of respiratory burst in teleost macrophages and neutrophils have relied on the use of crude cytokine-like preparations from mitogen-stimulated kidney leucocytes. These leucocyte supernatants have been shown to contain soluble mediators which both stimulate and deactivate antimicrobial responses of fish macrophages. Crude cytokine supernatants have been found to contain two distinct MAF activities that modulate respiratory burst activity (Neumann *et al.* 2001). One activity corresponds to a protein of 50 kDa, while the other 30 kDa factor may be similar to the one characterised by Graham and Secombes (1988) (1990b). From functional and physicochemical tests, this latter activity is believed to be IFN-γ: it has been demonstrated to be a product of surface Ig

negative lymphocytes (considered to be T-lymphocytes) and has been shown to be capable of upregulated expression following stimulation of T-lymphocytes *in vivo* or *in vitro* (Graham and Secombes, 1990b; Marsden *et al.* 1994; Sinigaglia *et al.* 1999). Recently, Zou *et al.* (2004) reported the presence of an IFN-γ-like molecule in the fugu (*Takifugu rubripes*) genome database. There is also evidence to suggest that a TNF-α-like molecule may be responsible for some of the MAF activity in these supernatants (Neumann *et al.* 2001) and human recombinant TNF-α (rTNF-α) synergises with crude cytokine preparations to enhance priming of respiratory burst activity in rainbow trout macrophages, while priming of the respiratory burst can be partly inhibited using anti-TNF-receptor mAbs (Hardie *et al.* 1994; Jang *et al.* 1995).

Significant progress has been made in recent years in the search for fish cytokine genes and TNF-α, members of the TGF-β superfamily, various interleukins, several chemokines and chemokine receptors have been cloned and sequenced (Hardie *et al.* 1998; Knight *et al.* 1998; Secombes *et al.* 2001; Hong *et al.* 2003; Zou *et al.* 2004). While advances in sequencing technology, the use of expressed sequence tag analysis and the generation of cDNA arrays will allow the identification, cloning and expression of many genes from the fish immune system, functional studies of the proteins will still be required if we are to elucidate the interactions and regulation of the teleostean cytokine network.

Lymphocytes

Research on the cell-mediated arm of the specific immune system has lagged behind that of the humoral response despite increasing evidence that CMI and/or local immunity play important roles in the protection gained following vaccination (Nakanishi *et al.* 1999) and, until relatively recently, there was some doubt about the existence of separate B- and T-

lymphocyte populations in teleosts (Graham and Secombes, 1990a; Partula, 1999; Kuzyk *et al.* 2001a). Studies were hindered by complications in thymectomising juvenile fish, the lack of inbred strains and the dearth of specific surface markers (Kuzyk *et al.* 2001a). On the functional level, research on fish specific immune responses concentrated mainly on Ab production, and the relatively few investigations of specific CMI centered on allogenicity (Dijkstra *et al.* 2001). However, mAbs raised to IgM allowed panning for surface Ig-positive (sIg+) lymphocytes and selective enrichment for putative B- and T-lymphocyte populations. Differential responses to mitogens (LPS, Concanavalin A (Con A) and phyto-haemagglutinin), behaviour in the mixed leukocyte response (MLR), responses to thymus-dependent and independent antigens, the use of Abs to determinants on T-like cells (eg. anti-Thy-1) and the production of lymphokines have been used to discriminate further between the two populations and indicate the existence of T- and B-lymphocytes in fish (Graham and Secombes, 1990a; Nakanishi *et al.* 1999). Fischer *et al.* (2003) were able to separate rainbow trout leukocytes by magnetic sorting and showed that surface IgM^+ ($sIgM^+$) lymphocytes were positive for IgM expression but not T-cell receptor (TcR) or CD8 expression, while $sIgM^-$ lymphocytes lacked expression of IgM but were positive for TcR and exhibited high levels of CD8 expression following allogeneic sensitisation. The presence of the membrane-associated antigen receptor (TcR), a member of the Ig superfamily, is a characteristic feature of mammalian T-lymphocytes, and RNA isolated from sea-bass gut-associated lymphoid tissue contains a transcript with significant homology to the T-cell receptor β chain (Scapigliati *et al.* 2000). The sequences of TcR α and TcR β chains from trout are also known (Van Lierop *et al.* 1998).

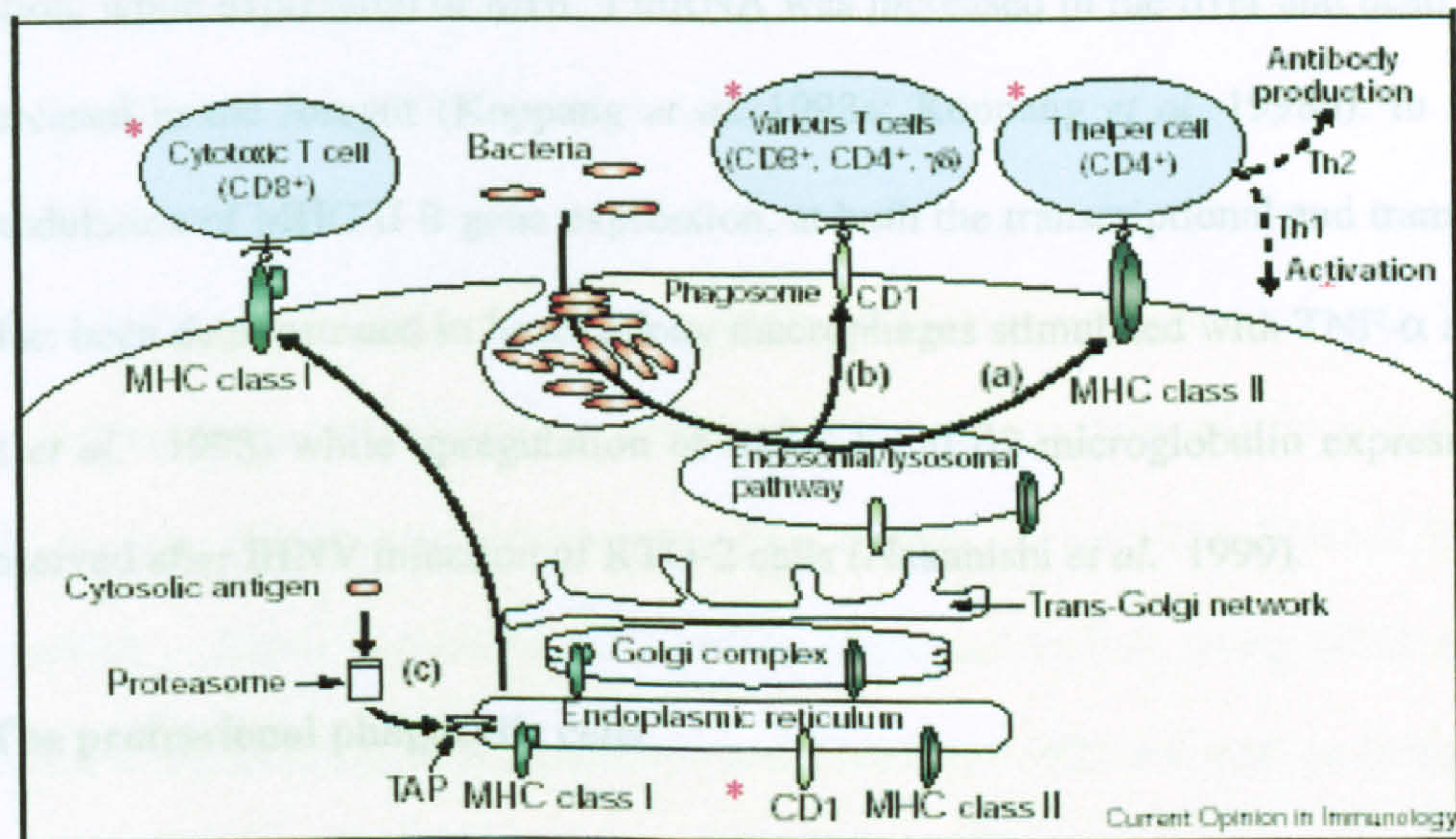
Major histocompatibility complex (MHC)

The MHC encodes two classes of structurally and functionally distinct glycoproteins that present antigenic peptides to T-lymphocytes and thus initiate specific immune responses (Nakanishi *et al.* 1999). Major histocompatibility (MH) receptors are Ig superfamily member proteins that interact with T-lymphocytes through a specific T-cell receptor (TcR) in order to initiate immune responses. Genes for the major histocompatibility Class I (MHC I) and Class II (MHC II) molecules, and β2-microglobulin have been identified which has considerably improved understanding of several aspects of the fish immune system (Reitan and Secombes, 1997; Knight *et al.* 1998; Koppang *et al.* 1998a; Koppang *et al.* 1998b). Teleosts are one of the most primitive groups possessing the MHC/TcR system. In teleosts, these genes appear to be present in several different linkage groups, rather than linked in a complex on a single chromosome; therefore, the word 'complex' is not always used (Dixon and Stet, 2001). There are two types of these highly polymorphic receptors. Class I MH receptors are composed of two non-covalently linked polypeptides encoded by different genes, a heavy chain (45 kDa) and the 12 kDa β2-microglobulin. Class II receptors comprise two non-covalently linked polypeptide chains called α and β, both of approximately 30 kDa. The outermost peptide domains of the two MH receptors fold into a groove structure which is used to carry peptides from pathogens out to the cell surface for presentation to T-cells. The presentation of these foreign peptides is one of the key events in T-cell activation (Dijkstra *et al.* 2001; Dixon and Stet, 2001). In higher vertebrates, the MHC II protein has a well documented role in the presentation of exogenous processed antigens to $CD4^+$ T-lymphocytes while the principal specific response to many viral disease agents is cell-mediated cytotoxicity involving classical MHC class I molecules. These

molecules present endogenous peptides at the cell surface for recognition by the TcR/CD8$^+$ receptor complex of cytotoxic T-lymphocytes (CTLs) (Koppang *et al.* 1998a; Koppang *et al.* 1998b; Dijkstra *et al.* 2001). Recognition can lead to stimulation and clonal expansion of the T-cell and to specific lysis of the peptide-presenting cells. The activated CTLs only recognise cells with the same MHC class I molecule and peptide as that with which they were initially stimulated.

Originally, it was thought that only endogenous proteins were presented by MHC class I at the cell surface. After being degraded to small peptides by the proteasome in the cytoplasm, these peptides are transported to the endoplasmic reticulum (ER), where they form a complex with MHC class I and β2-microglobulin, the resulting heterotrimer then being transported to the cell surface. In contrast, exogenous antigens were believed to be presented only by MHC class II molecules at the cell surface, stimulating an Ab response (Safley and Ziegler, 1994; Dijkstra *et al.* 2001). However, it is now known that exogenous antigens can be presented by MHC class I molecules via an alternative MHC class I processing pathway whereby phagocytosed protein antigens are transported from the endocytic pathway to the cytosol and, following proteasomal degradation, the resulting peptides are transported to the ER where they are bound to the newly synthesised MHC I molecules (Harty and Bevan, 1999; Dijkstra *et al.* 2001; Van Den Elsen and Rudensky, 2004) (Fig. 1.4).

Recently, modulation of MHC I and II mRNA expression in teleosts has been investigated by northern blot analysis, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or by fluorescence activated cell sorter (FACS) analysis with antisera against protein expressed in bacteria. High levels of MHC II expression have been



* adjacent to figure elements indicates that these molecules or pathways have not yet been identified in teleosts (Hansen and Zapata, 1998; Ohta *et al.* 2002). Modified from Pieters (2001).

Fig. 1.4. Phagocytosis of bacteria and the interaction of the phagosomal pathway with the adaptive immune system in mammals. Following internalisation of bacteria into phagosomes, bacteria and bacterial products are delivered to endosomal and lysosomal organelles, where they are degraded via bactericidal mechanisms. Subsequently, antigens can be presented to the adaptive immune system by different pathways:

- degraded exogenous antigens can be presented by MHC class II molecules. Complexes of MHC class II with peptide can be recognised by T helper cells which can either activate the presenting cell (Th1 cells) or stimulate the production of antibodies by B cells (Th2 cells).

- lipid moieties derived from the bacteria can be presented to various T cell subsets by CD1 molecules.

- MHC class I molecules can present peptides, derived from antigens located in the cytosol and processed by the proteasome and transporter associated with antigen processing (TAP), at the cell surface to $CD8^+$ cytotoxic cells. Activation of cytotoxic cells leads to rapid lysis of the presenting cell.

observed in the head kidney, spleen, hindgut and gills of Atlantic salmon following vaccination, while expression of MHC I mRNA was increased in the liver and head kidney, and decreased in the foregut (Koppang *et al.* 1998a; Koppang *et al.* 1998b). In rainbow trout, modulation of MHC II B gene expression, at both the transcriptional and translational levels, has been demonstrated in head kidney macrophages stimulated with TNF-α and LPS (Knight *et al.* 1998) while upregulation of MHC I and β2-microglobulin expression has been observed after IHNV infection of RTG-2 cells (Nakanishi *et al.* 1999).

1.2.3 The professional phagocytic cells

1.2.3.1 Neutrophils.

Neutrophils, together with eosinophils and basophils, belong to the granulocyte cell lineage. Because of morphological and histochemical variations, the use of different staining protocols and inconsistencies in nomenclature used to describe fish blood cells (Ellis, 1977; Ainsworth, 1992), the identification of leukocytes has been problematic. The existence of neutrophils, however, has been known for some time and the characteristics and functions of these cells have been well studied (Moritomo *et al.* 1988; Plytycz *et al.* 1989; Afonso *et al.* 1997; Afonso *et al.* 1998a; Afonso *et al.* 1998b; Neumann *et al.* 2001). Neutrophils are short-lived cells which are present in the blood and in reservoirs in haematopoietic tissues (Afonso *et al.* 1998a). While they are less actively phagocytic than monocytes/ macrophages (Griffin, 1983) and, to a degree, rely on opsonins for uptake of micro-organisms (Lofgren *et al.* 1983; Ainsworth and Chen, 1990; Lamas and Ellis, 1994a), they carry potent antimicrobial compounds in their granules and lysosomes, and can generate large quantities of reactive oxygen intermediates (ROIs) during the respiratory burst

(Higson and Jones, 1984; Secombes and Fletcher, 1992; Neumann *et al.* 2001; Allen, 2003). Neutrophils of carp and rainbow trout have also been shown to exhibit non-specific cytotoxicity (Kurata *et al.* 1995; Sasaki *et al.* 2002). Rainbow trout neutrophils are strongly peroxidase-positive, esterase-negative and their cytoplasm is rich in glycogen granules. In neutrophils, hydrogen peroxide (H_2O_2), a product of the respiratory burst, can be involved in the myeloperoxidase (MPO) reaction, where MPO catalyses the oxidation of halide ions by H_2O_2 to form hypohalites, chloramines and singlet oxygen. The MPO-H_2O_2-halide system, which is highly cytotoxic, has been demonstrated in fish, using MPO extracted from rainbow trout neutrophils (Secombes and Fletcher, 1992) As well as being highly efficient killers, neutrophils form a larger reserve in kidney and blood than monocytes and, therefore, are first to arrive in large numbers at a site of infection (Neumann *et al.* 2001; Afonso *et al.* 1997; Afonso *et al.* 1998a), summoned by microbial products and by cytokines secreted from resident macrophages, other neutrophils and activated lymphocytes (Paulnock, 1994; Gudmundsson and Agerberth, 1999). In addition to engulfing and destroying foreign particles, the neutrophils themselves may be engulfed and their antimicrobial compounds added by activated macrophages to their existing armoury (Hiemstra and van Furth, 1994; Afonso *et al.* 1998a; Afonso *et al.* 1998b).

1.2.3.2 Monocytes/macrophages.

Monocytes belong to the mononuclear cell lineage and are the circulating precursors of tissue macrophages (Ellis, 1977; Stafford *et al.* 2001a) although, under steady-state conditions, replenishment of tissue macrophages can occur through local proliferation of progenitor cells (Rutherford *et al.* 1993). After monocytes migrate from the blood and mature into tissue macrophages, they retain effective phagocytic capabilities which are

intrinsic to their role in bactericidal activities, antigen presentation and secretion of immunoregulatory compounds (Griffin, 1983; Secombes and Fletcher, 1992; Crawford *et al.* 1994; Dixon and Stet, 2001; Neumann *et al.* 2001). Tissue macrophages are a heterogenous group of cells whose characteristics appear to be determined largely by the tissue environment in which they are found (Rutherford *et al.* 1993; Crawford *et al.* 1994; Paulnock, 1994). Macrophages play an important role in specific as well as non-specific immune responses by means of their antigen-presenting function, secretion of cytokines and production of non-specific humoral defence components. In salmonids, blood-borne antigens are endocytosed by endothelial cells and macrophages of the kidney sinusoids and splenic ellipsoids. In addition, antigen uptake is carried out by epithelial cells and sub-epithelial phagocytes in the intestine, gills and skin (Dalmo *et al.* 1997; Press and Evensen, 1999). Following intracellular digestion, antigenic substances are processed and presented to T-lymphocytes by macrophages in their role as APCs, thereby initiating specific immune responses (Vallejo *et al.* 1992). In addition, monocytes/macrophages or factors released by them have been shown to be necessary as accessory cells in proliferative responses to T-cell mitogens (Con A) (Secombes and Fletcher, 1992) and the MLR (Nakanishi *et al.* 1999). While little is known about the surface markers of teleost macrophages, it appears that they express MHC II molecules, Ab (Fc) and complement receptors (Johnson and Smith, 1984; Secombes, 1996; Chen *et al.* 1998; Knight *et al.* 1998; Koppang *et al.* 1999; Barnes *et al.* 2002). The presence of macrophages in tissues throughout the body and their location in gill, skin, gut epithelium and lamina propria means that they are well placed for the early detection of invading microorgansims (Dalmo *et al.* 1997; Lin *et al.* 1999; Press and Evensen, 1999). Through release of cytokines by these resident tissue macrophages,

neutrophils, monocytes and other immune-responsive cells are rapidly attracted from blood and haematopoietic tissues to a site of infection (Gordon *et al.* 1988; Gordon, 1988; Do Vale *et al.* 2002). The circulating neutrophils and monocytes adhere to the endothelium of capillaries at the infection site and are attracted by chemotactic factors to diapedese through the capillary endothelium into the extravascular space to the focus of inflammation (Manthey and Vogel, 1994; Nielsen *et al.* 1994). The elicited monocytes differentiate into fully mature macrophages, engulf and process foreign invaders via the phagocytic pathway and display processed antigens on their surfaces in conjunction with major histocompatibility receptors (Safley and Ziegler, 1994). By exhibiting antigens in this way, these APCs trigger lymphocytes to release cytokines which stimulate further humoral and cell-mediated immune responses designed to clear the infection (Crawford *et al.* 1994; Harty and Bevan, 1999). In addition to attracting additional immune-responsive cells, some of these cytokines can prime neutrophils and macrophages to become 'activated', whereby their phagocytic capabilities and production of antimicrobial substances are increased, resulting in more efficient, though non-specific, killing (Chung and Secombes, 1987; Paulnock, 1994) Certain of the cytokines, or cytokine combinations, will also act as inhibitory signals, exerting suppressive effects on the immune response which may serve to limit tissue damage (Walker *et al.* 1991; Crawford *et al.* 1994; Neumann and Belosevic, 1996). Once the infection has been resolved, macrophages can act as scavengers, engulfing and removing tissue debris and dead cells (Nielsen *et al.* 1994).

Although macrophages can exert damage against parasites by external means (Whyte *et al.* 1989), killing of pathogens by both neutrophils and macrophages requires that the organism first be taken up by phagocytosis, a form of endocytosis characterised by

the uptake of particulate matter such as cells and cell debris, micro-organisms or macro-molecular aggregates (Neumann *et al.* 2001; Secombes, 1994). Opsonisation of particles with complement, C-reactive protein and Ab increases uptake by the phagocyte (Johnson and Smith, 1984; Rose and Levine, 1992; Bandin *et al.* 1993; Dalmo *et al.* 1997; Chen *et al.* 1998; Barnes *et al.* 2002).

The mechanisms used in phagocyte-mediated killing can be broadly classed as oxygen-dependent or oxygen-independent. In the oxygen-dependent mechanism, interaction between the organism and the phagocyte, via a receptor or by non-specific hydrophobic association, initiates local assembly of the NADPH-oxidase enzyme in the plasmalemma which invaginates to form a vesicle called a phagosome (Robinson and Badwey, 1994). Stimulation of the phagocyte membrane during phagocytosis triggers the production of ROIs which is accompanied by an increased demand for oxygen – termed the 'respiratory burst' (Higson and Jones, 1984; Chung and Secombes, 1988). Adherence of a particle to the phagocyte membrane appears sufficient to initiate the burst; ingestion does not appear to be necessary as soluble activating agents, such as phorbol myristate acetate (PMA), and by-products of phospholipase digestion are also capable of stimulating the burst (Chung and Secombes, 1988; Murphy *et al.* 1995; Silverman, 1997). During the respiratory burst, molecular oxygen is reduced to the superoxide anion (O_2^-) by the NADPH-oxidase enzyme complex, and the superoxide is transported into the phagosome (Robinson and Badwey, 1994). A portion of the O_2^- dismutates to H_2O_2, either spontaneously or catalyzed by the enzyme superoxide dismutase (SOD). Excess H_2O_2 can be converted into H_2O and O_2 by catalase. Singlet oxygen, which can be detected by the chemiluminescence emitted on its return to a stable form, is also produced during spontaneous dismutation of O_2^-, and

hydroxyl ions ($OH^{\bullet}$) can be produced by the reaction between O_2^- and hypochlorous acid (HOCl) or nitric oxide (NO) (Robinson and Badwey, 1994; Babior, 2004). The NO is produced by an inducible nitric oxide synthase (iNOS) enzyme, and both NO and reactive nitrogen intermediates (RNIs) such as peroxynitrite ($ONOO^-$), formed by the reaction of NO with O_2^-, have potent microbiocidal activity (Neumann *et al.* 2001). From recent work, it appears that one of the ways in which the NO response can be induced is through activation of macrophages by cleavage products of transferrin, an acute phase protein of which the increased expression in rainbow trout has been observed following bacterial infection (Stafford *et al.* 2001b). The bactericidal effect of many of these ROI and RNI products has been demonstrated both by inhibition studies and in cell-free systems (Sharp and Secombes, 1993; Skarmeta *et al.* 1995; Hardie *et al.* 1996).

There is little information available about oxygen-independent killing but it is thought that fish phagocytes may possess potentially bactericidal enzymes which could be released into the phagosome following fusion with lysosomes. The acidification of the phagosome, nutrient deprivation, release of lysosomal hydrolases and other antimicrobial peptides such as cathepsin, elastase, proteinase, cathelicidins and defensins may all be found to play a part in degradation of ingested organisms (Hiemstra and van Furth, 1994; Gudmundsson and Agerberth, 1999). Whatever the mechanism used, phagocytic killing mechanisms are an important frontline of defence against invading pathogens, serving both to remove the pathogen from the host organism and to destroy it in a manner such that constituent antigens may be presented, in conjunction with MHC molecules, to alert the immune system.

1.3 Using the immune system to protect against *P. salmonis*.

1.3.1 Immune responses to mammalian rickettsiae.

In mammals, humoral immune responses are observed in SFG and TG rickettsial infections. However, control of the infection is highly dependent on cellular immunity and a crucial role has been identified for T-lymphocytes. Both $CD4^+$ and $CD8^+$ T-lymphocyte subsets have a part to play in dealing with the infection, either through stimulation of intracellular killing by the target cells or through CTL-mediated clearance (Díaz-Montero *et al.* 2001; Walker *et al.* 2001).

The intracellular killing process is known to be mediated in mice by IFN-γ and TNF-α. These cytokines act in concert to activate endothelial cells, the major target cells of the rickettsiae, and other minor target cells to kill the organisms by a NO synthesis mechanism. Both $CD4^+$ and $CD8^+$ T-lymphocytes are potentially good sources of IFN-γ and it is hypothesized that the sources of the protective cytokines are the T-lymphocytes and macrophages which infiltrate the perivascular space surrounding vessels with infected endothelium (Walker *et al.* 2001).

In vaccination experiments with mice, Díaz-Montero *et al.* (2001) achieved some protection against challenge with *R. conorii* following immunisation with soluble recombinant protein. As it was then considered that soluble proteins did not effectively enter the MHC I presentation pathway, it was considered that this indicated a role for $CD4^+$ T-lymphocytes in the immune response to rickettsiae. However, Walker *et al.* (2001) found that depletion of $CD4^+$ cells had no effect on the outcome of infection in a mouse model of

infection while $CD8^+$-depleted mice experienced persistent infection and mortality after receiving a sublethal dose of *R. conorii.*

$CD8^+$ T-lymphocytes have two major functions – production of cytokines, such as IFN-γ, and CTL activity. In addition to its role in combating murine infections, IFN-γ has been shown to inhibit rickettsial growth in human macrophages, macrophage-like cell lines, fibroblasts and endothelial cells *in vitro* (Hickman *et al.* 1993). However, by using MHC-1 or IFN-γ gene knockout mice and by studying MHC-1-restricted CTL activity, Walker *et al.* (2001) demonstrated the importance of CTL activity in providing immunity to rickettsial infection, and suggested that CTL activity may be more important than production of IFN-γ and may operate by induction of apoptosis in infected liver cells, with subsequent phagocytosis of these cells by perivascular macrophages.

While *P. salmonis* conforms to the historical definition of a rickettsia as being an obligate intracellular bacterium, 16S rRNA analysis indicates that *P. salmonis* is phylogenetically unrelated to the majority of *Rickettsiales* (Fryer *et al.* 1992; Mauel *et al.* 1996). However, a role for $CD8^+$ T-cells, $CD4^+$ T-cells, IFN-γ and TNF-α has also been suggested in mammalian immunity to other intracellular bacteria such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Harty and Bevan, 1999). Given the increasing similarities being revealed between the mammalian and teleost immune systems, it seems likely that somewhat similar mechanisms may be involved in mounting an immune response to intracellular bacteria. Thus, it would seem advisable to examine specific cell-mediated responses, in addition to serological responses, to determine if correlation can be found between the response and effective immunity.

1.3.2 Vaccination against *P. salmonis*.

P. salmonis infections respond poorly to antibiotic treatments and for many years there has been considerable interest in developing a vaccine (Smith *et al.* 1997; Kuzyk *et al.* 2001a). Design of an effective vaccine against *P. salmonis* requires further investigation to elucidate which antigens, presented in association with MHC molecules, stimulate the immune response. As discussed above, elements of CMI, particularly CTLs, are required for resolution of infections which remain cell-associated in the pathogenic process and an MHC I-restricted CTL response has been shown to be important in the control of several intracellular bacteria infecting mammals (Leong *et al.* 1997; Dijkstra *et al.* 2001). However, in fish, the identification of such T-cell epitopes has not been possible since T-cell markers have not been available (Leong *et al.* 1997). By way of overcoming this problem, Kuzyk *et al.* (2001a) reported on the use of promiscuous T-cell epitopes (TCEs) incorporated into fusion protein constructs to enhance the immunogenicity of other epitopes within the contruct. His team cloned a 17 kDa putative outer surface protein of *P. salmonis* OspA, and constructed chimeric fusion proteins with OspA using promiscuous TCEs from measles virus fusion protein (MVF) and *Clostridium tetani* tetanus toxin P2 epitope. These xenobiotic TCEs are strong T_H cell epitopes which are MHC class II restricted and are able to bind MHC class II molecules from a variety of haplotypes. In this study, it was demonstrated that the addition of TCEs, particularly MVF, dramatically improved the protective response generated by vaccination with OspA alone. The greatest protective effect was obtained when salmon were vaccinated with an OspA construct including both TCEs. Given the intracellular life-cycle of *P. salmonis*, it was felt that the increased

protection obtained by incorporating T-cell epitopes into their vaccine could be attributed to improved CTL effector responses (Kuzyk *et al.* 2001a).

Vaccine efficacy can be defined as the degree to which it induces protective immunity in the target host (Reitan and Secombes, 1997). The influence of different antigen delivery systems on the Ab isotype and lymphokine profile is known in mammals. Certain Ab isotypes activate complement, promote phagocytosis, and mediate Ab-dependent cellular toxicity and are more protective than other isotypes. Likewise, adjuvants can affect the immune response that is elicited. The basis for this difference in immune response is not fully explained but seems to be dependent on the cytokines induced by the different adjuvants and delivery systems. While isotype-switching has not been observed in fish, cytokines have been shown to augment the immune response and thus fish cytokines may be useful as enhancers for vaccines (Leong *et al.* 1997). In addition, priming for release of cytokines that stimulate non-specific defences is of significance where relatively crude vaccines are used and where protective epitopes are not well defined. Cytokines, such as MAF, can be released by gut lymphocytes as well as by blood and kidney leucocytes and, therefore, additional protection may be gained by enhancing non-specific defences through priming of mucosal sites by oral vaccination (Secombes *et al.* 1996).

Reliable and reproducible methods to evaluate vaccines are needed to substitute for expensive and unpredictable challenge tests. With the increased use of vaccines, studies on the role of immune responses in fish have advanced and reagents to monitor defined aspects of the immune system have become available (Reitan and Secombes, 1997). The detection of a variety of lymphocyte responses following immunisation may be a useful way of analysing whether a particular vaccination programme has been successful, especially if

such responses correlate with disease resistance. These studies have usually been confined to the analysis of Ab production or lymphocyte proliferation following exposure to T-cell mitogens (Marsden *et al.* 1994).

With products where serology can be correlated with protection, the production of significant Ab titres can be used instead of challenge testing. It has been shown that vaccination against a number of bacterial diseases in fish is frequently followed by a significant increase in humoral responses against the pathogen (Reitan and Secombes, 1997). Obtaining samples for serology is relatively easy and assays can be adapted for high throughput analysis by ELISA. Furthermore, ELISA specificity has been improved by the availability of mAbs to fish Igs of an increasing number of fish species. However, the specificity of the assays for humoral responses is not always well documented and the association between an immune response and protection is not always consistent (Fryer *et al.* 1990; Marsden *et al.* 1994; Reitan and Secombes, 1997). The reliability of serological methods depends on a correlation between humoral Abs and protection, which is not yet well established for many fish diseases (Reitan and Secombes, 1997). For example, Kuzyk *et al.* (2001a) found no correlation between elevated Ab titres to OspA and increased protection against *P. salmonis*.

As Ab titres often do not correlate with protection against disease, it is perhaps surprising that more studies on CMI have not been carried out (Marsden *et al.* 1994). However, assays for CMI in fish have been hindered because they are less specific than Ab assays and there can be large variations between individuals (Reitan and Secombes, 1997). While most studies of CMI in fish have looked at lymphokine release following stimulation with T-lymphocyte mitogens, Marsden *et al.* (1994) examined lymphokine (MAF)

production in rainbow trout in response to a specific antigen. MAF was chosen, as it is released from T-lymphocytes and capable of being specifically primed by vaccination. It was demonstrated that peak production of MAF by antigen-primed cells corresponded with Ab titres. In addition, the released MAF was found to increase phagocyte bactericidal activity. Thus, not only would lymphokine release appear to be an alternative to Ab titre as an *in vitro* indicator of immune response following vaccination, but monitoring of lymphokine production may also indicate that effective stimulation of the CMI has been achieved.

1.4 Aims of the project:

The overall aim of this project was to examine the interaction between the fish pathogen *P. salmonis* and its host, with a view to vaccine development. The project is divided into three main parts.

Part 1: In Chapter 2, the aim was to confirm the identity of a RLO, isolated from diseased European seabass. On identification of the organism as *P. salmonis*, phylogenetic analyses were conducted to determine the relationship between the seabass isolate and the salmonid isolates from the Americas and Europe.

Part 2: In Chapters 3 and 4, the focus of the research was to examine the ability of *P. salmonis* to survive in the fish host and to investigate some mechanisms by which the pathogen might evade the host immune response.

Part 3: Schering-Plough Aquaculture Ltd. has produced a recombinant vaccine against *P. salmonis*. The ability of the vaccine to induce an immune response in salmonids was investigated by examining the stimulation of:

a) non-specific immunity through measurement of iNOS expression and the end products of NO metabolism in serum (Chapter 5).

b) specific cell-mediated immunity through measurement of MAF-production by immunised lymphocytes (Chapter 6).

c) specific humoral immunity through measurement of the levels of serum antibody against *P. salmonis* (Chapter 7).

Chapter 2 - Confirmation of *P. salmonis* as a pathogen in European sea bass *Dicentrarchus labrax* and phylogenetic comparison with salmonid strains

2.1 Introduction

Piscirickettsia salmonis, the causative agent of piscirickettsiosis or SRS, was first identified as a pathogenic agent in disease outbreaks amongst farmed coho salmon in Chile, during 1989 (Fryer *et al.* 1990; Branson and Diaz-Munoz, 1991; Cvitanich *et al.* 1991). Subsequently, the organism was confirmed as the agent responsible for clinical and chronic disease amongst farmed salmonids from both the Pacific and Atlantic coasts of Canada, Ireland, Norway and Scotland (Brocklebank *et al.* 1992; Palmer *et al.* 1996; Olsen *et al.* 1997; Birrell *et al.* 2003). The disease has appeared primarily to affect salmonids; chinook salmon, sakura salmon, rainbow trout, pink salmon *O. gorbuscha* and Atlantic salmon are all susceptible, albeit to differing degrees (House *et al.* 1999). While RLOs have been observed in non-salmonid hosts such as the blue-eyed plecostomus *Panaque suttoni* (Khoo *et al.* 1995), five species of cultured tilapia in Taiwan (Chen *et al.* 1994; Chern and Chao, 1994) and in Hawaiian tilapia (Mauel *et al.* 2003), to date the only non-salmonids in which a piscirickettsia-like organism (PLO) has been confirmed using serological methods are the white seabass *Atractoscion nobilis* in California, USA (Chen *et al.* 2000a) and the grouper, *Epinephelus melanostigma* in Taiwan (Chen *et al.* 2000b). Rickettsia-like organisms have also been identified in European sea bass (Comps *et al.* 1996) but, while antigenic similarities have been confirmed (Steiropoulos *et al.* 2002), the relatedness of these organisms to *P. salmonis* has not yet been determined.

Based on the sequence of its 16S rRNA gene, the *P. salmonis* type-strain, LF-89 (ATCC VR 1361), was placed among the Gammaproteobacteria (Fryer *et al.* 1992). Subsequently, Mauel *et al.* (1999) used comparisons of 16S rRNA gene sequences and ITS sequences to determine the relatedness of other *P. salmonis* isolates from Chile, Canada and Norway. They established that these strains formed a monophyletic group within the Gammaproteobacteria, although one Chilean isolate, EM-90, had diverged sufficiently to allow differentiation from the other isolates based on restriction fragment length polymorphism (RFLP) (Mauel *et al.* 1996). This group found only one ITS sequence in the isolates examined but, following polyacrylamide gel analysis of amplified ITS regions, Casanova *et al.* (2001), have suggested that *P. salmonis* may contain at least two rRNA (rrn) operons, as is commonly the case for other Gram-negative bacteria (Gürtler and Stanisich, 1996; Crosby and Criddle, 2003). More recently, Reid *et al.* (2004) have extended the information on regional variation of *P. salmonis* isolates through their comparison of 16S rDNA and ITS sequences from Scottish and Irish isolates.

In this study, routine diagnostic histopathological examination was conducted on European sea bass presenting with clinical signs of nervous disease. In paraffin sections an RLO was seen to be associated with the encephalitic lesions. Serological analyses, IFAT and IHC, were then used to confirm the tentative diagnosis and to confirm whether or not the organism was antigenically related to the *P. salmonis* type-strain, LF-89. The DNA sequences of the 16S rDNA and ITS region were then compared with those of published *P. salmonis* strains to establish whether or not the sea bass piscirickettsia-like organism (SBPLO) might be another strain of *P. salmonis* and how closely genetically related it was to the salmonid pathogens. Furthermore, it was sought

to establish if the sea bass isolate possessed at least two ITS regions, one of which contained tRNA genes in the 16S-23S spacer region.

2.2 Materials and methods

2.2.1 Fish

Juvenile sea bass from a farm in Greece which were exhibiting abnormal swimming and whirling behaviour, and which were experiencing low levels of mortality, were euthanized by terminal anaesthesia in 2-phenoxyethanol and sampled for histological analysis and screening by PCR for suspected *P. salmonis* infection. Samples of whole fish were fixed in 10% neutral buffered formalin (NBF) for histology, and samples of brain and mid-gut were preserved in 100% and 70% ethanol for subsequent extraction of genomic DNA for DNA analysis.

2.2.2 Examination of tissue sections

2.2.2.1 Histology.

Formalin fixed tissues for histological examination were embedded in paraffin, sectioned at 5.0 μm and stained with haematoxylin and eosin.

2.2.2.2 Serological analyses.

Slides were prepared from 5.0 μm paraffin-wax embedded tissue sections taken from the brain and midgut of infected sea bass. Sections prepared from the liver and kidney of rainbow trout *O. mykiss* experimentally infected with *P. salmonis* LF-89 (type-strain ATCC VR 1361) were used as a positive control, while sections from non-infected sea bass were used as negative controls. Sections were dewaxed in two successive xylene

baths (5 min each) and rehydrated in a 100% ethanol bath for 5 min followed by a 70% ethanol bath for 3 min. After rinsing with distilled water, tissue sections were encircled with wax using a PAP pen.

Indirect fluorescent antibody test (IFAT)

Sections were subjected to additional fixing in 95 % methanol for 5 min, washed with 0.01 M phosphate buffered saline (PBS, pH 7.4) and non-specific binding sites were blocked by incubation in goat serum (diluted 1/10 in PBS) for 10 min at room temperature (RT; approx. 21 °C). After washing in PBS, tissue sections were incubated with rabbit anti-*P. salmonis* serum (kindly provided by Professor J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, USA), diluted 1/100 in PBS. Tissue sections incubated with PBS only were also used as additional negative controls. Slides were washed again in PBS, then incubated with fluorescein isothiocyanate (FITC)–labelled, goat, anti-rabbit serum diluted 1/100 in PBS, for 30 min at RT. After a final washing step, slides were mounted in 50 % (v/v) glycerol and examined under oil immersion with an Olympus BX50 fluorescent microscope.

Immunohistochemistry (IHC)

The IHC procedure was carried out essentially as described by Alday-Sanz *et al.* (1994). The sections were incubated in 10 % (v/v) hydrogen peroxide in methanol for 10 min at RT, to block endogenous peroxidase activity. After washing with Tris-buffered saline (0.2 M TBS, pH 7.2), non-specific binding sites were blocked by incubation in goat serum, diluted 1/10 in TBS, for 10 min at RT. Slides were washed in TBS, tapped dry and incubated with anti-*P. salmonis* polyclonal antibody (pAb), diluted 1/100 in TBS, for 60 min in a humid chamber. Excess primary antibody was removed by washing in TBS and slides were incubated in goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, diluted 1/50 in TBS, for 30 min in a humid chamber.

Sections were then incubated with 1 % (v/v) hydrogen peroxide in 1.5 mg 3,3-diaminobenzidine tetrahydrochloride (DAB) and 10 ml TBS for 10 min. The reaction was stopped by immersing the slides in tap water and slides were then counterstained with haematoxylin for 3 min. Excess stain was removed by incubating the slides in tap water for 10 min followed by dehydration in 70% ethanol (3 min), 100% ethanol (5 min) and two successive xylene baths (2 x 5 min). Slides were mounted in Pertex and examined under a light microscope.

Concurrently, infected sea bass tissues were screened with an anti-*Photobacterium damselae* subsp. *piscicida* mAb in IFAT and IHC. Tissues from sea bass, positive and negative for *Ph. damselae* subsp. *piscicida*, were used as controls. The mAb was used according to the manufacturer's instructions, and either goat anti-mouse IgG-HRP or goat anti-mouse IgG-FITC diluted 1/100 in PBS was used as secondary antibody. The incubations with these antibodies were as described above.

2.2.3 DNA analysis

2.2.3.1 Isolation of genomic DNA.

Brain tissue (0.5 g), preserved in 70 % or 100 % ethanol, was excised using sterile scalpels and genomic DNA was extracted using a Nucleon ST Kit. Brain tissue from two fish was combined for each sample and two samples were extracted on two different days. As a control for possible contamination during the extraction process, concurrent extraction procedures were carried out in the absence of any tissues. Genomic DNA, extracted from cell monolayers infected with *P. salmonis* LF-89 or with *P. salmonis* strain SLGO-95 (kindly provided by Dr. P.A. Smith, Faculty of Veterinary Sciences, University of Chile, Santiago, Chile) was used as a positive control. Extracted DNA was stored at –20 °C until required for PCR.

2.2.3.2 PCR amplification of 16S rDNA.

Initially, a nested PCR amplification was carried out using the bacterial 16S primers, EubA and EubB, in the first-round amplification and Piscirickettsia-specific primers, PS2S and PS2AS, in the second-round amplification (Table 1) (Mauel *et al.* 1996). PCR amplifications were performed using 0.2 ml Ready-to-Go PCR beads, following the manufacturer's instructions, with a final concentration of 1 µM of the appropriate primer pair and 1 µl template DNA. For the second-round amplification, 1 µl of the first-round reaction was used as template. Cycling conditions were modified from Corbeil *et al.* (2003). Briefly, for primary PCR the mixture was denatured at 95 °C for 5 min and amplified with 35 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 40 s with a final extension step of 72 °C for 5 min. For nested PCR, the mixture was denatured at 95 °C for 5 min followed by amplification with 35 cycles of 94 °C for 30 s, 61 °C for 40 s, 72 °C for 40 s and a final extension step of 72 °C for 5 min. Subsequently, the nested PCR was carried out using primer pairs (Table 1) designed to amplify the region between nucleotides 225 and 1475, namely PS2S and 860R (nucleotides 226 – 860), PS2S and 1283R (226 – 1283) and 860F and 1470R (860 – 1476). The numbering corresponds to the published sequence for the *P. salmonis* type-strain LF-89 (GenBank accession number U36941) (Mauel *et al.* 1999). For these amplifications, cycling parameters were as described above except that each primer was used at 1 µM final concentration and an annealing temperature of 56 °C was used in the PCR cycle. All amplifications were performed using a Biometra T Gradient thermocycler (Anachem Ltd., Bedfordshire, UK) and 5 µl of each amplification reaction was examined for specificity by electrophoresis on a 1 % agarose gel containing 0.5 $mg.ml^{-1}$ ethidium bromide. Molecular weight markers (DNA Molecular Weight Marker VI or DNA Molecular Weight Marker XIV) were added to the gel as a reference.

2.2.3.3 PCR amplification of the ITS region.

A nested PCR amplification was carried out, using conditions and cycling parameters as described above for the 16S rDNA PCR. However, primers PS16SA and PS23SB were used in the first round reaction and primers PS16SH and PS23SC used in the second round as previously described by Mauel *et al.* (1999) (Table 2.1). Subsequently, using primers ITSUF and ITSUR, complimentary to *P. salmonis* LF-89 16S and 23S rDNA sequences (Table 2.1), direct PCR amplification was carried out on DNA isolated from infected sea bass. These primers were also used for nested PCR amplification of first round products obtained with primers PS16SA and PS23SB. Amplification conditions were as described above for nested 16S rDNA PCR except that each primer was used at 0.5 μM with an annealing temperature of 58 °C. A faint secondary product produced by this amplification was excised from the gel and re-amplified using 30 cycles and either primer pair PS16SH / PS23SC or primer pair ITSUF / ITSUR.

2.2.3.4 DNA sequencing.

In order to obtain template DNA for sequencing, the GFX™ PCR DNA and Gel Band Purification Kit was used to clean amplification reactions or to clean fragments excised from gels. Sequencing reactions were carried out using a DYEnamic™ ET terminator kit Cycle Sequencing Kit and following the manufacturer's instructions. Primers used for the nested PCR reactions were diluted to 5 pmol and used as sequencing primers. Each segment of DNA was sequenced in the forward and reverse direction, following at least three separate PCR amplifications on different days, with the exception of the secondary ITS fragment which was amplified in a single run using two different primer pairs, as described above. Sequencing reactions were run on the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Cheshire, UK), were viewed using BioEdit software (Hall, 1999) and aligned using Clustal X (Thompson *et al.* 1994) with manual

Table 2.1. Sequences and specificity of primers used for 16S rRNA gene and internal transcribed spacer (ITS) region PCR and sequencing. F: forward; R: reverse

Primer/location	Sequence (5'-3')	Specificity
Eub B (27F)[a]	AGAGTTTGATCMTGGCTCAG	Eubacterial
Eub A (1518R)[a]	AAGGAGGTGATCCANCCRCA	Eubacterial
PS2S (223F)[a]	CTAGGAGATGAGCCCGCGTTG	*P. salmonis* 16S
PS2AS (690R)[a]	GCTACACCTGCCAAACCACTT	"
850F (851F)[b]	GGATTCCCTTGAGGAGTTTAGTGG	"
850R (828R)[b]	CCACTAAACTCCTCAAGGGACTCC	"
1280R (1283R)[b]	CTTTCTCAGGTTCGCTCCAC	"
1490R (1487R)[b]	CTTCACCCCAGTCATGACCC	"
PS16SA (1387F)[a]	GCCTTGTACACAACCGCCC	*P. salmonis* ITS
PS23SB (507R)[c]	CCTTTCCCTCACGGTCAT	"
PS16SH (1519F)[b]	CCTGCGGCTGGATTACCT	"
PS23SC (203R)[c]	TAGATGTTTCAGTTCCCC	"
ITSUF (1430F)[b]	AGTGAATTGCACCAGAAGGG	"
ITSUR (303R)[c]	ATCACCCTCTATCGCCACAC	"

[a] Numbering corresponds to *Escherichia coli* 16S rRNA gene

[b] Numbering corresponds to *P. salmonis* LF-89 16S rRNA gene

[c] Number of bases from the 5' end of the *P. salmonis* 23S rRNA gene

editing. Sequences obtained for the SBPLO 16S rDNA were compared with published *P. salmonis* sequences, sequences from fish pathogens or from other members of the Gammaproteobacteria (see Table 2.2), while sequences obtained for ITS DNA were compared amongst *P. salmonis* strains only. Phylogenetic trees were constructed using PHYLIP version 3.6 (Felsenstein, 1989). Distance matrices generated by DNADIST were determined using the assumptions of Kimura (1980). These matrices were used to generate dendrograms using the neighbour-joining method (Saitou and Nei, 1987). Dendrograms were also constructed using the parsimony programme DNAPARS and the maximum-likelihood programme DNAML, in the PHYLIP software package. The bootstrap values were obtained from 1000 trees generated with SEQBOOT and CONSENSE within PHYLIP.

Table 2.2. Bacterial species and strains used in this study and the GenBank accession numbers for their 16S rDNA and ITS DNA sequences

Species/strain	GenBank Accession Number	DNA sequence
Piscirickettsia salmonis SBPLO	AY542956	16S rDNA
Piscirickettsia salmonis LF-89	U36941	"
Piscirickettsia salmonis SLGO-94	U55015	"
Piscirickettsia salmonis NOR-92	U36942	"
Piscirickettsia salmonis EM-90	U36940	"
Piscirickettsia salmonis ATL-4-91	U36915	"
Piscirickettsia salmonis IRE-91A	AY498633	"
Piscirickettsia salmonis IRE-98A	AY498634	"
Piscirickettsia salmonis IRE-99D	AY498637	"
Piscirickettsia salmonis SCO-95A	AY498636	"
Piscirickettsia salmonis SCO-02A	AY498635	"
Piscirickettsia salmonis SBPLO (ITS_0)	AY607584	ITS and 23S rDNA
Piscirickettsia salmonis SBPLO (ITStRNA)	AY607585	"
Piscirickettsia salmonis LF-89	U36943	"
Piscirickettsia salmonis EM-90	U36944	"
Piscirickettsia salmonis ATL-4-91	U36945	"
Piscirickettsia salmonis NOR-92	U36946	"
Piscirickettsia salmonis SLGO-94	U62104	"
Piscirickettsia salmonis C1-95	U62103	"
Piscirickettsia salmonis IRE-91A	AY498625	"
Piscirickettsia salmonis IRE-98A	AY498624	"
Piscirickettsia salmonis IRE-99C	AY498632	"
Piscirickettsia salmonis IRE-99D	AY498631	"
Piscirickettsia salmonis SCO-95A	AY498621	"
Piscirickettsia salmonis SCO-98B	AY498630	"
Piscirickettsia salmonis SCO-98C	AY498629	"
Piscirickettsia salmonis SCO-02A	AY498628	"
Piscirickettsia salmonis SCO-02D	AY498622	"
Piscirickettsia salmonis SCO-02E	AY498623	"
Piscirickettsia salmonis SCO-02F	AY498626	"
Piscirickettsia salmonis SCO-02G	AY498627	"
Vibrio anguillarum	X16895	16S rDNA
Photobacterium damselae subsp. *piscicida*	AB026844	"
Aeromonas salmonicida	X60405	"
Methylophaga marina	X95459	"
Beggiatoa sp.	AF035956	"
Piscirickettsia grp. Clone LA7-B48N	AF513949	"
Tilapia parasite	AF206675	"

2.3 Results

2.3.1 Histology

In general, lesions found in infected tissue sections were necrotizing and granulomatous. Liver, kidney, gastro-intestinal tract, pancreas, muscle and subdermis were involved to variable degrees from one fish to another, but the organ most consistently affected was the brain. Here, lesions were also necrotizing and granulomatous, involving mostly brain stem, but in some fish they extended also to involve the tegmentum and olfactory lobes and tracts. In most fish, lesions were present in the third ventricle, involving the ciliated ependymal cells lining this, or those cells immediately beneath. The ventricular lumen sometimes was filled with debris and large numbers of eosinophilic foamy macrophages, some of which contained dense, basophilic, spherical, 1 µm-sized RLOs. These large foamy gemistocyte-like cells dominated the inflammatory response in the brain.

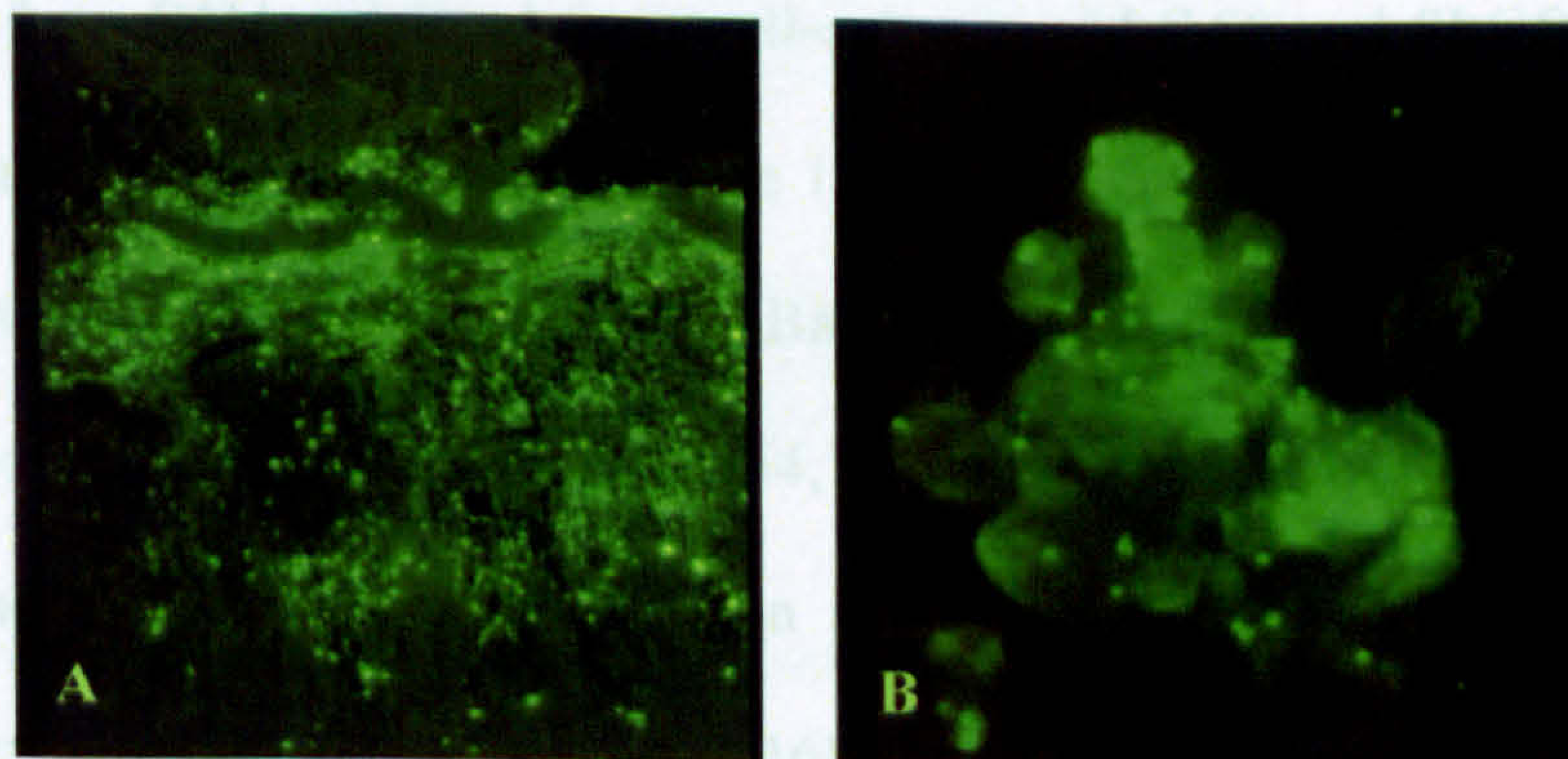


Fig. 2.1. Indirect fluorescent antibody test, using anti-*P. salmonis* polyclonal serum, to detect (A) sea bass piscirickettsia-like organisms (SBPLOs) in the brain of European sea bass (*D. labrax*) (B) *P. salmonis* strain SLGO-95 in CHSE-214 cell culture smear. Magnification: a = x100; b=x1000.

2.3.2 Serological analyses

In sections of sea bass brain tissue, ring-shaped organisms were detected by IFAT in the tegmentum using anti-*P.salmonis* pAb as primary antibody (Fig.2.1A). The appearance of these organisms was similar to the *P. salmonis* type-strain LF-89, seen in IFAT of cell culture smears (Fig.2.1B). No organisms were detected in the non-infected tissue sections or in sections incubated with anti-*Ph. damselae* subsp. *piscicida* mAb (not shown). In IHC, DAB deposition corresponded with areas of necrosis in the medulla oblongata (Fig. 2.2 B) and the sensory epithelium of the olfactory organ (Fig. 2.2 C, D & E). No organisms were detected in the non-infected tissue or in sections incubated with anti-*Ph. damselae* subsp. *piscicida* mAb (Fig. 2.2 A).

2.3.3 Nested PCR and sequence analysis

2.3.3.1 16S rDNA.

Nested PCR of DNA extracted from sea bass samples, using *P. salmonis*-specific primers PS2S and PS2AS, amplified a product of approximately 470 bp. A fragment of similar size was amplified from DNA extracted from cell-cultures of LF-89 and SLGO-95. Sequence analysis confirmed the sea bass product to be 16S rDNA, corresponding to nucleotides 226 – 673 of the published LF-89 sequence (GenBank accession number U36941) and differing from this sequence at positions 448, 450-454, 464-468, 470 and 641. Subsequent nested PCR and sequencing of the region between nucleotides 226-1475 confirmed the initial findings and revealed that the sea bass RLO 16S rDNA also differed from LF-89 rDNA at a further 10 positions (859, 876, 918, 928, 960, 1005, 1289, 1402, 1084, 1103). A sequence

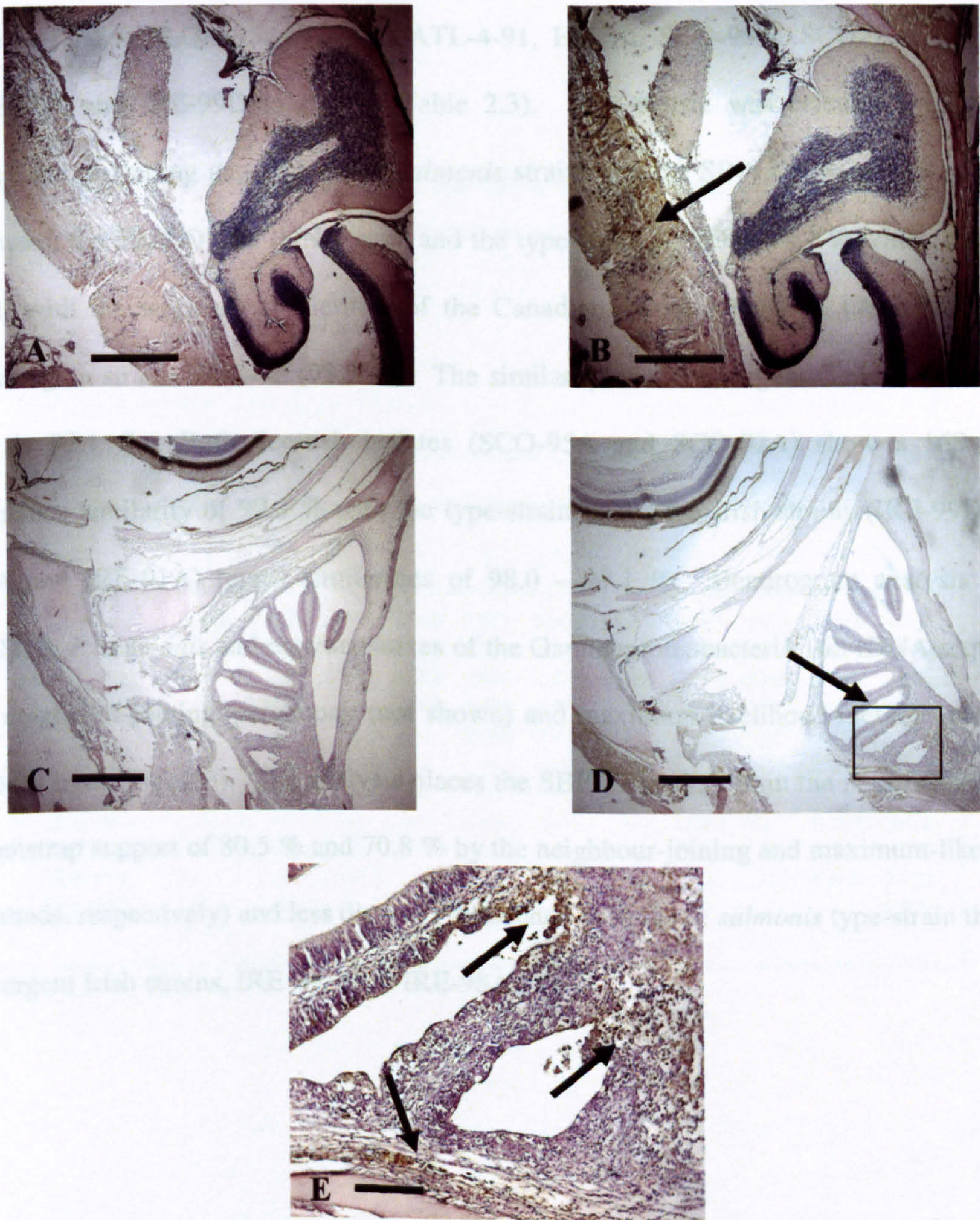


Fig. 2.2. Immunohistochemical staining of sea bass brain sections using anti-*Ph. damselae* subsp. *piscicida* mAb (A); and rabbit anti-*P. salmonis* serum (B) & (C). Scale bar = 500 µm. Arrows indicate areas of DAB deposition. Boxed area in (D) indicates area enlarged in (E). Scale bar = 100 µm.

identity matrix, constructed from the 16S rDNA sequence of the SBPLO, and *P. salmonis* strains LF-89, SLGO-95, NOR-92, ATL-4-91, EM-90, SCO-95A, SCO-02A, IRE-91A, IRE-98A and IRE-99D is shown (Table 2.3). This matrix was calculated from 1297 positions (including gaps) of the *P. salmonis* strains and the SBPLO. Sequence similarity between the SBPLO 16S rDNA gene and the type-strain LF-89 is 98.2 % which compares well with the sequence similarities of the Canadian strain, ATL-4 91 (98.7 %) and the Norwegian strain, NOR-92 (98.9 %). The similarity of the divergent Chilean strain, EM-90, is 97.1 %. Both Scottish isolates (SCO-95A and SCO-02A) show a 16S rDNA sequence similarity of 99.3 % with the type-strain, while the Irish strains (IRE-99D, IRE-98A and IRE-91A) reveal similarities of 98.0 – 97.1 %. Dendrogram analysis of the SBPLO, *P. salmonis* and representatives of the Gammaproteobacteria 16S rDNA sequences by neighbour-joining, parsimony (not shown) and maximum likelihood methods produced similar trees (Fig. 2.3). The analysis places the SBPLO strain within the *P. salmonis* group (bootstrap support of 80.5 % and 70.8 % by the neighbour-joining and maximum-likelihood methods, respectively) and less distantly associated with the *P. salmonis* type-strain than the divergent Irish strains, IRE-91A and IRE-98A.

Table 2.3. Sequence similarities of partial 16S rDNA sequences between 10 isolates of *P. salmonis* and the SBPLO isolate. The comparison was made using 1297 nucleotide positions, including gaps.

	% similarity to										
Isolates	1	2	3	4	5	6	7	8	9	10	11
1. LF-89	1.000	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
2. SLGO-94	---	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
3. ATL-4-91	---	---	1.000	0.989	0.971	0.976	0.988	0.988	0.966	0.973	0.976
4. NOR-92	---	---	---	1.000	0.972	0.977	0.989	0.989	0.966	0.974	0.976
5. EM-90	---	---	---	---	1.000	0.960	0.972	0.972	0.959	0.959	0.975
6. SBPLO	---	---	---	---	---	1.000	0.988	0.988	0.976	0.983	0.974
7. SCO-95A	---	---	---	---	---	---	1.000	1.000	0.977	0.985	0.986
8. SCO-02A	---	---	---	---	---	---	---	1.000	0.977	0.985	0.986
9. IRE-91A	---	---	---	---	---	---	---	---	1.000	0.991	0.972
10. IRE-98A	---	---	---	---	---	---	---	---	---	1.000	0.972
11. IRE-99D	---	---	---	---	---	---	---	---	---	---	1.000

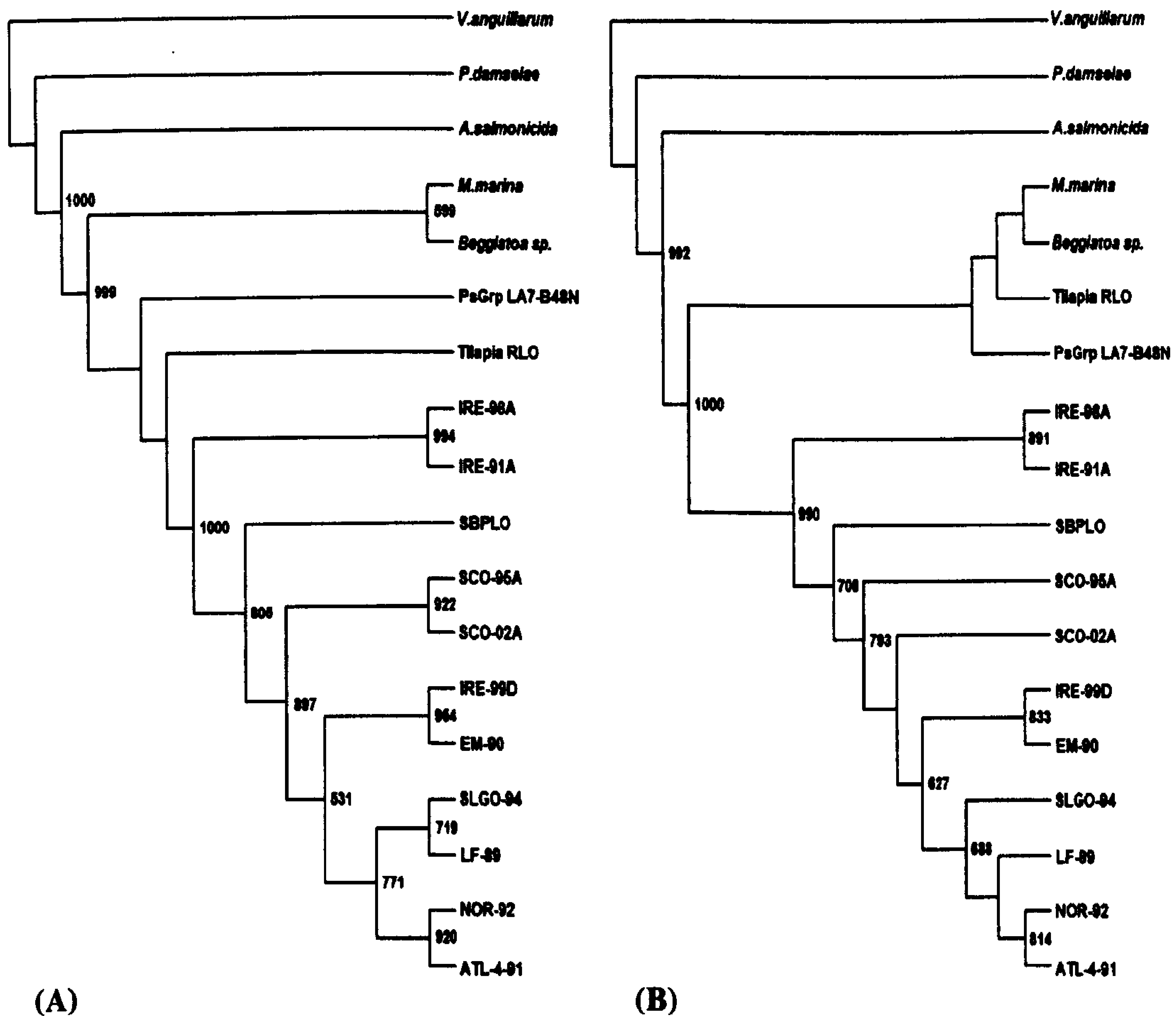


Fig. 2.3 Phylogenetic relationships of partial 16S rDNA sequences between the SBPLO, *P. salmonis* strains and members of the Gammaproteobacteria (Table 2.2) inferred from the neighbour-joining (A) and maximum-likelihood (B) methods using *V. anguillarum* (GenBank accession no. X16895) as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50 %. Dendrograms were inferred from 1132 positions, between nucleotide positions 226-1350 of *P. salmonis* LF-89.

2.3.3.2 ITS region.

Nested PCR of the sea bass RLO ITS region using primer pair PS16SA/PS23SB in the first round and primer pair PS16SH/PS23SC in the second round amplifications was successful on only one occasion and produced readable sequence from an amplified product of approximately 520 bp. Subsequent direct or nested PCR amplifications using primers ITSUF and ITSUR produced two fragments, a major product of approximately 750 bp and a faint, secondary product of approximately 1050 bp (Fig. 2.4). When sequenced, the major SBPLO product (ITS_0) confirmed our initial sequence obtained using primers PS16SA/PS23SB and PS16SH/PS23SC. This sequence also corresponded overall with the LF-89 ITS sequence (GenBank accession number U36943) found by Mauel *et al.* (1999) and with the LF-89 ITS B sequence reported by Casanova *et al.* (2001). The SBPLO ITS_0 differed at 32 positions from LF-89, mainly as the result of a 17-base gap in the SBPLO sequence between positions 223-239 (numbering calculated from the 5' end of the LF-89 ITS sequence) (Fig. 2.5). Comparison of the complete ITS_0 DNA sequence (between positions 1-292 and including gaps) reveals a similarity of 87.7 % with the corresponding LF-89 sequence (Table 2.4). If the 17-base gap region is excluded from the analysis for all strains, the SBPLO/LF-89 sequence similarity is increased to 93.8 % but the position of the SBPLO within the range of similarities is not significantly altered (data not shown).

The sequence identity matrix shows that the SBPLO ITS_0 sequence exhibits a higher sequence identity with other members of the *P. salmonis* group than the Irish isolates IRE-91A and IRE-98A (76.8 % and 77.4 %, respectively). The Scottish isolates and the remaining Irish isolates, IRE-99C and IRE-99D, show a sequence divergence from the type-strain of less than 10 %.

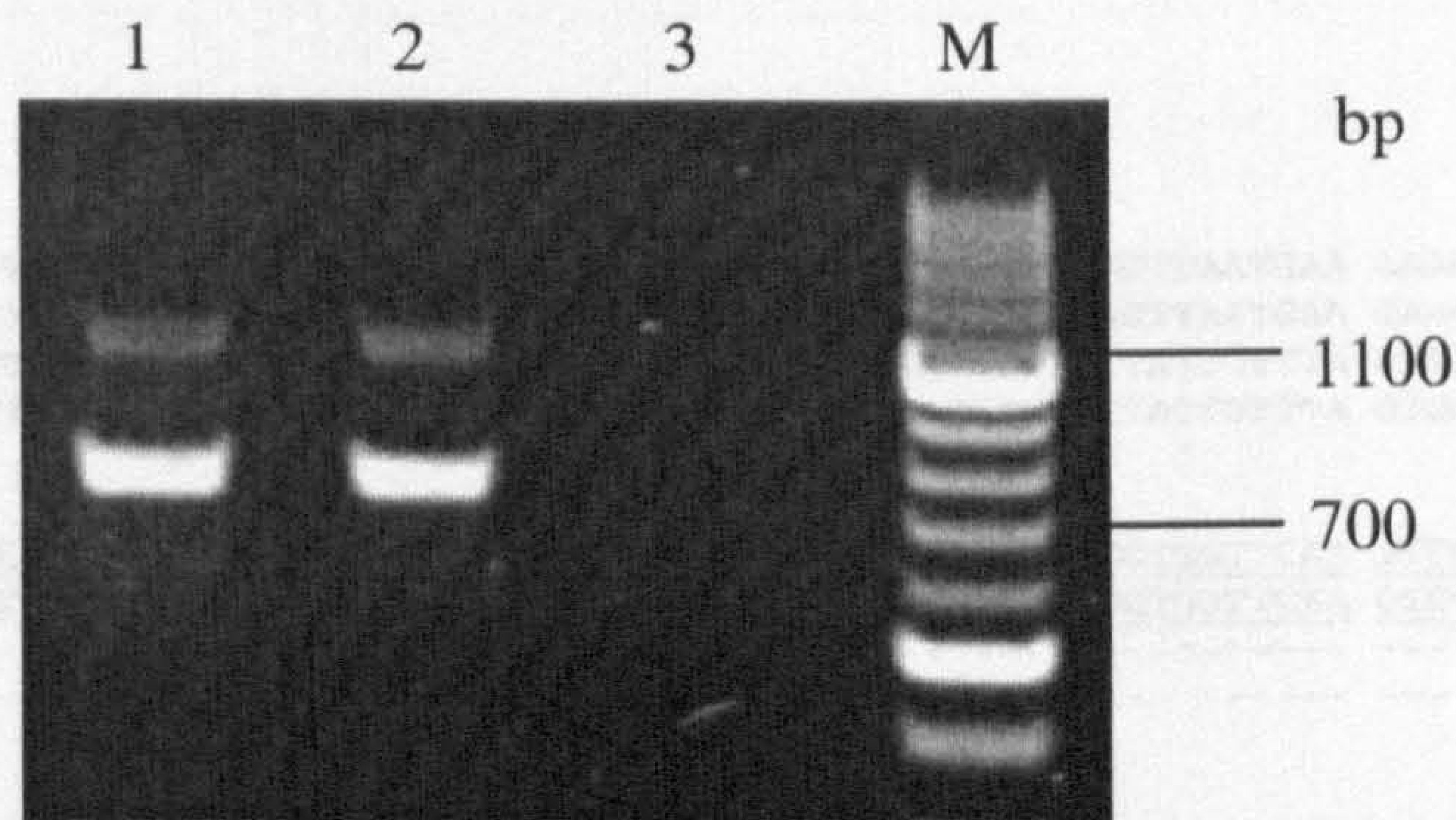


Fig. 2.4 Products of direct PCR, using primer pair ITSUF/ITSUR, for amplification of internal transcribed spacer region (ITS) DNA. Lanes 1 & 2: DNA isolated from SBPLO-infected sea bass; Lane 3: negative control; M: molecular weight marker.

Dendrograms inferred from the SBPLO ITS_0 and *P. salmonis* ITS sequences (nucleotide positions 49 – 310), obtained with either distance or parsimony tree-building methods, produced similar trees (Fig. 2.6). This analysis again placed the SBPLO strain within the *P. salmonis* group and, while this strain is divergent from most of the group, it is more closely related to the type-strain than the Irish isolates, IRE-91A and IRE-98A. Bootstrap support for this position was 100 % and 99.1 % for neighbour-joining and parsimony methods respectively. Following excision from the gel, re-amplification and sequencing, the secondary SBPLO product (ITS_{tRNA}) was found to contain genes coding for $tRNA^{ile}$ and $tRNA^{ala}$, and to correspond essentially with the LF-89 ITS A sequence described by Casanova *et al.* (2001). Both SBPLO ITS sequences (ITS_0 GenBank accession number AY607584; ITS_{tRNA} GenBank accession number AY607585) are shown in Figure 2.5.

```
                                                                                        70
SBPLO ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGT-C ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
LF-89 ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
SBPLO ITSo       ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA
LF-89 ITS        ATTTAY-GAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA

                                                                                       140
SBPLO ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
LF-89 ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
SBPLO ITSo       TA-------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        TA-------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                       210
SBPLO ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
LF-89 ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                       280
SBPLO ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
LF-89 ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                       350
SBPLO ITStRNA    AATGCCGTGA AATGATTATT AG--ATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
LF-89 ITStRNA    AATGCCGTGA AATGATTATT TATAATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- -------TTT
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- -------TTT

                                                                                       420
SBPLO ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG
SBPLO ITSo       GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITS        GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG

                                                                                       490
SBPLO ITStRNA    ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITStRNA    GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
SBPLO ITSo       ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITS        GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT

                                                                                       560
SBPLO ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG
SBPLO ITSo       GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITS        GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG

                                  580
SBPLO ITStRNA    ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITStRNA    ATATAAGACT TCTTGGGGTT ATATGA
SBPLO ITSo       ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITS        ATATAAGACT TTNTGGGGTT ATATGA
```

Fig. 2.5 Sequences of the complete SBPLO ITS_0 and ITS_{tRNA} aligned with the corresponding sequences from the *P. salmonis* type-strain, LF-89. Sequences corresponding to the $tRNA^{ile}$ and $tRNA^{ala}$ genes are underlined. Dashes indicate gaps.

Table 2.4. Sequence similarities of partial ITS DNA sequences between 18 isolates of *P. salmonis* and the SBPLO isolate. The comparison was made using 269 nucleotide positions, including gaps.

	% similarity																		
Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. LF-89	1.000	0.962	0.965	0.958	0.958	0.958	0.877	0.950	0.950	0.950	0.946	0.950	0.943	0.954	0.954	0.768	0.774	0.942	0.931
2. SLGO-94	—	1.000	0.988	0.973	0.973	0.939	0.867	0.966	0.965	0.965	0.962	0.965	0.958	0.969	0.969	0.755	0.761	0.920	0.909
3. C1-95	—	—	1.000	0.969	0.969	0.935	0.856	0.962	0.962	0.962	0.958	0.962	0.954	0.965	0.965	0.751	0.757	0.916	0.905
4. ATL-4-91	—	—	—	1.000	0.992	0.931	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.755	0.761	0.924	0.912
5. NOR-92	—	—	—	—	1.000	0.939	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.763	0.768	0.931	0.920
6. EM-90	—	—	—	—	—	1.000	0.877	0.924	0.924	0.924	0.920	0.924	0.916	0.928	0.928	0.761	0.759	0.938	0.927
7. SBPLO	—	—	—	—	—	—	1.000	0.867	0.867	0.867	0.863	0.871	0.859	0.871	0.871	0.833	0.830	0.858	0.847
8. SCO-95A	—	—	—	—	—	—	—	1.000	0.992	0.992	0.988	0.992	0.984	0.996	0.996	0.760	0.765	0.931	0.920
9. SCO-98B	—	—	—	—	—	—	—	—	1.000	0.992	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
10. SCO-98C	—	—	—	—	—	—	—	—	—	1.000	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
11. SCO-02A	—	—	—	—	—	—	—	—	—	—	1.000	0.988	0.996	0.992	0.992	0.755	0.761	0.927	0.916
12. SCO-02D	—	—	—	—	—	—	—	—	—	—	—	1.000	0.984	0.996	0.996	0.763	0.768	0.931	0.920
13. SCO-02E	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.988	0.988	0.751	0.757	0.923	0.912
14. SCO-02F	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	1.000	0.763	0.768	0.935	0.923
15. SCO-02G	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.763	0.768	0.935	0.923
16. IRE-91A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.966	0.765	0.753
17. IRE-98A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.770	0.759
18. IRE-99C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.988
19. IRE-99D	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000

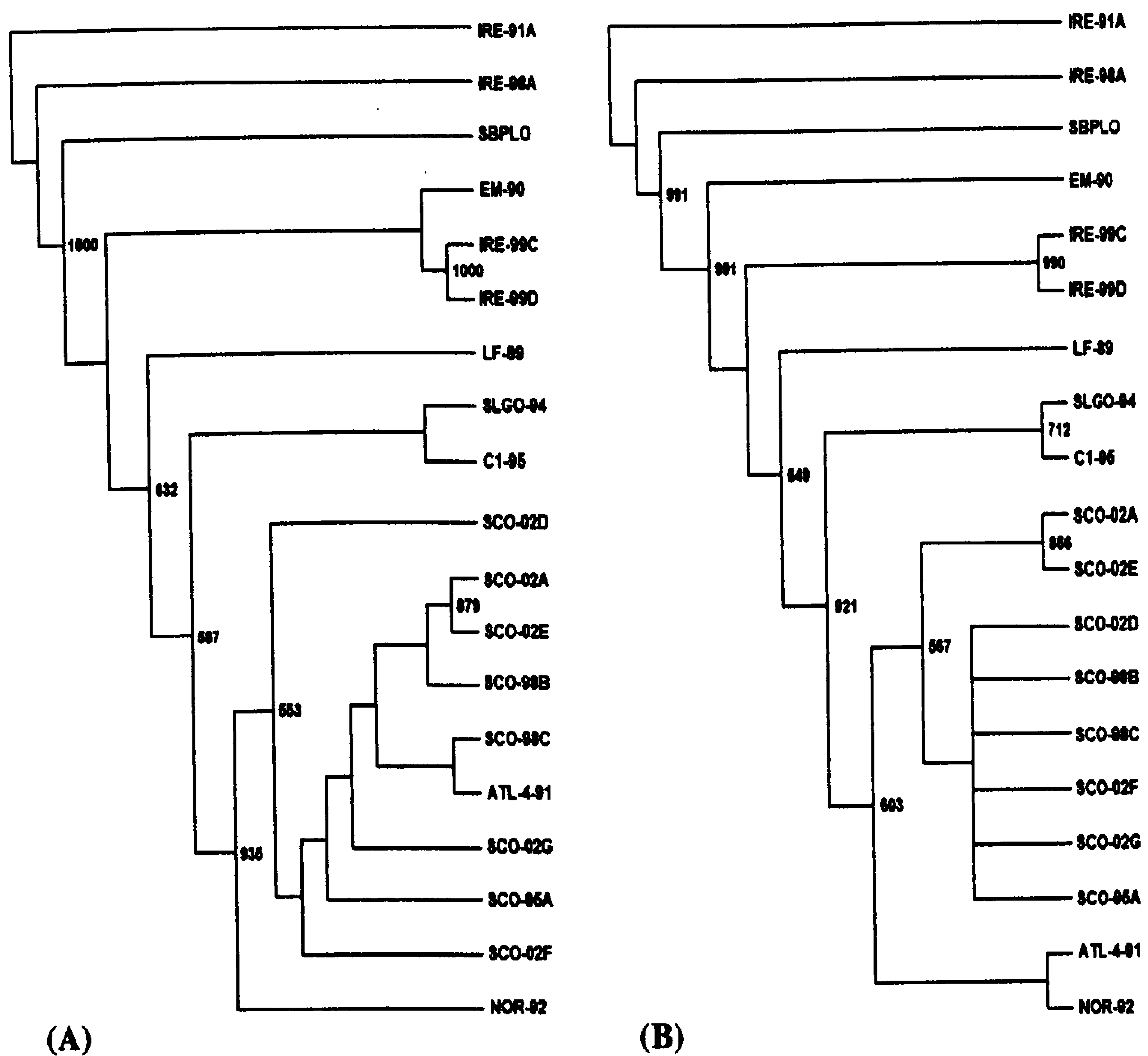


Fig. 2.6 Phylogenetic relationships of partial ITS sequences between the SBPLO and *P. salmonis* strains inferred from the neighbour-joining (A) and parsimony (B) methods, using strain IRE-91A as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50 %. Dendrograms were inferred from 260 positions, between nucleotides 50 – 310 from the 5' end of the *P. salmonis* LF-89 ITS sequence.

2.4 Discussion

Histological analysis of tissues from European sea bass revealed the presence of RLOs in macrophages in the brain, which was the organ most consistently affected in diseased fish. Necrotic lesions in the anterior medulla oblongata and the presence of 1 μm basophilic organisms in cytoplasmic vacuoles have previously been described for RLO infection of sea bass (Comps *et al.* 1996). The morphological similarity between organisms detected in the sea bass medulla oblongata and *P. salmonis* LF-89 cell culture smears by IFAT suggested that the sea bass pathogen was related to *P. salmonis*. Furthermore, the association of DAB deposition with areas of necrosis seen in IHC strengthened the argument that a *P. salmonis*-related organism was responsible for the pathology found in the sea bass. While initial, on-farm diagnosis suggested that *Ph. damselae* subsp. *piscicida* might have played a role in the disease evidence for involvement of *Ph. damselae* in the sea bass pathology was not found, as IHC using anti-*Ph. damselae* mAb was negative.

In salmonids, *P. salmonis* produces a systemic disease and, while pathological changes are found in heart, brain, intestine, ovary and gill, the most characteristic pathology is severe inflammation and necrotic lesions of the kidney, liver and spleen (Fryer *et al.* 1990; Rodger and Drinan, 1993; Palmer *et al.* 1996; Olsen *et al.* 1997). Likewise, in diseases of white seabass (Chen *et al.* 2000a) and grouper (Chen *et al.* 2000b), both associated with PLOs, and in diseases of tilapia (Chen *et al.* 1994; Chern and Chao, 1994) which were associated with RLOs, the kidney, spleen and liver also exhibited the most marked pathology, although lesions were commonly found in all tissues, including the brain. In the European sea bass, however, the most significant pathology has been associated with the brain (Comps *et al.* (1996) and this study) and, while antigenic

similarities between the sea bass RLO and *P. salmonis* have been intimated by cross-reaction with anti-*P. salmonis* antiserum in IHC (Steiropoulos *et al.* 2002), the different pathology suggested that the organisms might not be related. Of possible interest in this regard is a RLO which was associated with brain lesions and encephalitis in Scottish farmed Atlantic salmon, but which did not react in a latex agglutination test for *P. salmonis* (Grant *et al.* 1996).

Since the development of molecular genetic techniques, several methods have been used to differentiate between genera, species and strains of bacteria (Grayson *et al.* 1999; Romalde *et al.* 1999; Gürtler and Mayall, 2001; Houpikian and Raoult, 2001). For phylogenetic studies, where differentiation between strains is used to determine their inter-relatedness and how they may have evolved from a common ancestor, methods such as DNA-DNA hybridisation or pulse-field gel electrophoresis allow very fine discrimination between isolates (Leclerc *et al.* 2000; Houpikian and Raoult, 2001; Le Roux *et al.* 2004). However, as these methods are not practical for routine use and may prove too sensitive, their application might be most useful in differentiating between strains where a close relationship has already been demonstrated (Leclerc *et al.* 2000; Gürtler and Mayall, 2001; Le Roux *et al.* 2004).

Another method widely used for phylogeny is the comparison of aligned nucleotide sequences of conserved genes, which have been termed 'molecular chronometers' (Fournier *et al.* 1998). The rRNA genes are highly conserved across the bacterial kingdom and the 16S rRNA gene within the rrn operon contains highly conserved regions as well as regions that vary according to species, genera and family. Therefore, comparison of 16S rDNA

sequence has become widely used for the classification of organisms (Ruimy *et al.* 1994; Spröer *et al.* 1999; Gürtler and Mayall, 2001).

However, in some cases, the level of conservation between strains is such that differentiation between them on the basis of 16S rDNA sequence analysis is not possible. This is the case, for example, with mammalian rickettsiae (Stothard *et al.* 1994) and, in these situations, workers have looked to sequencing other, less-highly conserved, genes (Roux *et al.* 1996; 1997; Fournier *et al.* 1998). Another alternative is the ITS region DNA found between the 16S and 23S rRNA genes in the rrn operon (García-Martínez *et al.* 1999; Leclerc *et al.* 2000; Houpikian and Raoult, 2001; Hamid *et al.* 2002). Bacteria frequently possess several copies of the rrn operon in their genome (Gürtler and Stanisich, 1996; Crosby and Criddle, 2003) although there are exceptions such as the Mycobacteria and Mycoplasma where, as a rule, only one or two copies are present (Gürtler and Stanisich, 1996). It has been found that there can be considerable variation in the length and sequence of the copies of the 16S-23S ITS region even within a single genome and therefore, the scope for comparison between strains using ITS sequences is increased (Gürtler and Stanisich, 1996; Gürtler and Mayall, 2001; Hamid *et al.* 2002). The main source of variation is the number and type of tRNA genes found in the region between the 16S and 23S rRNA genes (Gürtler and Stanisich, 1996). The majority of Gram-positive bacteria do not possess tRNA genes within the ITS region but either $tRNA^{ala}$ or $tRNA^{ile}$ or both genes may be present. In Gram-negative bacteria, however, it is common to find genes for both $tRNA^{ile}$ and $tRNA^{ala}$, or only $tRNA^{glu}$ (Gürtler and Stanisich, 1996; Christensen *et al.* 2000). A further major source of variation between the ITS regions of different strains is in the form of insertion/deletions believed to have arisen from recombination events (Peréz-

Luz *et al.* 1998; Andersson *et al.* 1999; García-Martínez *et al.* 1999; Gürtler and Mayall, 2001). Because considerable variation in ITS sequence can occur not only between different strains of a genus but also between the operons of a single organism (intercistronic variation), the strength of ITS analysis may lie in fine discrimination between strains of bacteria where 16S rDNA analysis has already suggested a close relationship exists (Ruimy *et al.* 1994; Perez-Luz *et al.* 1998; García-Martínez *et al.* 1999). Furthermore, the limited number of published ITS sequences compared with the number of 16S rDNA sequences, means that initial phylogenetic placement of an organism will, most likely, be based on analysis of its 16S rRNA.

In the study of fish pathogens, differences in 16S rDNA sequences have been used for classifying isolates (Romalde *et al.* 1999; Reid *et al.* 2003). Using 16S rDNA sequences, Mauel *et al.* (1999) demonstrated a monophyletic relationship between strains of *P. salmonis* from Chile, Canada and Norway, with similarities ranging from 99.7 to 98.5 %. These workers also examined the DNA sequence of the ITS and of the 23S rRNA and found that, while these also showed low levels of divergence between isolates, the greater variability of these regions was reflected in the wider range of similarities; 95.2 – 99.7 % and 97.9 - 99.8 % respectively. Nevertheless, the phylogenetic trees derived from all three data sources, 16S rDNA, ITS and 23S rDNA, were in close agreement. Recently, Reid and Birkbeck (2003) compared 16S rDNA and ITS sequences from Scottish and Irish isolates and found that the Scottish isolates formed a homogenous group which was closely related to the Chilean isolates, LF-89 and SLGO-94. Irish isolates from 1999 clustered with EM-90, hitherto the most distantly related strain, while Irish isolates from 1991, 1995 and 1998 formed a new divergent group. However, while these groups found only a single rrn

operon, Casanova *et al.* (2001), using different PCR primers, identified two ITS sequences in the type-strain, LF-89 and in EM-90, by polyacrylamide gel electrophoresis of PCR amplified ITS region DNA. One ITS sequence was the same as that described by Mauel *et al.* (1999), while the second ITS contained genes for $tRNA^{ile}$ and $tRNA^{ala}$. Based on this observation, it was proposed that more than one rrn operon might exist in these Chilean isolates.

In this study, and in work by Steiropoulos *et al.* (2002), serological analysis had established the likelihood of a relationship between the organism found in European sea bass and those organisms identified as *P. salmonis* from Chilean, Canadian and Norwegian salmonids. However, detailed analysis of genetic similarities has not previously been undertaken. It has been confirmed, through phylogenetic analysis of 16S rDNA and ITS sequences, that the organism found in European sea bass is a member of the *P. salmonis* genus which clusters with other members of this genus for which sequence information is available. Sequence similarity between the sea bass 16S rRNA gene and the *P. salmonis* type-strain is 98.2 %, which compares well with sequence similarities of the Canadian and Norwegian strains. The greater variability of the 16S-23S spacer region is reflected in the lower sequence similarity of the ITS_0 sequence, but this does not affect the overall placement of the SBPLO isolate within the range of ITS sequence similarities exhibited among the currently available *P. salmonis* sequences. Although it is more divergent than the Scottish isolates and Irish isolates from 1999, both sequence similarity and dendrograms for 16S rDNA and ITS sequences suggest that the SBPLO isolate is more closely related to the majority of *P. salmonis* strains than the Irish isolates from 1991 and 1998.

The present study was conducted on a single isolate from one site in Greece. A more robust phylogenetic analysis will require the availability of further isolates from sea bass in the Mediterranean to determine whether or not these form a separate cluster within the *P. salmonis* group. Detailed comparisons of the tRNA-containing ITS sequence, between *P. salmonis* strains, may yield useful information about the geographical spread of the organism, while sequence comparisons of other genes coding for shared antigens, evident from serological analysis, may help to further elucidate relationships among the group. Ultimately, DNA-DNA hybridisation studies may suggest the requirement for creation of separate species.

The finding of RLOs in widely-differing fish species, from numerous locations world-wide, suggests that these are ubiquitous parasites, adapted to utilise whatever hosts are available locally. As with other pathogens, it will be important to determine whether it is environmental changes or the availability of naïve hosts which result in reduced host immunity or increased pathogenicity (host susceptibility), leading to the establishment of conditions which encourage disease outbreaks. Given the rapidly expanding aquaculture industry, with the frequent introduction of new species for farming, it seems likely that PLOs may, in the future, pose disease problems. As antibiotic treatment of disease caused by this intracellular pathogen is both costly and relatively inefficient (Fryer and Hedrick, 2003), successful combatting of this disease will rely largely upon the availability of vaccines. Therefore, it will be crucial to understand the mechanisms the organism uses to evade the host immune system and, using this knowledge, develop more effective vaccines.

Chapter 3 - Survival of *P. salmonis* in rainbow trout head kidney macrophages

3.1 Introduction

In natural outbreaks of disease, the presence of *P. salmonis* organisms has been reported in cytoplasmic vacuoles of hepatocytes and macrophages in liver, in macrophages in kidney and spleen and in macrophages in the peripheral blood (Branson and Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Almendras *et al.* 2000). The organism has also been described in macrophages associated with the inflammatory response in the brain of sea bass (Chapter 2). While the presence of *P. salmonis*, and occasionally of degenerating organisms and debris, within macrophages may simply be a result of macrophage activity in resolving the infection, it is also possible that *P. salmonis* is capable of replicating within macrophages, in common with other intracellular parasites.

Mycobacterium tuberculosis, *Legionella pneumophila*, *Listeria monocytogenes* and mammalian rickettsiae are mammalian pathogens which have developed strategies to avoid eradication by the macrophage bactericidal mechanisms (Turco and Winkler, 1983; De Chastellier and Berche, 1994; Raupach and Kaufmann, 2001; Radulovic *et al.* 2002; Allen, 2003). Among other survival strategies, mycobacteria prevent acidification of the phagosome and phagosome-lysosome fusion, thereby precluding digestion by lysosomal contents (D'Arcy Hart *et al.* 1972; Orme *et al.* 1993; Fenton and Vermeulen, 1996; Raupach and Kaufmann, 2001; Bannantine and Stabel, 2002; Tooker and Coussens, 2004; Weiss *et al.* 2004). *Legionella pneumophila*, likewise, avoids lysosomal enzyme digestion by preventing maturation of the phagosome and acquisition of lysosomal characteristics (Allen,

2003). On the other hand, while phago-lysosomal fusion does occur in *L. monocytogenes* infections, the organism can escape destruction by disruption of the phagosome membrane and escape into the cytoplasm (De Chastellier and Berche, 1994; Harty and Bevan, 1999). Once in the cytoplasm, the organism recruits host cell filamentous actin (F-actin) and propels itself across the cell by assembling actin filaments at one pole. Upon reaching the cell membrane, the bacterium forms a membrane protrusion which can be internalised by an adjacent cell, again followed by escape into the cytoplasm, thereby achieving direct cell-to-cell spread (Heinzen *et al.* 1993; Van Kirk *et al.* 2000; Vazquez-Boland *et al.* 2001). Actin-based motility (ABM) is also used by SFG rickettsiae *R. conorii* and *R. rickettsia* but is not used by the TG rickettsia, *R. prowazekii*, which escapes from the phagosome and mutiplies to high numbers in the cytoplasm of the host cell (Popov *et al.* 1987; Van Kirk *et al.* 2000).

While several fish pathogens have been reported to resist killing by macrophages (Evelyn, 1996; Chen *et al.* 1998), relatively few have been demonstrated to survive and multiply in these cells. While *Edwardsiella ictaluri* was killed by channel catfish (*Ictalurus punctatus*) macrophages (Shoemaker *et al.* 1997), virulent *E. tarda* strains were capable of survival and replication in blue gourami (*Trichogaster trichopteris* Pallas) adherent head kidney phagocytes, at least up to 6.5 h post-infection (Rao *et al.* 2001). *Photobacterium damselae* subsp. *piscicida* appeared to prevent phagosome-lysosome fusion and numbers of intracellular bacteria increased over the 18 h observation period, in head kidney macrophages from hybrid striped bass (*Morone saxatilis*) (Elkamel, 2002). Likewise, live *Mycobacterium* spp. were able to cause some inhibition of phagosome-lysosome fusion in rainbow trout (Chen *et al.* 1998). Meanwhile, the interaction of *Renibacterium*

salmoninarum with rainbow trout macrophages has been well studied and the bacterium has been shown to survive for 3-4 days in rainbow trout head kidney macrophages (Bandin *et al.* 1993; Bandin *et al.* 1995; Hardie *et al.* 1996; Gutenberger *et al.* 1997).

In this chapter, the following work was undertaken to investigate if *P. salmonis* was capable of survival and multiplication in rainbow trout (*O. mykiss*) macrophages and, if so, to explore possible reasons for this capability:

(a) to ascertain if *P. salmonis* is capable of long-term survival in *in vivo*-infected head kidney macrophages, culture supernatants from adherent macrophage monolayers infected with *P. salmonis* were inoculated onto CHSE-214 cells.

(b) the continued presence of the pathogen within macrophages was confirmed by TEM at various times following infection of macrophage monolayers, derived from normal fish, exposed to *P. salmonis in vitro.*

(c) the effect of vaccination on the persistence of the pathogen in macrophage monolayers was assessed by TEM on Day 6 following exposure of macrophages from immune and naïve fish to *P. salmonis in vitro.*

(d) the use of ABM by *P. salmonis* as a means of dissemination was investigated in CHSE-214 cells and in macrophage monolayers by TEM and confocal microscopy, respectively. Ruthenium red was used in TEM to stabilise actin filaments against oxidative fragmentation by osmium tetroxide (OsO_4) (Heinzen *et al.* 1993), while fluorescently-labelled phalloidin, a bicyclic peptide which specifically binds only cytoskeletal structures made of F-actin (Clerc and Sansonetti, 1987; Heinzen *et al.* 1993) was used to visualise actin by confocal microscopy.

3.2 Materials and Methods

3.2.1 Piscirickettsiae

Piscirickettsia salmonis LF-89 (ATCC VR 1361), the type-strain, was propagated in CHSE-214 cells (ATCC CRL 1681) (Lannan *et al.* 1984). Cell monolayers were grown at 22°C in sealed 25 cm^2 (T-25) tissue culture flasks containing Minimal Essential Medium with Earle's salts (EMEM) plus 10% foetal bovine serum (FBS), supplemented with MEM non-essential amino acids (5 ml (100X) NEAA per 500 ml EMEM) and 2 mM L-glutamine (sEMEM). After 48 h (≥80 % confluency), medium was removed and the monolayer inoculated with rickettsial culture supernatant from spent monolayers displaying a 90-100 % CPE. The volume of inoculum added was one fifth of the original culture medium volume. Rickettsiae were allowed to adsorb to the cells for 2 h at 15 °C, after which time the inoculum was aspirated, monolayers were rinsed with PBS (0.01M, pH7.4) to remove unattached organisms and cell debris, and the culture medium was replaced. Cultures were then incubated at 15 °C to allow a full CPE to develop, approximately 15 - 22 days later. Control flasks were inoculated as described, using supernatant from uninfected monolayers. Approximate viable counts were determined from $TCID_{50}$ results calculated by end-point dilution according to the method of Reed and Muench (1938).

3.2.2 Fish

Rainbow trout obtained from local fish farms, were held in tanks supplied with aerated flow-through dechlorinated water at the Aquatic Research Facility, University of Stirling, Stirling, UK and acclimatised to a water temperature of 15-17°C. The fish were fed daily on

a dry pelleted commercial diet. Fish were euthanised using 4-ethyl-aminobenzocaine (10 % (w/v) in 100 % ethanol) and ex-sanguinated by withdrawing blood from the caudal vein. To reduce the red blood cell contamination of kidney tissue samples, gill arteries were severed prior to explantation of head kidney using sterile technique.

3.2.3 Isolation of rainbow trout head kidney macrophages

Macrophage cell suspensions were prepared from head kidney tissue largely according to the method of Secombes (1990). Head kidney was removed aseptically and a cell suspension prepared by pushing the tissue through sterile 100 μm nylon mesh into Leibovitz-15 medium (L-15, Sigma). Suspensions (10 ml) prepared from each fish were layered carefully onto 2 x 51% (v/v) Percoll cushions and centrifuged at 400 x g for 30 min at 4 °C. The bands of cells at the medium/51 % interface were collected, pooled and washed in L-15 by centrifugation at 800 x g for 10 min. Cells were suspended to a concentration of $2 \times 10^7.ml^{-1}$ in L-15 plus 0.1 % FBS and 100 μl volumes aliquoted into T-25 flasks, 6-well tissue culture plates or onto sterile coverslips in 6-well plates, as appropriate. Cells were incubated at 18 °C. After 3-5 h, non-adherent cells were removed by washing with L-15 or PBS and monolayers were incubated in L-15 with 5 % FBS at 18 °C until required.

3.2.4 Re-isolation of *P. salmonis* from *in vivo*-infected macrophage monolayers

Fish (approximately 1.5-2 kg) were anaesthetised using 4-ethyl aminobenzocaine and were challenged by injecting 0.5 ml *P. salmonis* culture supernatant (approx. 10^5 $TCID_{50}$) intraperitoneally. Macrophage-enriched fractions were obtained from the head kidneys of a recent mortality (<2 h) and from a moribund fish, 9 d and 16 d post-challenge, respectively.

Head kidney (HK) cell suspensions (10 ml), prepared as in Section 3.2.3, from each fish were layered onto 2 x 10 ml 34/51 % Percoll gradients and centrifuged at 400 x g for 30 min at 4°C. The bands of cells at the 34/51% interface were collected, pooled separately for each fish and washed in L-15 by centrifugation at 800 x g for 10 min. Cells were suspended to a concentration of 2 x $10^7.ml^{-1}$ in L-15 plus 0.1% FBS and adherent cell monolayers were prepared by seeding 100 μl aliquots in T-25 flasks. After overnight incubation, unattached cells were removed by washing and monolayers were cultured for 22 d or 15 d, respectively. Supernatants from these cultures were inoculated onto CHSE-214 monolayers. Filtered (0.2 μm) supernatants or supernatants of macrophage monolayers from an uninfected fish were inoculated onto additional CHSE-214 monolayers, as controls. The CHSE-214 cells were monitored daily for development of characteristic *P. salmonis* CPE (Chapter 1; Fig. 1.2).

3.2.5 Survival of *P. salmonis* in macrophages infected *in vitro*

Macrophage monolayers were prepared, on separate occasions, from normal head kidney tissues, as described above (Section 3.2.4) and incubated at 18°C for 24 or 48 h. Supernatants were removed and monolayers were incubated at 15°C with rickettsial culture supernatant (approx. 5 x $10^{3.7}$ $TCID_{50}$) for 1-3 h prior to washing with PBS and fixing for TEM (Section 3.2.7). In other cases, *P. salmonis* supernatants were removed by washing after 2 h and monolayers were re-supplemented with L-15/ 5 % FBS and incubated at 15°C for an additional 24 h or 5 d before fixing. Control inocula were L-15 culture medium or *P. salmonis* culture supernatants inactivated by heating at 70°C for 1.5 h. Inactivation was confirmed by failure to produce CPE on CHSE-214 cell monolayers.

3.2.6 Effect of vaccination on survival of *P. salmonis* in macrophages infected *in vitro*

Fish (approximately 100 g) were anaesthetised using 4-ethyl aminobenzocaine, as before. Vaccinated fish received 0.1 ml SRS/4 vaccine (Schering-Plough Aquaculture, Essex, UK) injected i.p. while control fish received 0.1 ml ERM (Enteric Redmouth) vaccine (Schering-Plough Aquaculture). The SRS/4 vaccine is an experimental recombinant vaccine currently being field tested by Schering-Plough Aquaculture. After 7 weeks, fish were euthanised as described above and head kidney explanted. Cell suspensions were prepared from head kidney tissues, as described above (Section 3.2.4), and cells from 5 vaccinated fish and 7 control fish were pooled separately and seeded at 2 x 10^7 cells.ml^{-1} in 3 wells of a 6-well plate. Non-adherent cells were removed by washing after 3 h and monolayers were re-supplemented with L-15/ 5 % FBS. After incubation at 18°C for 24 h, supernatants were removed and monolayers were incubated at 15 °C with rickettsial culture supernatant (approx. 5 x $10^{3.7}$ $TCID_{50}$). *P. salmonis* supernatants were removed by washing after 2 h and monolayers were re-supplemented with L-15/ 5 % FBS and incubated at 15°C for an additional 6 d before fixing for TEM.

3.2.7 Preparation of cell monolayers for TEM

Culture medium was decanted from cells and the appropriate volume of cold 2.5 % glutaraldehyde in 0.075 M cacodylate buffer, pH 7.4 was added to fix the cells, namely 2.5 ml per T-25 flask or 0.8 – 1.0 ml per well of a 6-well plate. Monolayers were scraped from the attachment surface using a rubber policeman and the suspension centrifuged at 3650 x g for 15 min to pellet the cells. After a minimum of 1 h incubation at 4 °C, the pellets were gently dislodged from the cone of the centrifuge tube and incubated for a further 1 h in

glutaraldehyde. Once fixation was complete, the glutaraldehyde was aspirated off and the pellets were incubated at 4 °C in cacodylate buffer, pH 7.4, for at least twice the length of the fixation time or, more usually, overnight. Pellets were post-fixed *en bloc* in 1.0 % cacodylate buffer in cacodylate buffer (pH 7.4) for 1 h, washed for 3 x 10 min with dH_2O and stained with 2.0 % uranyl acetate in 30 % (v/v) acetone for 1 h, in the dark. Pellets were dehydrated through the following acetone series at RT: 60 % acetone for 40 min; 90 % acetone for 40 min; 100 % acetone for 1 h; 100 % acetone for 1 h. Pellets were then incubated with rotation o/n in equal parts 100 % acetone: Spurrs resin, rotary incubated for a further 24 h in Spurrs resin and polymerised at 60 °C for 48 h or 70°C for 16 h. Sections (90-100 nm) were cut using a Reichert Ultracut E ultramicrotome, mounted on a 200 mesh copper grid, double-stained with uranyl acetate and Reynolds Lead Citrate and examined using a Philips 301 transmission electron microscope at 80 kV.

3.2.8 Examination of actin in *P. salmonis*-infected CHSE-214 cells by TEM

P. salmonis was propagated on CHSE-214 cell monolayers in T-25 flasks as normal. On Day 5 post-inoculation, medium was decanted from the flasks and actin in infected cells was fixed and stained using 3 methods:

(a) Detergent and ruthenium red: cells were permeabilized by flushing once with 5ml Triton X-100 solution (1% (v/v) Triton X-100, 3 mM $MgCl_2$, 50 mM phosphate buffer, pH 6.8) at 4 °C followed immediately by rinsing briefly with cold 0.05 M cacodylate buffer, pH 7.4. Cells were fixed by adding 5 ml 2.5% (v/v) glutaraldehyde, 0.075 M cacodylate buffer, pH 7.4 to each flask, scraping off the monolayer into the fixative and centrifuging at 3650 x g for 15 min at 4 °C. Pellets were incubated for 1 h at 4 °C in 2.5 % (v/v)

glutaraldehyde, 0.075 M cacodylate buffer and 1 % (w/v) ruthenium red, pH 7.4. The pellets were then rinsed twice in cold 0.05 M cacodylate buffer and post-fixed in 1 % (v/v) OsO_4 in 0.05 M cacodylate buffer (pH 7.4) containing 1 % (w/v) ruthenium red for 30 min at 4 °C. Pellets were rinsed again, as above, and stained *en bloc* overnight in uranyl acetate prior to dehydration in acetone and processing, as described above.

(b) Quick fix with ruthenium red: cell monolayers were fixed for 5 min in 4 ml ice-cold fixing-staining solution (1 ml 8.0 % glutaraldehyde, 2 ml 0.2 M phosphate buffer, pH 6.8, 1 ml distilled water (dH_2O) and 0.04 g clarified ruthenium red). The fixing-staining solution was decanted, 5 ml ice-cold 2.5 % (v/v) glutaraldehyde, 0.075 M cacodylate buffer, pH 7.4 was added to each flask, the monolayer was scraped into the fixative and centrifuged at 3650 x g for 10 min at 4 °C. The supernatants were decanted and the pellets were post-fixed on ice for 20 min in 4 ml 2 % (v/v) OsO_4. After rinsing in dH_2O, the pellets were stained *en bloc* overnight in uranyl acetate prior to dehydration and processing.

(c) Detergent and quick fix with ruthenium red: cells were permeabilized by flushing once with 5ml Triton X-100 solution at 4 °C followed immediately by rinsing briefly with cold 0.05 M cacodylate buffer, pH 7.4. Monolayers were subsequently fixed and stained as for method (b).

3.2.9 Preparation of *P. salmonis*-infected rainbow trout macrophages for confocal microscopy

A sterile, circular glass coverslip was placed in each well of a 6-well plate and wells were seeded with macrophage-enriched cell suspensions (2×10^7 cells.ml^{-1}), prepared as described in Section 3.2.4. After 3 h incubation at 18 °C, non-adherent cells were removed

by washing with L-15 medium. Macrophage monolayers were inoculated with *P. salmonis* at this time, or were incubated for an additional 24 h at 18 °C, in L-15/5 % FBS, before inoculating. In the former case, macrophages were inoculated with live *P. salmonis* culture supernatant or with culture supernatant which had been subjected to freezing at –70°C in order to inactive the rickettsiae. Both live and frozen *P. salmonis* supernatants were washed to remove potentially toxic extracellular products by centrifugation at approx. 16,000 x g in a microcentrifuge and resuspension in an equal volume of L-15 culture medium. Macrophage culture supernatants were removed by aspiration and the monolayers were inoculated with 400 µl of the appropriate *P. salmonis* preparation. Rickettsiae were allowed to adsorb to the cells for 2 h at 15°C, after which time the inoculum was aspirated, wells were rinsed gently with PBS to remove unattached organisms and cell debris, and the monolayers were re-supplemented with L-15/5 % FBS culture medium. After incubation at 15 °C for 48 h, the monolayers were fixed for confocal microscopy (Section 3.2.10). In the case of macrophages grown for 24 h following isolation, monolayers were inoculated with live *P. salmonis* culture supernatant. Heat-killed (70 °C for 1.5 h) *P. salmonis* supernatant or contemporaneous uninfected CHSE-214 cell suspension, were used as negative controls. No non-biohazard positive controls were available. All supernatants used for inoculation were centrifuged at approx. 16,000 x g for 5 min and resuspended in an equal volume of L-15 medium. Macrophage monolayers were infected with 400 µl of the appropriate inoculum for 2 h, as described above, and incubated at 15 °C for 4 d before fixing for microscopy (Section 3.2.10).

3.2.10 Double-fluorescent staining of F-actin and *P. salmonis* for confocal microscopy

Coverslips were removed carefully from culture wells and rinsed gently to remove unattached cells and debris. To fix the attached cells and effect preliminary actin staining, each coverslip was flooded with phalloidin solution (200 µl NBF, 5 µl Triton X-100, 5 µl Alexa Fluor® 488 phalloidin) and incubated for 5 min. Coverslips were then flooded with rabbit anti-*P. salmonis* serum (kindly provided by the late Professor J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, USA), diluted 1/1000 in PBS, and incubated for 30 min. After washing with PBS, coverslips were incubated with Alexa Fluor 633® goat anti-rabbit IgG (4 $\mu g.ml^{-1}$ PBS) for 30 min, washed once more with PBS, and mounted in phalloidin solution on glass slides. After sealing with nail varnish, the slides were inverted and examined using a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope. All incubations were carried out in the dark

3.3 Results

3.3.1 Re-isolation of *P. salmonis* from *in vivo*-infected macrophage monolayers

Four days after inoculation of CHSE-214 cells with supernatants from *in vivo*-infected macrophages, CPE characteristic of *P. salmonis* was observed (Chapter 1; Fig. 1.2). No CPE developed in CHSE-214 cells inoculated with filtered supernatants or with supernatant of macrophages from the uninfected fish.

3.3.2 Survival of *P. salmonis* in macrophages infected *in vitro*

Intact *P. salmonis* organisms could be observed inside macrophages by 1 h post-infection (Fig. 3.1). On occasion, macrophages had engulfed the remains of CHSE-214 cell vacuoles, and both intact or degrading *P. salmonis* could be seen among the contents of these large vacuoles (Fig. 3.1A). Where individual organisms had been taken up, these were frequently found to be contained in a closely-apposed vacuole membrane, within the host cell (Figs. 3.1B & C). However, in some cases, membranes appeared not to completely enclose the organism and, where a vacuole membrane was not visible, there were instead small vesicles or granules (Fig. 3.1D). By 3 h post-infection, some rickettsiae were still contained within membrane-bound vacuoles in the host cell, although vacuoles containing degrading organisms sometimes appeared to be more swollen than those containing intact or dividing organisms (Figs. 3.2A & B). The membranes of several rickettsia-containing vacuoles appeared not to be complete and, again, small vesicles or granules were located at the gaps (Fig. 3.2A).

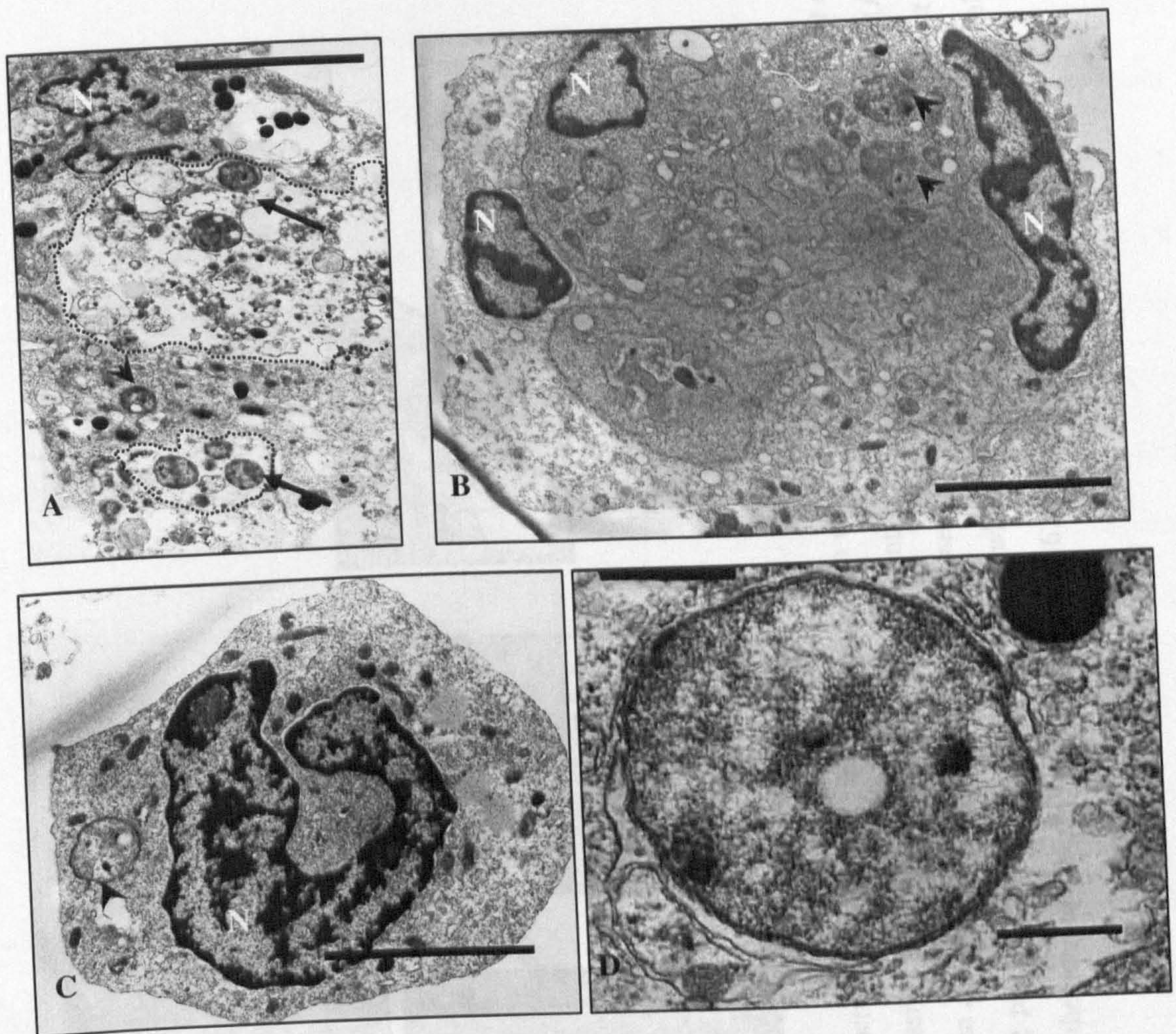


Fig. 3.1. Transmission electron microscope (TEM) photographs of rainbow trout head kidney (HK) macrophages, 1 h after inoculation with *P. salmonis in vitro*, showing individual organisms present within closely apposed vacuoles (arrowheads) or in larger vacuoles (solid arrows) containing debris from CHSE-214 cells (A, B & C). Dotted lines delineate vacuoles in (A). Nuclei of macrophages are indicated by N. In (B), rickettsiae are visible inside a macrophage which has, in turn, been engulfed by a second macrophage. In (D), an intact organism is visible, partly surrounded by vacuole membrane and partly by small vesicle-like structures. Scale bars represent (A) 4.65 μm; (B) 2.67 μm; (C) 3.39 μm; (D) 0.46 μm.

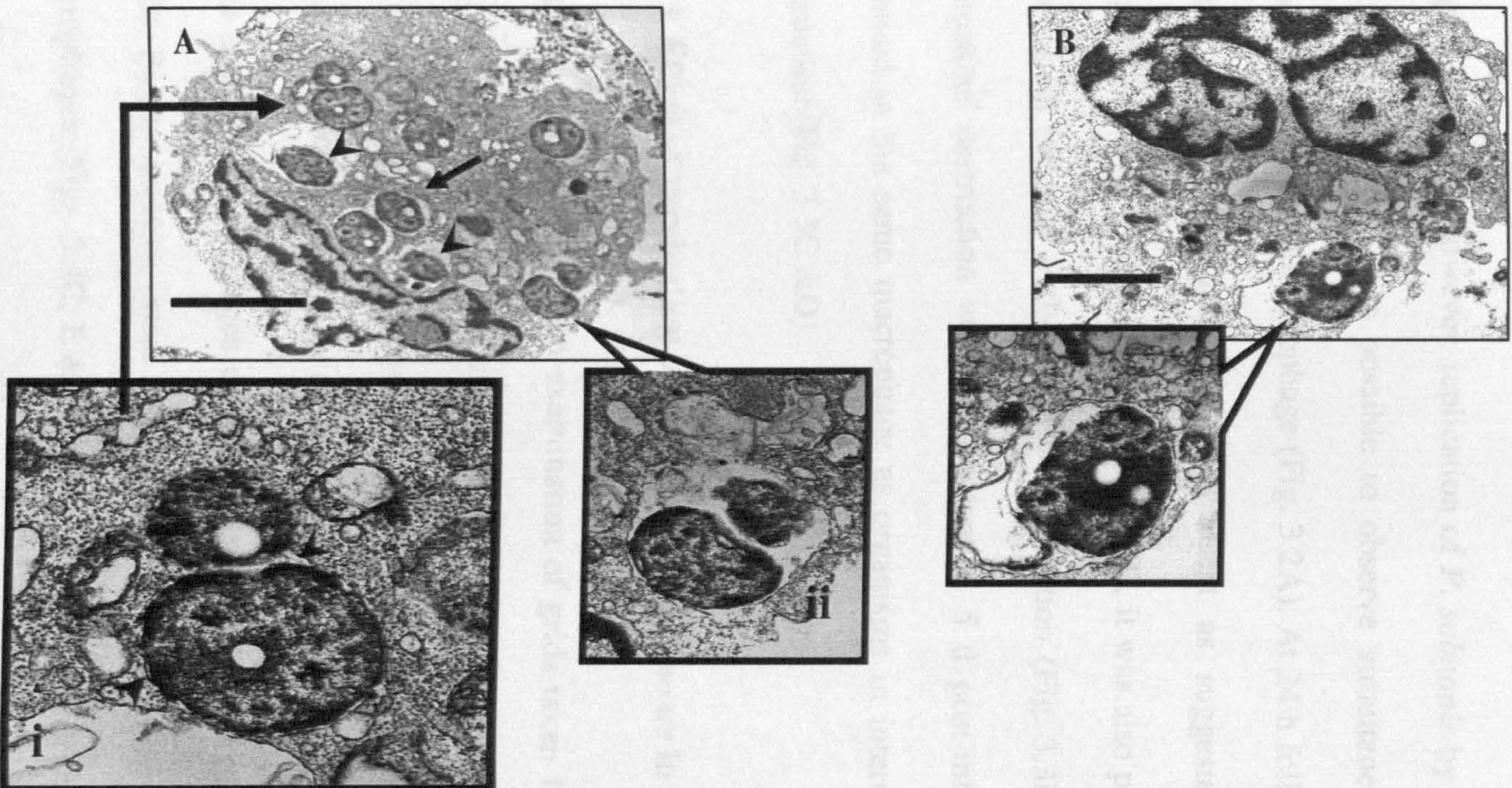
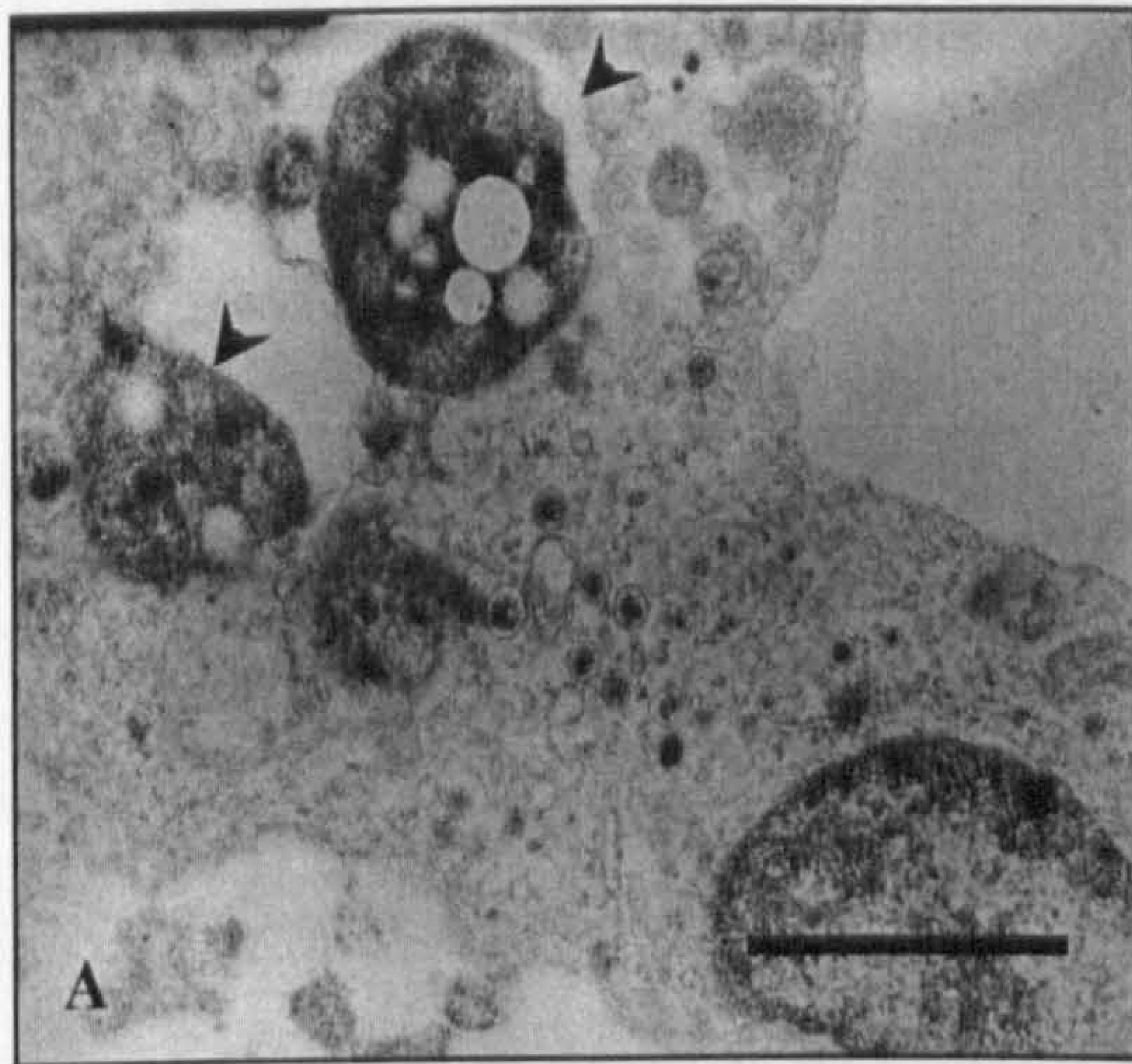


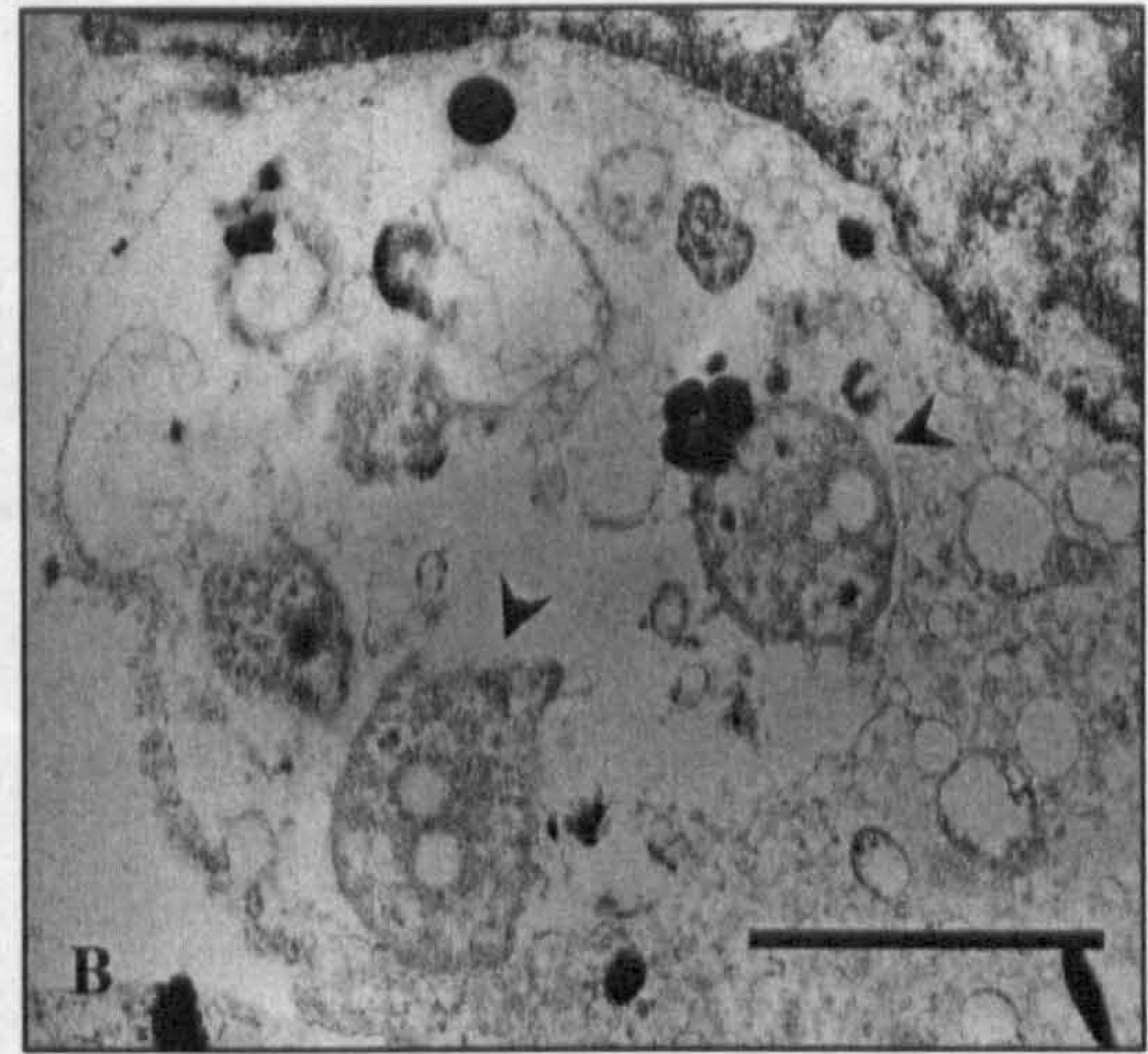
Fig. 3.2. TEM photographs of rainbow trout HK macrophages, 3 h after *in vitro* inoculation with *P. salmonis*. Although there is evident degradation of some organisms (A (arrowheads) & B), it is also possible to see organisms which have recently completed replication by binary fission (A, solid arrow) or are undergoing binary fission (Inset i). Replication and destruction are occurring simultaneously in the same macrophage (A). In Inset i, the host-cell vacuole in which *P. salmonis* replication is taking place is clearly visible (arrowheads) while, in Inset ii, rickettsiae are only partially enclosed in membrane and vesicle-like structures are present in the openings. Scale bars represent (A) 2.67 μm; (B) 1.54 μm.

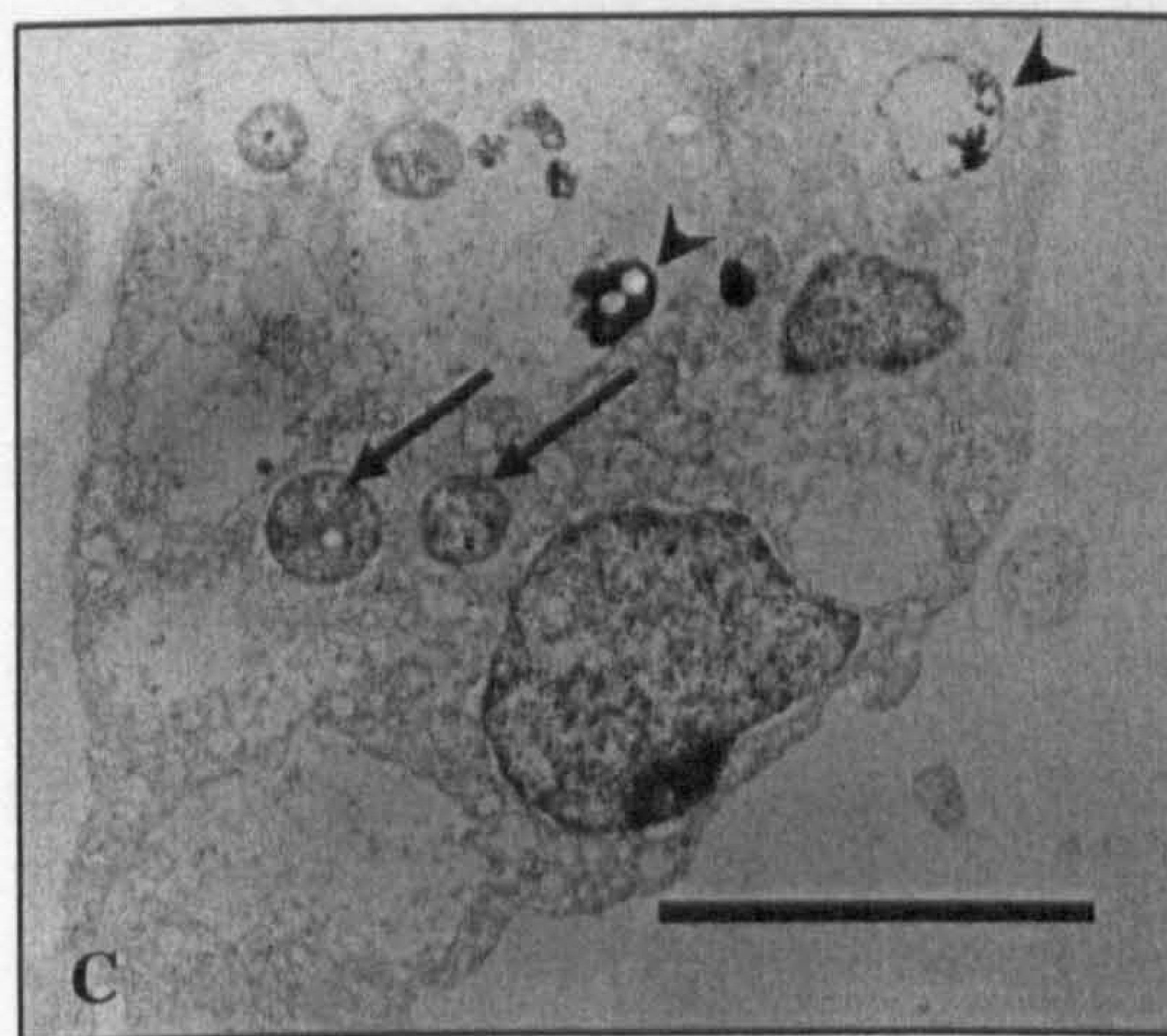
Loss of membrane integrity, membrane 'blebbing' or extreme undulation/ dissociation of the rickettsial outer membrane suggested destruction of the organism by the macrophage (Figs. 3.2A & B). However, replication of *P. salmonis* by binary fission was also apparent at this time, and it was possible to observe simultaneous replication and destruction occurring in the same macrophage (Fig. 3.2A). At 24 h following infection, while advanced destruction of the organism was apparent as suggested by extreme deformation or congestion of cell contents (Figs. 3.3A & B), it was also possible to find virtually intact *P. salmonis* in the very early stages of destruction (Fig. 3.3B). This pattern of simultaneous replication/ destruction was still evident at 5 d post-infection, with intact *P. salmonis* observed in the same macrophage as organisms in intermediate and advanced stages of destruction (Fig. 3.3C &D).

3.3.3 Effect of vaccination on survival of *P. salmonis* in macrophages infected *in vitro*

Observations were based on examination of grids taken from 3 discontiguous sections of pellets from vaccinated and control fish. While intact *P. salmonis* could be found among cell debris and in the remains of vacuoles from the infected CHSE-214 cells used as inoculum, an intact organism was found only in one immune macrophage (Fig. 3.4A) while other immune macrophages contained *P. salmonis* in various stages of destruction (Fig. 3.4B). By comparison, intact and replicating *P. salmonis* were commonly found in control macrophages (Figs. 3.4C, E & F) although degrading organisms were also seen (Figs. 3.4C - F). Once again, it was possible to observe intact *P. salmonis* in the same macrophage as organisms in intermediate and advanced stages of destruction (Figs. 3.4C, E & F).







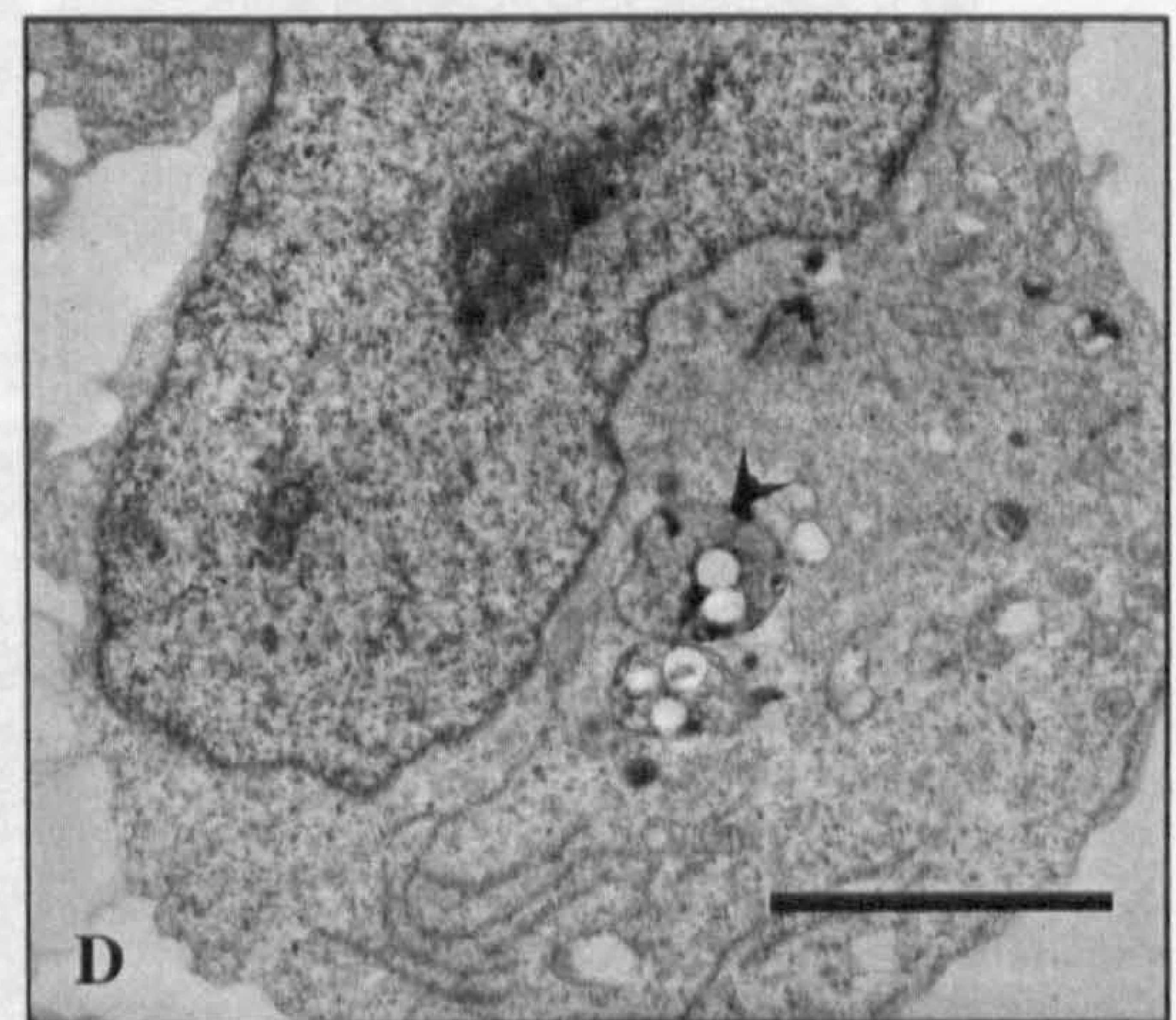


Fig. 3.3. TEM photographs of trout HK macrophages 24 h (A & B) and 5 d (C & D) after *in vitro* inoculation with *P. salmonis*. Destruction of *P. salmonis* is visible at both times (arrowheads). However, it is still possible to find intact *P. salmonis* 5 d after exposure of the organisms to macrophages (C, solid arrows). Replication and destruction are occurring simultaneously in the same macrophage (C). Scale bars represent (A) 1.54 µm; (B) 2.04 µm; (C) 4.65 µm; (D) 2.67 µm.

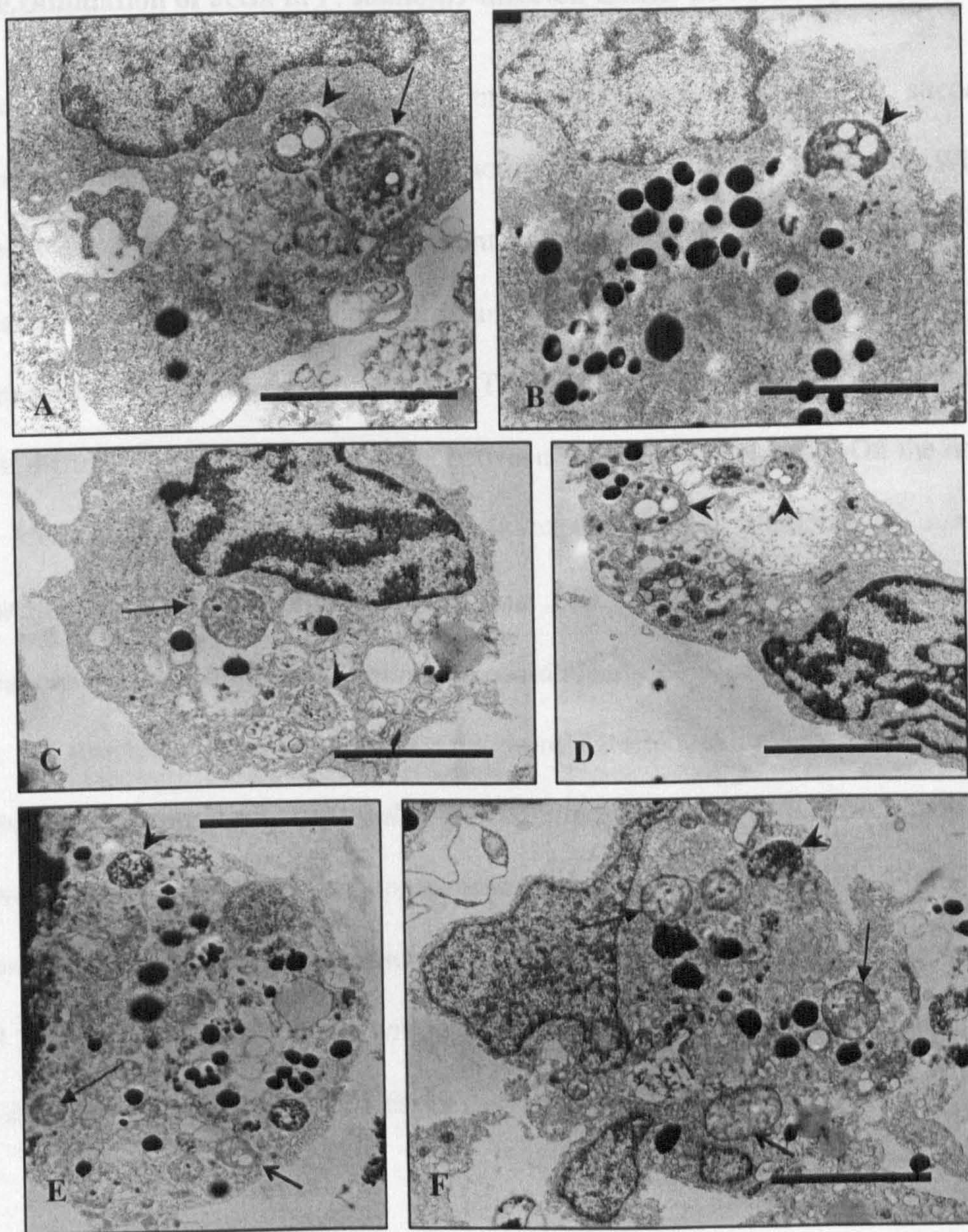


Fig. 3.4. TEM photographs of macrophages pooled from 5 vaccinated fish (A & B) or 7 control (vaccinated with ERM vaccine) fish (C – F), 6 d after *in vitro* inoculation with *P. salmonis*. Intact organisms are indicated by solid arrows, organisms which are undergoing destruction are indicated by arrowheads and organisms which are replicating by binary fission are indicated by open arrows (E & F). Scale bars represent (A, B, C, D, F) 2.67 μm; (E) 3.39 μm.

3.3.4 Examination of actin in *P. salmonis*-infected CHSE-214 cells by TEM

Method (a), which incorporated detergent permeabilisation, was very successful in achieving fixation and clear visibility of actin filaments (Figs. 3.5A & B). While actin bundles were frequently observed adjacent to, and partially surrounding, *P. salmonis* organisms, nothing approaching the structure of tails was detected. However, rickettsiae were exceedingly prone to destruction by Triton X-100, as were cell membranes, which made it difficult to interpret associations between actin and *P. salmonis*. On the other hand, while Method (b) was not so successful in exposing actin filaments, *P. salmonis*, its membranes, and the membranes of the host cell were excellently preserved so that associations between actin and *P. salmonis* could clearly be observed (Figs. 3.6A-D). Once again, no structures resembling actin tails were detected. Although actin bundles were observed in close proximity to *P. salmonis*-containing vacuoles (Fig. 3.6A), they were also observed adjacent to non *P. salmonis*-associated vacuoles (Fig. 3.6B). Furthermore, *P. salmonis* organisms were never observed free in the host cell cytoplasm but were always found at least partially enclosed within a vacuole membrane, whether replicating (Figs. 3.6A & C) or apparently in the process of degradation (Fig. 3.6D).

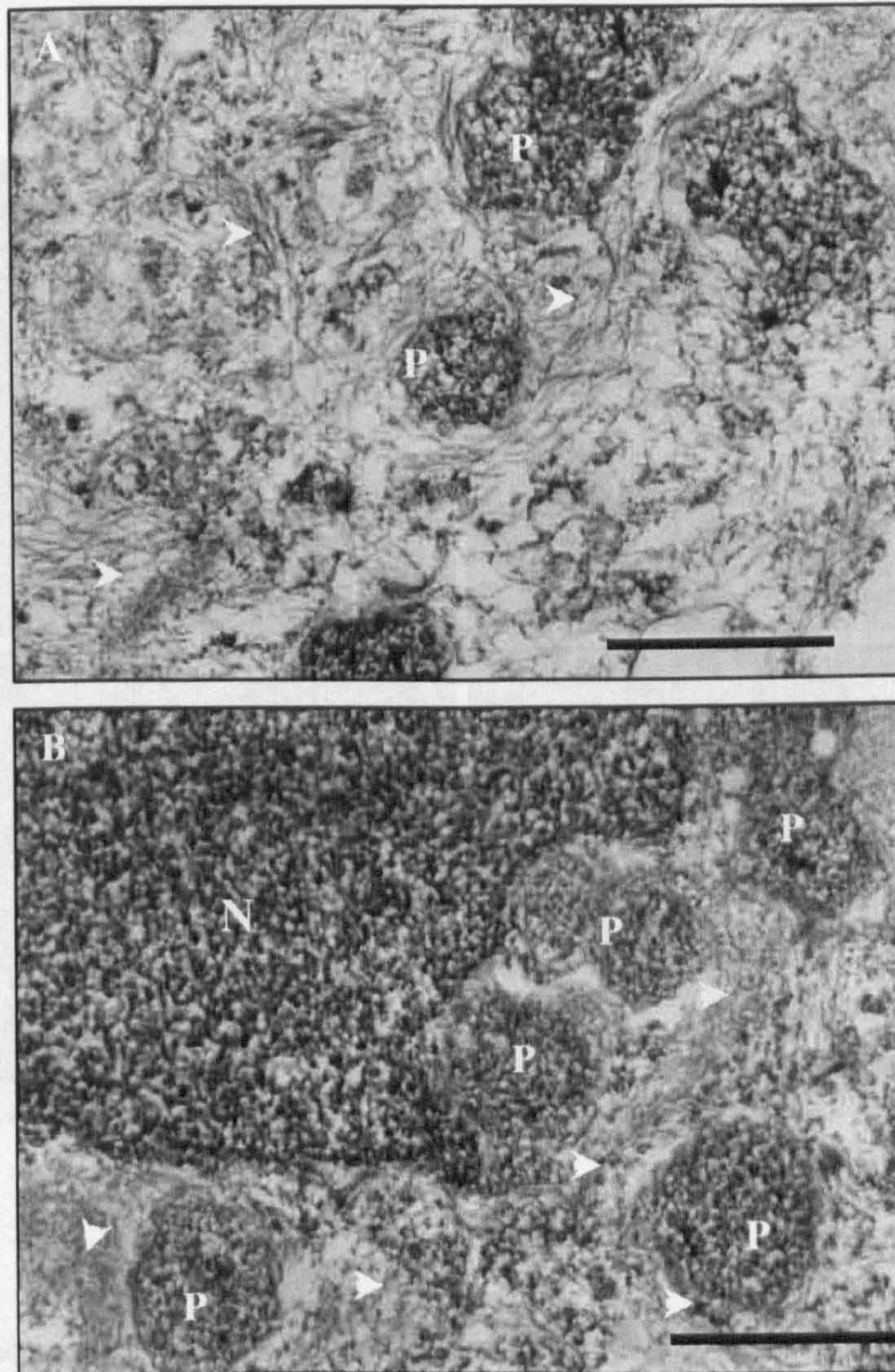


Fig. 3.5. TEM photographs of *P. salmonis*-infected CHSE-214 cells, fixed and stained using Method (a) Detergent and ruthenium red. The location of actin filament bundles is indicated by arrowheads. The remains of Triton X-100 degraded *P. salmonis* organisms are indicated by P. N is the CHSE-214 cell nucleus. Scale bars represent 910 nm.

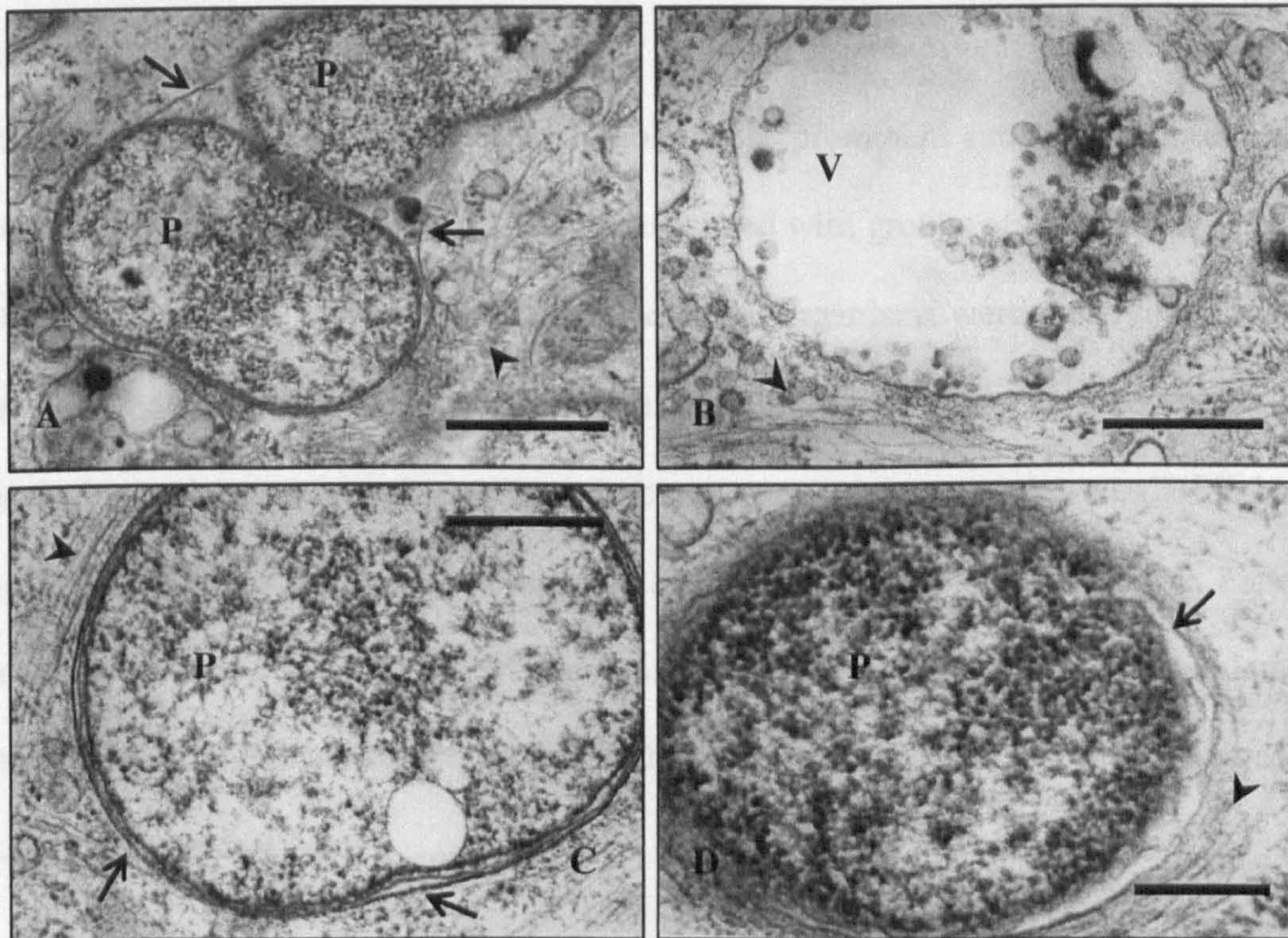


Fig. 3.6. TEM photographs of *P. salmonis*-infected CHSE-214 cells, fixed and stained using Method (b) Quick fix with ruthenium red. The location of actin filament bundles is indicated by arrowheads. Membranes of vacuoles surrounding *P. salmonis* organisms, P, are indicated by open arrows (A, C & D). Actin bundles are visible both in the vicinity of *P. salmonis*-containing vacuoles and of a vacuole, V, not associated with *P. salmonis* (B). Replication of organisms by binary fission is occurring inside *P. salmonis*-associated vacuoles (A & C). Scale bars represent (A) 560 nm; (B) 470 nm; (C) 340 nm; (D) 270 nm.

3.3.5 Examination of F-actin in *P. salmonis*-infected macrophages cells by confocal microscopy

In order to achieve optimal staining of actin, it was found best to incubate fixed cells with phalloidin solution both before and after indirect immunofluorescence staining of *P. salmonis*. Using this approach, it was possible to see prominent actin stress fibres and other cytoskeletal structures, sometimes closely associated with groups of *P. salmonis* (Figs. 3.7A - D). The fluorescent antibody-labelled *P. salmonis* organisms were clearly visible, both in groups and as individuals, and the specificity of this staining was verified by the lack of signal obtained from coverslips of macrophages exposed to non-infected CHSE-214 cells (Fig. 3.7E). Similar patterns of actin localisation were found in macrophages exposed to both live and dead (not shown) *P. salmonis*. No polar structures which articulated with individual organisms or fibrillar material along the sides of the organisms, suggestive of actin tails, were observed.

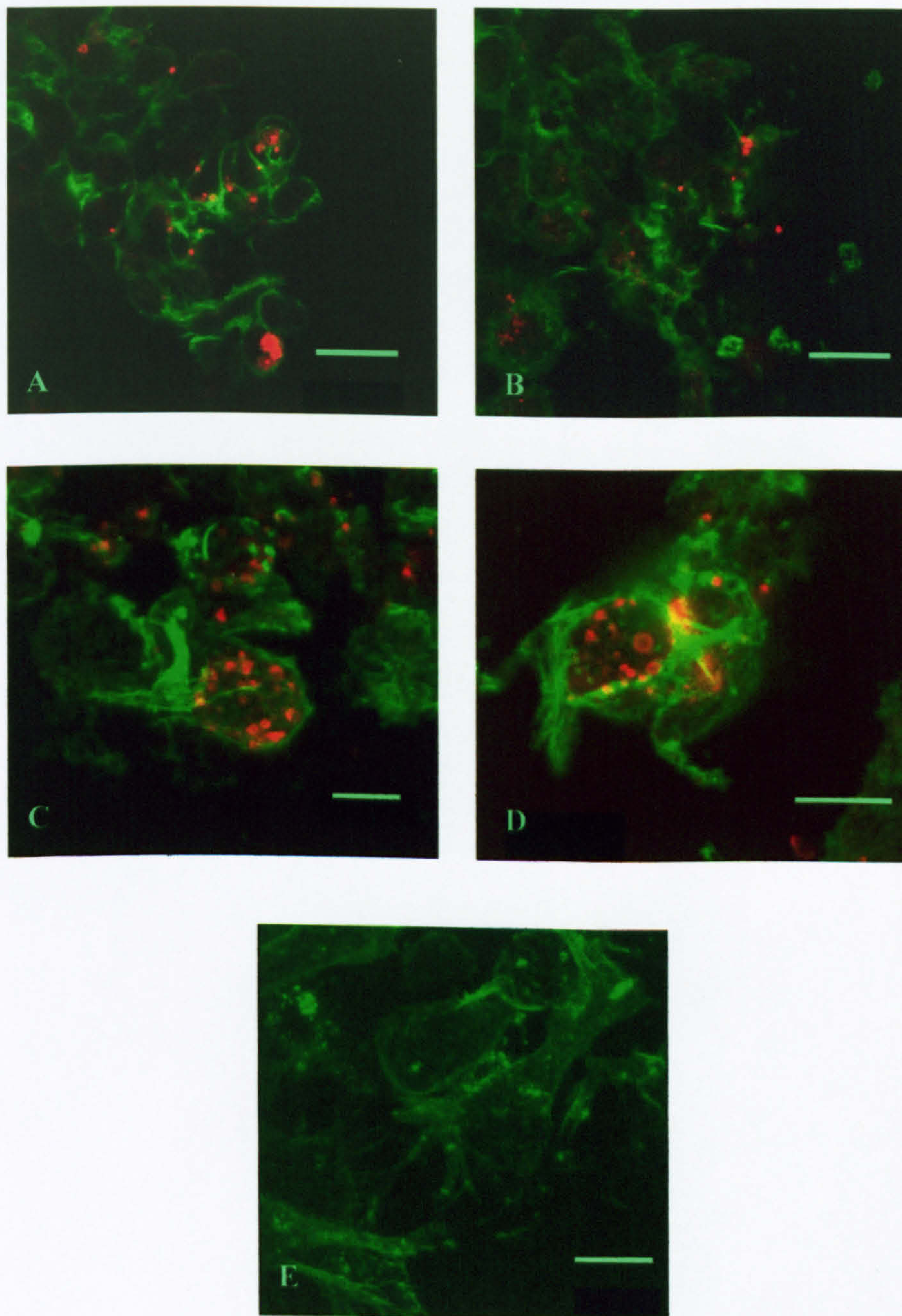


Fig. 3.7. Confocal microcope images of actin filaments (green) in head kidney (HK) macrophages infected with *P. salmonis* (red) for 2 d (A & B), 4 d (C & D) or uninfected (E). Figures are 2-dimensional (2D) representations of 3D revolving images in which it is clear that actin bundles do not articulate with *P. salmonis* organisms. Scale bars represent 20 μm (A & B) or 10 μm (C-E).

3.4 Discussion

The adherent cell population obtained from rainbow trout HK cell suspensions separated on 34/51 % Percoll gradients, is considered to be comprised of macrophages (Secombes, 1990; Garduno and Kay, 1992; Shoemaker *et al.* 1997; Afonso *et al.* 1998a; Boesen *et al.* 2001; Stafford *et al.* 2001a). Neutrophils are not thought to survive in culture for more than 96 h (Lamas and Ellis, 1994a). Therefore, the ability to re-isolate *P. salmonis* from adherent cells, which had been cultured for 15 or 22 d following HK explantation from trout infected *in vivo*, suggests that the pathogen is capable of surviving in the presence of macrophages. In order to confirm if the organism was capable of survival and replication within the macrophage, or merely maintaining viability in compromised macrophages or cell debris, macrophage monolayers were infected *in vitro* and survival of *P. salmonis* was examined by TEM at various times following infection. Interpretation of the interactions between *P. salmonis* and macrophages was complicated somewhat by the ability of the macrophages to engulf swollen, membrane-enclosed CHSE-214 vacuoles or other cell debris from the infected CHSE-214 cell inoculum. Presumably, *P. salmonis* organisms within this engulfed debris were not in direct contact with macrophage killing systems, which may falsely have extended survival times. However, by 3 h post-infection, it appeared that the macrophages were exerting some form of piscirickettsiacidal effect, as *P. salmonis* organisms could be observed in various stages of disintegration. Nevertheless, despite this indication of functional macrophage killing mechanisms, *P. salmonis* was capable of survival and replication in the same cell. This concurrent destruction and replication could still be observed at 5 d post-infection, which implies that *P. salmonis* may be able to survive and

reproduce in functional macrophages and suggests that survival of *P. salmonis* within macrophages is a matter of the pathogen being able to multiply more quickly than the macrophage killing systems can destroy it. Alternatively, as is the case with *L. monocytogenes*, a portion of the *P. salmonis* may avoid destruction by escaping from the phagosome into the cytoplasm of the macrophage (De Chastellier and Berche, 1994).

In the normal course of events, the macrophage phagosome in which a pathogen has been taken up goes through a maturation process leading towards the destruction of the pathogen. The phagosome becomes acidic following acquisition of a membrane H^+-ATPase pump. Early markers are shed and the maturing phagosome acquires late endosomal markers and finally becomes a fully matured phagolysosome, following fusion with lysosomes (Tooker and Coussens, 2004). Mycobacteria have developed an array of defensive mechanisms to prevent phagosome acidification and phagosome-lysosome fusion (Pieters, 2001; Bannantine and Stabel, 2002; Flynn and Chan, 2003; Tooker and Coussens, 2004; Weiss *et al.* 2004). Another survival mechanism, proposed for *M. tuberculosis*, is budding out of the phagosome into a closely-apposed vesicle which does not undergo subsequent lysosomal fusion (Fenton and Vermeulen, 1996). Likewise, *L. monocytogenes* can restrict the acquisition of lysosomal characteristics by its phagosome, or can avoid destruction by escape into the host cell cytoplasm (De Chastellier and Berche, 1994). In her study of *R. salmoninarum*, Gutenberger reported that some bacteria appeared to escape from the phagosome into a vacuole with a tightly-apposed membrane (Gutenberger *et al.* 1997) and considered that this might allow the bacterium to exist in a vacuole which does not fuse with lysosomes. Gutenberger also described *R. salmoninarum* organisms which were only partly enclosed by membrane and which were considered to be escaping into the

macrophage cytoplasm. In the present study, *P. salmonis* was observed in vacuoles with closely-apposed membranes but was also found in incomplete vacuoles, where small vesicles or granules were located at membrane gaps. It was not clear if the piscirickettsiae in these apparently incomplete vacuoles were escaping into the macrophage cytoplasm or if the vacuole membrane was, instead, being breached by lysosomal vacuoles. By cytochemical staining for acid phosphatase, it should be possible to determine if the presence of these vacuoles signifies lysosomal fusion and the transfer of hydrolytic enzymes to the phagosome (De Chastellier and Berche, 1994). Alternatively, treatment with 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) followed by anti-dinitrophenol immunogold labelling would allow the identification of acidic phagosomes and lysosomes. The DAMP accumulates in acidic cellular compartments where it links to proteins in the presence of aldehyde fixatives. The presence of immunogold labelling could then be used to differentiate organisms within acidified phagosomes from those free in the cytoplasm or enclosed within non-acidic vacuoles (De Chastellier and Berche, 1994). The acidification of *P. salmonis*-containing vacuoles could also be examined by confocal microscopy, using fluorescent labelling of *P. salmonis* with Lysotracker Red (Molecular Probes) staining to identify acidic phagosomes (Weiss *et al.*, 2004).

Orme *et al.* (1993), reported that *M. avium* in control cells tended to occupy discrete tight vacuoles while those in macrophage inhibition factor (MIF)-treated cells were often grouped into common vacuoles, many of which had fused with dense lysosomal compartments. Where a macrophage was refractory to MIF, bacteria remained viable and killed the cell. In macrophages infected with *P. salmonis*, in addition to tight vacuoles, the organism was also found in more spacious vacuoles. Frequently, these were occupied by an

organism undergoing destruction, often accompanied by an intact organism. As these spacious vacuoles were also observed to contain organisms which, it appeared, had recently undergone replication, it may be that the newly formed daughter cell was more resistant to degradation and, therefore, could remain intact while the mother cell was being destroyed. However, until such time as lysosomal staining is carried out in macrophages, infected synchronously with *P. salmonis*, it will not be possible to determine if the organism's survival is dependent on escape into a tight non-phagosomal vacuole or the ability to replicate rapidly before being overwhelmed by phagolysosomal digestion.

In studies on mice, Kokorin *et al.* (1980) reported that macrophages taken from convalescent animals were able to clear *Coxiella burnetti* and *R. conorii* infections more effectively than macrophages from naïve animals or those immunised with killed vaccine. In *R. conorii* infections, fewer macrophages from convalescent animals contained rickettsiae and the majority of these rickettsiae were degraded. These findings lend support to the observations from the present study where only a single intact *P. salmonis* was found in macrophages from vaccinated fish infected *in vitro*, while intact and replicating *P. salmonis* organisms were commonly found in macrophages from control fish. The fact that these macrophages were obtained from fish 7 weeks following vaccination suggests that the macrophages have been activated, presumably by T-lymphocytes responding to the vaccination (Graham and Secombes, 1990a; Marsden *et al.* 1994). While the observation is merely qualitative and the macrophages from vaccinated fish were pooled from only 5 fish, compared with 7 control fish, the stark difference in results warrants that this work be repeated in a quantitative experiment to determine if there is a statistical improvement in the ability of macrophages from immunised fish to clear *P. salmonis* infection. Furthermore, the

specificity of the response should be evaluated, by examining the ability of the macrophages to degrade other organisms such as *R. salmoninarum* or *A. salmonicida*. Should a specific response be found, it would suggest that vaccination has produced a population of lymphocytes which are capable of specifically priming macrophages to clear *P. salmonis* infection. It is possible, therefore, that the macrophage cultures also contained sensitized T-lymphocytes which, on contact *in vitro* with *P.salmonis*, released MAF and activated the macrophages.

One of the methods which intracellular pathogens use to avoid phagolysosomal digestion in macrophages is to escape into the cytoplasm and recruit host-cell actin for ABM (Gouin *et al.* 1999; Heinzen *et al.* 1999; Van Kirk *et al.* 2000; Stamm *et al.* 2003). However, despite using three methods to stabilise and stain actin, no evidence of the production of actin tails by *P. salmonis* was found in the present study. Methods used for staining actin in CHSE-214 cells for TEM required considerable adjustments to the published methods of Tilney and Tilney (1994) in order to reveal actin filaments, while keeping *P. salmonis* and membrane structures relatively intact. In fact, the published procedure for ruthenium red fixation in detergent-extracted cells (adapted to produce Method (a)) may contain a typographical error as the concentration of glutaraldehyde in the published fixation solution is given as 0.25 %, rather than the more likely 2.5 % glutaraldehyde found to be required for fixation of *P. salmonis*. In addition, it was found necessary to reduce the recommended concentration of Triton X-100 by a factor of ten, to 0.1 %, and to reduce exposure of cells to the detergent to the absolute minimum, in order to preserve any detail of *P. salmonis* or cell structures. The alternative published method (Tilney and Tilney, 1994), for fixation of intact infected cells, was adapted to produce

Methods (b) and (c) and was found to preserve *P. salmonis* and membrane structures admirably. However, inclusion of ruthenium red in the fixation solution was necessary to improve visibility of actin filaments and, once again, Triton X-100 was found to be extremely destructive of *P. salmonis* and cell structures. In fluorescent staining of macrophage actin, double staining with phalloidin, both before and after immunofluorescence staining of *P. salmonis*, was found to give optimal results. Having optimised all of these methods, actin filaments could be seen plainly in both CHSE-214 cells and macrophages, and the relative positions of actin bundles and *P. salmonis* organisms was clear.

The potential rate of progress of *P. salmonis* infection was taken into account when deciding how long after infection to fix cells, to allow time for actin tail formation. In mammalian rickettsial infections, Heinzen *et al.* (1993) observed tail production as early as 30 min after infection of Vero cells with *R. rickettsia*, while Gouin *et al.* (1999) found tail production by 24 h post-infection of Vero and Hep2 cells with *R. conorii*. Furthermore, these organisms are likely to initiate tail production more quickly following infection, because of their higher growth temperatures and more rapid growth rates. Given that foci of CPE can be observed, in our laboratory, by Day 3 after infection of CHSE-214 monolayers with *P. salmonis*, but that development of 100 % CPE usually takes 14-18 days, perhaps 2-3 times slower than mammalian rickettsiae (Nacy and Osterman, 1979), it was considered that 4-5 d post-infection would be an appropriate time to look for tail production. However, despite close associations of actin bundles with *P. salmonis*, no evidence of actin tail production or ABM was found.

It could be argued that, in the absence of a positive control, the lack of evidence for actin tail formation may be due to inadequate methodologies or incorrect timing of fixation. Previously, the organisms reported to use ABM were restricted to serious human pathogens (*L. monocytogenes*, *S. flexneri*) which could not be cultured in our laboratory. Recently, however, Stamm *et al.* (2003) have demonstrated ABM in *M. marinum*, a fish pathogen, and this discovery may allow development of a model for ABM by other fish pathogens, which could be carried out at the lower incubation temperatures used for cultivating these pathogens and reflect their slower growth.

In summary, it appears that *P. salmonis* is capable of survival, and even replication, within naïve fish head kidney macrophages. This survival may depend on ability to escape destruction within phagolysosomes, but it would not appear to utilise ABM as a means of evasion and intercellular spread. While degradation of some pathogens does take place soon after entry to the host cell, it is obvious that not all organisms are damaged by the macrophage, as replicating and apparently intact organisms could be observed at various times following infection.

A primary defence of the macrophage is production of ROIs via the respiratory burst upon phagocytosis of pathogens (Section 1.2.3.2). As not all *P. salmonis* organisms seem to be damaged during entry to the macrophage, it suggests that the pathogen may be able to resist or evade the cytotoxic effects of these ROIs. Alternatively, *P. salmonis* may avoid triggering the respiratory burst during uptake and this possibility is addressed in the following chapter, Chapter 4. Meanwhile, the more rapid clearance of internal *P. salmonis* by macrophages from vaccinated fish compared with naïve macrophages implies that more

effective defence mechanisms may be present or upregulated in immune fish. The effect of vaccination on macrophage defences is examined in Chapters 5 & 6.

Chapter 4 - Respiratory burst in rainbow trout head kidney macrophages

4.1 Introduction

Given that the macrophage is one of the cell types which the pathogen is likely to encounter soon after entry to the host and its prominent role as an effector cell in the immune response, it is not surprising that certain pathogens have adapted to live inside the macrophage for part of their life-cycle, using the cell as a shield against other cell-mediated and humoral immune responses (Kaufmann, 1993). As the tissue macrophage is a long-lived cell (Kaufmann, 1993; Allen, 2003), this affords the pathogen plenty of time in which to express virulence factors or multiply within the host. However, in order to survive within the macrophage, the pathogen must avoid being killed by the cell's numerous defences. Pathogens have evolved a variety of survival or escape tactics which include resistance to killing by reactive intermediates, inhibition of phagosome-lysosome fusion, prevention of phagosome acidification, egress from the phagosome into the macrophage cytoplasm etc. (Gordon *et al.* 1988; Brodsky *et al.* 1999; Allen, 2003; Tooker and Coussens, 2004) (discussed more fully in Chapter 3).

Since production of ROIs during the respiratory burst is one of the first defences mounted by the macrophage against the invading parasite, the ability to resist or avoid their bactericidal effects is a prerequisite for pathogen survival. The salmonid pathogen, *R. salmoninarum*, which has been shown to survive within macrophages for up to 5 days (Bandin *et al.* 1993), is sensitive to, and can be killed by, H_2O_2, one of the products of the respiratory burst (Hardie *et al.* 1996). However, the pathogen avoids exposure to ROIs in

non-immune fish by exerting a time and dose-dependent inhibitory effect on the respiratory burst, either directly or by exhausting the burst prior to entering the phagocyte (Campos-Peréz *et al.* 1997). Another fish pathogen, *V. anguillarum* serogroup O2a, has also been found to inhibit the respiratory burst in rainbow trout macrophages (Boesen *et al.* 2001). Meanwhile, some mammalian pathogens achieve 'silent' uptake by utilising certain host cell receptors, e.g. complement receptors, which are not linked to the respiratory burst pathway (Bermudez *et al.* 1991; Pieters, 2001).

As *P. salmonis* has been observed within macrophages of infected fish in natural outbreaks of the disease (Cvitanich *et al.* 1991), and has been found capable of replication within macrophages *in vitro* (this study, Chapter 3), it suggests that this obligate intracellular pathogen may also have developed some means of evading the bactericidal ROIs, either by avoiding or resisting their toxic effects, or by exerting some inhibitory effect.

The respiratory burst can be measured readily, using one of a number of assays to quantify one of the products (Bass *et al.* 1983; Higson and Jones, 1984; Chung and Secombes, 1988; Moritomo *et al.* 1988; Plytycz *et al.* 1989). A standard method used for measuring respiratory burst in rainbow trout macrophages is by the intracellular superoxide anion (O_2^-)-mediated reduction of nitroblue tetrazolium (NBT) to form a blue formazan precipitate, which can then be solubilised and the absorbance values read spectrophotometrically (Anderson *et al.* 1992). Measurement of the burst has been used as an indicator of immune response to a range of antigens/ immunostimulants in teleosts (Skarmeta *et al.* 1995; Whyte *et al.* 1989; John *et al.* 2002; Pedersen *et al.* 2003) and to

evaluate the stimulatory effect of cytokine-containing supernatants on macrophages *in vitro* (Graham and Secombes (1988) and Chapter 6).

In the present work, stimulation of respiratory burst in rainbow trout, *O. mykiss*, head kidney macrophages by *P. salmonis* was measured by NBT reduction. Unlike most other fish pathogens, the obligately intracellular *P. salmonis* must be grown in fish cell monolayers and supernatants from these cultures will contain tissue culture medium and significant amounts of host cell (CHSE-214) debris, which is difficult to separate from the rickettsial fraction (see Chapter 7). Furthermore, infection and lysis of host cells by *P. salmonis* may result in the expression and release of toxins or 'danger signals' into the medium (Dixon and Stet, 2001). As these factors may have exerted a stimulatory/ inhibitory effect on the respiratory burst it was necessary to assess their effects. In initial assays, the level of respiratory burst elicited by media components and host cell debris in supernatants from lysed, uninfected cell cultures was compared with that elicited by supernatants from *P. salmonis*-infected cultures. In subsequent assays, steps were taken to control for host cell contribution to the respiratory burst by lysing CHSE-214 cell controls to mimic lysis of infected cells, and washing *P. salmonis*/CHSE-214 preparations and CHSE-214 control preparations to remove toxins. The number of cells in the macrophage monolayers used for the assay and the detachment of monolayer during methanol fixing were also explored as sources of variation/error in the assay.

4.2 Materials and Methods

4.2.1 Fish

Rainbow trout, obtained from local fish farms, were held in tanks supplied with aerated flow-through dechlorinated water at the Aquatic Research Facility, University of Stirling, Stirling, UK. The fish were fed daily on a dry pelleted commercial diet. Fish were euthanised using 4-ethyl-aminobenzocaine and ex-sanguinated by withdrawing blood from the caudal vein. To reduce the red blood cell contamination of kidney tissue samples, gill arteries were severed.

4.2.2 Isolation of head kidney macrophages

Except where otherwise stated, pooled macrophage cell suspensions were prepared from head kidney (HK) tissue using 34/51% Percoll gradients as described in Section 3.2.4. Cell suspensions were also prepared by using only a 51% Percoll layer and collecting the band of cells at the medium/51% Percoll interface. Cells were suspended to a concentration of 2 x $10^7.ml^{-1}$ in L-15 plus 0.1 % FBS and 100 µl volumes aliquoted into a 96-well flat-bottomed plate. Cells were incubated at 18 °C. After 3 h or 24 h, non-adherent cells were removed by washing with L-15 or PBS and monolayers were incubated in L-15 with 5 % FBS at 18 °C until use.

4.2.3 *Piscirickettsia salmonis* antigen suspensions

Piscirickettsia salmonis was propagated in CHSE-214 cell monolayers as described previously (Section 3.2.1). Titres of *P. salmonis* were calculated by end-point dilution using

the method of Reed and Muench (1938). Antigen suspensions used for stimulation of the respiratory burst were prepared as described in each assay section. Macrophage monolayers were subjected to periodic microscopic examinations following exposure to antigen suspensions.

4.2.4 NBT reduction

NBT tablets (10 mg) were dissolved in L-15 to give a final concentration (2X) of 2 $mg.ml^{-1}$ (w/v). The solution was sterilised and filtered by passing through a 0.2 μm filter. Macrophage monolayers were covered with NBT solution to give a final concentration of $1mg.ml^{-1}$ (w/v) NBT in 100 μl volume. As a positive control, phorbol myristate acetate solution (PMA, 1 $\mu g.\mu l^{-1}$ (w/v) in 100 % ethanol) was added to NBT at 1 $\mu l.ml^{-1}$ (v/v). As a control for specificity of the reaction, superoxide dismutase solution (SOD, 300 $IU.\mu l^{-1}$ in phenol red-free Hank's Balanced Salt Solution (PRF-HBSS)) was added to NBT/PMA solutions at 1 μl $.ml^{-1}$ (v/v), where required. Monolayers were incubated with NBT for 20-30 min, or until blue formazan deposits could be discerned by eye in positive control wells. Except where stated, medium was decanted by inverting the plate and cells were fixed in 100 % methanol for 5 min, washed twice in 70 % methanol and air-dried. The reduced formazan was solubilised in 120 μl 2M potassium hydroxide (KOH) and 140μl dimethylsulphoxide (DMSO) and the absorbance measured at 630 nm (A_{630}) in a Dynex MRX II ELISA reader.

In some cases, washed but otherwise untreated monolayers were used to obtain cell counts. The medium was removed and 100 μl lysis buffer containing 0.1 M citric acid, 1 %

Tween 20 and 0.05 % crystal violet was added. After 2 min, the nuclei from a minimum of 3 wells were counted in a haemocytometer.

4.2.5 Statistical analysis

The data were analysed using Student's t-test or ANOVA, as appropriate, and significance was set at $p<0.05$.

4.2.6 Respiratory burst assays

4.2.6.1 Respiratory burst stimulation by PRF-HBSS.

The stimulatory effect of PRF-HBSS, used to wash cell monolayers, was investigated because of a series of anomalous results during preliminary assays.

Pooled HK monolayers were prepared from 2 x 700 g fish (water temperature approximately 15 °C) as described in Section 3.2.4, dispensed into 96-well plates and non-adherent cells removed by washing with L-15 following overnight incubation at 18 °C. Cells were then fed with L-15 supplemented with 5 % FBS and incubation continued at 18 °C. After 24 h, the cells were washed three times with L-15 to remove residual non-adherent cells. Cells were incubated in the last wash for 15-20 min prior to beginning the assay. PRF-HBSS was mixed with an equal volume of 2X NBT solution and 100 µl volumes were dispensed into each well, following aspiration of medium from the monolayers. For control wells, L-15, PMA and PMA/SOD were added to 2X NBT, as appropriate.

4.2.6.2 Respiratory burst stimulation by *P. salmonis*-infected cell supernatants, lysed CHSE-214 cell supernatants and growth medium

The level of stimulation by lysed CHSE-214 cell supernatants was measured as a control for the effect of cell debris and sEMEM in *P. salmonis* antigen preparations.

Monolayers were prepared from 5 x 200 g fish (water temperature approximately 6°C) as described in 4.2.6.1. However, in this case, monolayers were washed with PBS after 6 h in order to remove unattached cells. Cells were then incubated in L-15 with 5 % FBS at 18 °C. At 48 h, monolayers were washed three times with PBS to remove residual non-adherent cells and cells were incubated in the last wash for 15-20 min, as above. Uninfected CHSE-214 cell monolayer was frozen and thawed to disrupt the cells, in order to approximate lysis of infected cells. This cell suspension and a similar volume of supernatant from a contemporaneous batch of CHSE-214 cells, with 100% *P. salmonis* CPE, were both clarified (CL) by centrifugation at 1000 x g for 5 min to give $CHSE_{CL}$ and $Psal_{CL}$. The PBS was aspirated from the macrophage monolayers and the monolayers were covered with 50 µl $Psal_{CL}$, 50 µl $CHSE_{CL}$ or 50 µl L-15. As controls, PMA or PMA and SOD were added to 50 µl L-15 for some wells. After 30 min, 50 µl 2X NBT solution was added to the wells and incubated for 30 min prior to methanol fixing.

4.2.6.3 Optimising the methanol fixation step

From visual comparisons of formazan deposits, before and after the methanol fixation step, it appeared that cell monolayer could be detached and lost during this procedure. Therefore, results obtained when the medium was removed by inverting the plate (decanting) were compared with those obtained when medium was removed by aspiration.

HK monolayers were prepared and cells were incubated for 24 h before washing to remove unattached cells. Monolayers were then incubated for a further 24 h at 18 °C in L-15 with 5 % FBS and residual non-adherent cells were removed prior to use by washing with PBS. Monolayers were stimulated with 50 µl clarified *P. salmonis* supernatants ($Psal_{CL}$), 50 µl clarified, lysed CHSE-214 cell supernatants ($CHSE_{CL}$), 50 µl $Psal_{CL}$/SOD or 50 µl L-15, in replicate halves of the plate, and then incubated with 50 µl 2X NBT as described above. For one half of the plate, medium was decanted before fixing in fresh 100 % methanol. For the other half of the plate, medium was aspirated before adding 100% methanol to the wells.

4.2.6.4 Comparison of respiratory burst stimulation by washed *P. salmonis* antigen and washed CHSE antigen

Assay 1 – using delayed NBT addition.

Monolayers were prepared from 5 x 350 g fish (water temperature approximately 14 °C). Head kidney cell suspensions (10 ml) prepared from each fish were layered onto two 10 ml 51% (v/v) Percoll cushions and centrifuged at 400 x g for 30 min at 4 °C. The bands of cells at the medium/Percoll interface were collected, pooled and washed in L-15 by centrifugation at 800 x g for 10 min. Cells were suspended to a concentration of $2 \times 10^7.ml^{-1}$ in L-15 plus 0.1 % FBS and 100 µl volumes aliquoted into two 96-well plates and incubated at 18 °C. After 3 h, non-adherent cells were removed by washing with PBS and monolayer cell density in the two plates was compared by eye, using an inverted microscope. The plates were then incubated in L-15 with 5 % FBS at 18 °C. At 48h, the monolayers were washed three times with PBS, the last wash was aspirated off and the monolayers were covered with 30 µl L-15.

Pisicirickettsia salmonis supernatant from 100 % CPE culture was vortexed vigorously with 0.1 mm sterile glass beads in an attempt to release organisms from the CHSE-214 cells. Beads and cell debris were allowed to settle and the supernatant was aspirated off for use as neat *P. salmonis* antigen (Psal). A cell control was prepared from contemporaneous uninfected CHSE-214 monolayer by freeze-thawing, vortexing the suspension with glass beads in an attempt to further disrupt the cells, allowing beads and large debris to settle and aspirating off the supernatant (CHSE). Aliquots (3 ml) of these neat antigen preparations were washed (W) by centrifugation in 10 ml sEMEM at 20,000 x g for 35 min and resuspended in 3 ml sEMEM to give $Psal_W$ and $CHSE_W$. Macrophage monolayers, covered with L-15, were stimulated by adding 100 µl of each antigen preparation. To control for the stimulatory effect of the cell growth medium, sEMEM was added to some wells. Three wells per plate were retained for use as KOH/DMSO background controls and three wells were retained for estimating monolayer cell numbers. After incubating for 25 min, 50 µl 2X NBT was added to the wells and incubated for a further 20 min. To effect preliminary fixing of the monolayer, 100 % methanol was added in excess without first removing the supernatants. After preliminary fixing for 5 min, supernatants were decanted gently and monolayers were fixed in fresh 100 % methanol for 5 min prior to washing with 70 % methanol. The levels of formazan reduction by each group of cells was observed by eye, both before and after methanol fixing to confirm that relative levels had not been affected during this step. The formazan was then solubilised and the results measured spectrophotometrically, as described in Section 4.2.4.

Assay 2 – using simultaneous addition of NBT.

Monolayers were prepared from 4 x 250 g fish (water temperature approximately 11 °C) as before. *P. salmonis* 100 % CPE culture was centrifuged at 180 x g for 1 min and the supernatant taken off and retained. The pellet was vortexed vigorously with 0.1 mm sterile glass beads in 1ml L-15, the beads were allowed to settle and cell debris was collected by washing the beads in several changes of L-15. These washes were combined with the culture supernatant and the piscirickettsiae washed twice by centrifugation in 30 ml L-15 at 20,000 x g for 35 min. A cell control was prepared from uninfected CHSE-214 monolayer by freeze-thawing, vortexing the suspension with glass beads, washing and centrifuging as for piscirickettsiae. The *P. salmonis* and CHSE pellets were then resuspended to their original volumes in L-15 (12 ml and 10 ml respectively). Washed *P. salmonis* antigen ($Psal_W$) and washed CHSE antigen ($CHSE_W$) were mixed with an equal volume of 2X NBT and 100 µl aliquots added to the macrophage monolayers for 20 min. As controls, 2X NBT was mixed with L-15, L-15/PMA or L-15/PMA/SOD. Two wells were retained for use as KOH/DMSO background controls and four wells were retained for estimating cell numbers. Monolayers prepared from a fifth fish were cultured in a separate 96-well plate and stimulated with $Psal_W$/ NBT, $CHSE_W$/NBT or with L-15/NBT for 20 min. Methanol fixation and measurement of formazan levels were as above.

Assay 3 –using concentrated antigen and simultaneous addition of NBT.

Monolayers were prepared from 5 x 200-250 g fish (water temperature approximately 7.5 °C) as described above and incubated for 48 h before use. *P. salmonis* antigen was prepared from 100 % CPE cell culture washed once by centrifugation in 2 volumes L-15 at 20,000 x g for 35 min. A cell control was prepared from uninfected CHSE-214 monolayer by freeze-

thawing several times, washing and centrifuging as for piscirickettsiae. The *P. salmonis* and CHSE pellets were then resuspended to 1/3 their original volumes in L-15 (5 ml). Washed *P. salmonis* antigen ($Psal_W$) and washed CHSE antigen ($CHSE_W$) were mixed with an equal volume of 2X NBT and 100 µl aliquots added to the macrophage monolayers. As controls, 2X NBT was mixed with L-15, L-15/PMA or L-15/PMA/SOD. Four wells were retained for estimating cell numbers. Monolayers were incubated for 30 min prior to methanol fixing and formazan levels were measured as above.

4.3 Results

4.3.1 Effect of *P. salmonis* antigen suspensions on macrophage monolayers.

Observation of macrophage monolayers microscopically revealed that, within 5 min of exposure to *P. salmonis*-containing preparations, cells in the centres of some wells had 'rounded up' and detached from the surface (Fig. 4.1). The degree of this detachment was highly variable and inconsistent between adjacent wells.

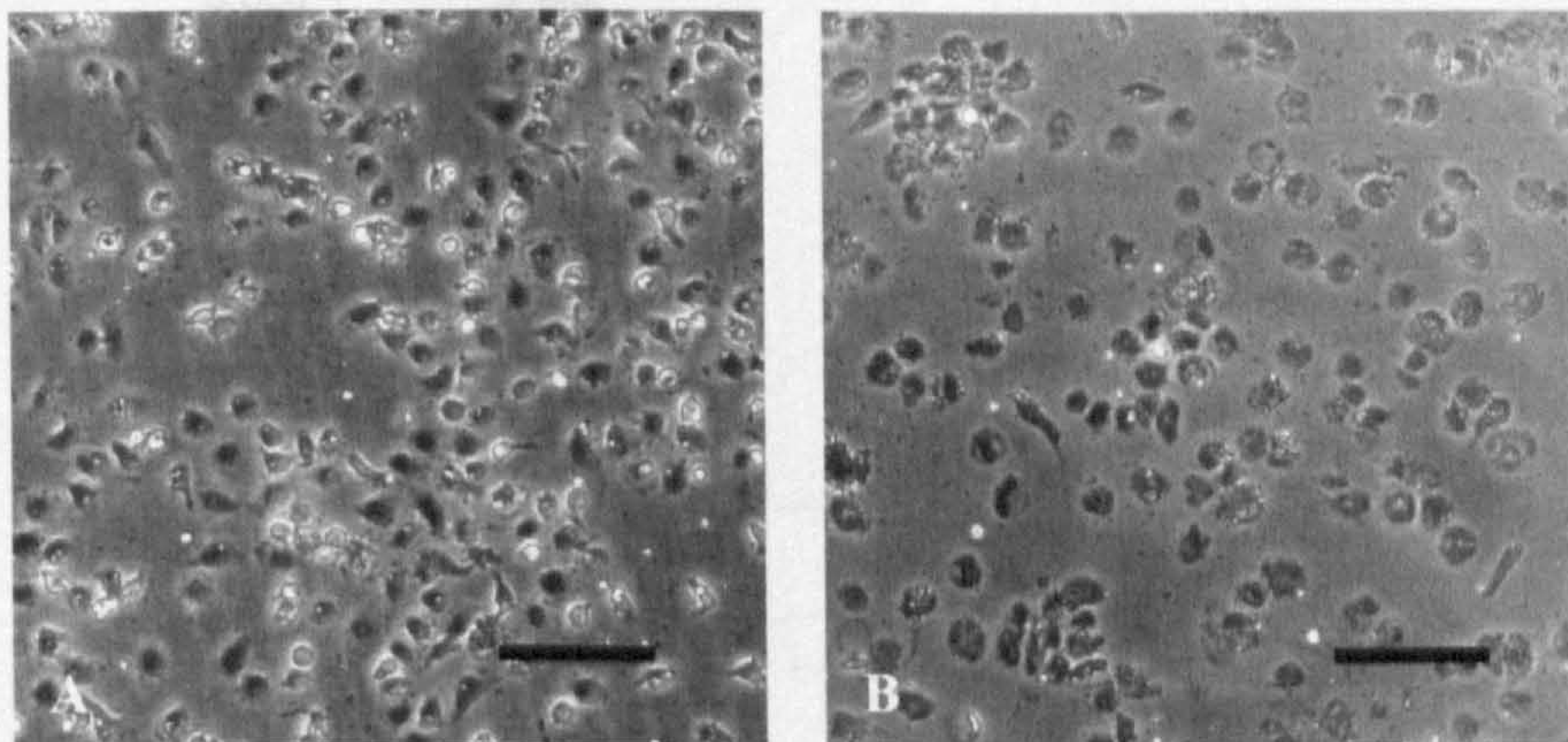


Fig. 4.1. Macrophage monolayers showing the effect of exposure to *P. salmonis*. Macrophages were isolated and incubated for 48 h as described in Section 3.2.3. Dark cells in (A) are spreading cells attached in the centre of the culture well. In (B), 15 min after exposure to *P. salmonis* culture supernatant, the cells are swollen, have a grainy, ghost-like appearance, and many have detached from the surface. Photographs were taken using an Olympus CK2 inverted microscope with phase contrast. Scale bar represents 100 µm.

4.3.2 Respiratory burst stimulation by PRF-HBSS.

Macrophage monolayers exposed to PRF-HBSS alone (n=12 wells) exhibited very high stimulation of respiratory burst, between 6 and 9 times higher than the level found in wells containing L-15 alone (n=15) and almost twice as high as that found in wells stimulated with PMA (n=8) (Fig. 4.2). SOD caused inhibition of respiratory burst in control wells (n=8).

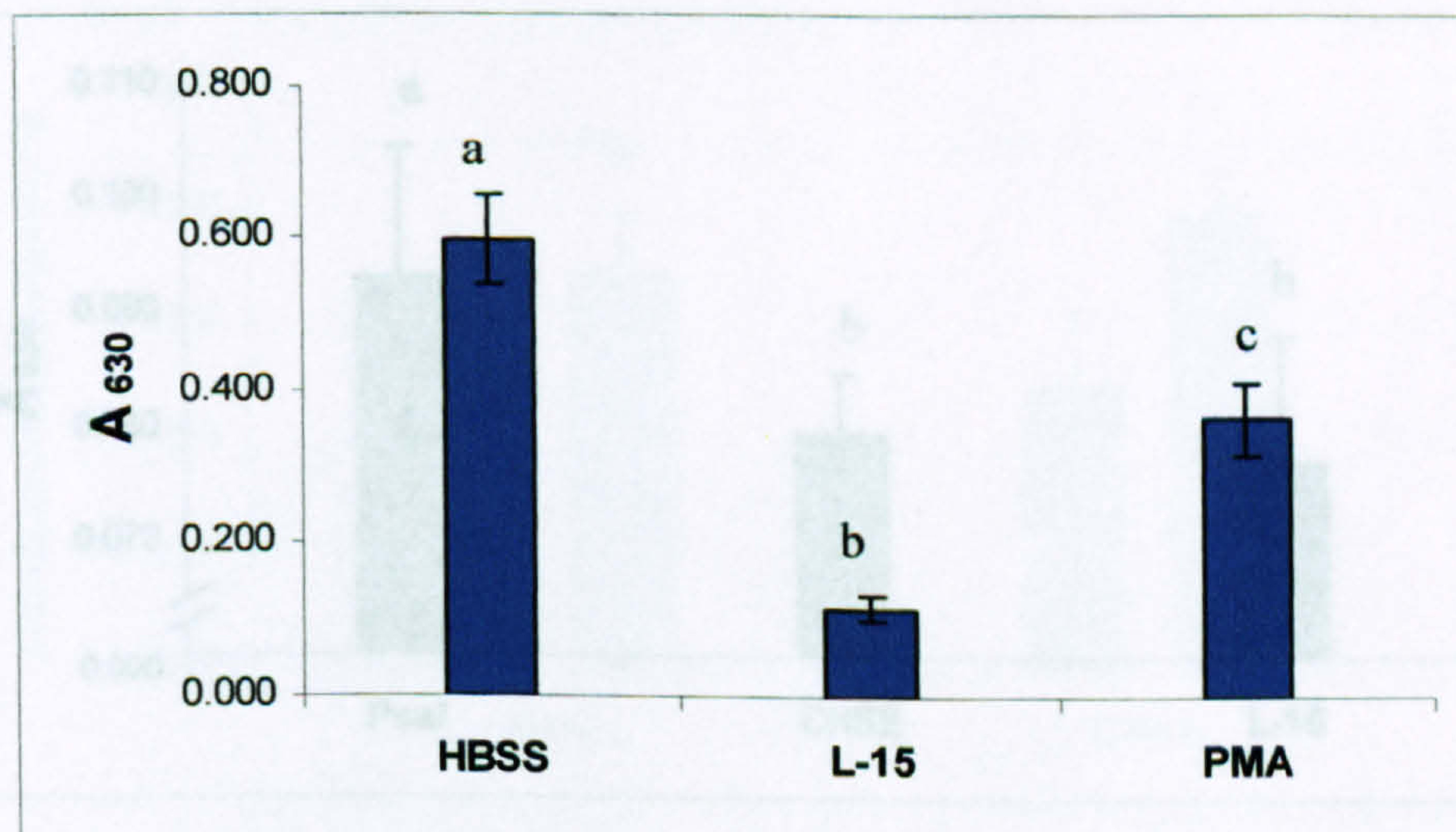


Fig. 4.2. Respiratory burst activity of rainbow trout head kidney macrophages following stimulation with PRF-HBSS, measured as the reduction of NBT after 30 min incubation. Macrophages were stimulated with PRF-HBSS (HBSS, n=12 wells), with L-15 culture medium (L-15, n=15) or with PMA (1 μg.ml^{-1} L-15, n=8) in the presence of NBT. Data represent the mean absorbance values of replicate wells at 630 nm (A_{630}) ± standard deviation (SD). Different letters denote a significant difference ($p<0.05$) between groups.

4.3.3 Respiratory burst stimulation by *P. salmonis*-infected cell supernatants, lysed CHSE-214 cell supernatants and growth medium

Macrophages stimulated with $Psal_{CL}$ exhibited significantly higher A_{630} values than those stimulated with $CHSE_{CL}$ or L-15 (n=8) (Fig. 4.3). Low values were observed for both PMA-stimulated wells (A_{630} = 0.070) and PMA/SOD-stimulated wells (A_{630} = 0.068), which were lower than values for L-15-stimulated wells (A_{630} = 0.079)(n=8). These low values were found to result from expiry of this batch of PMA.

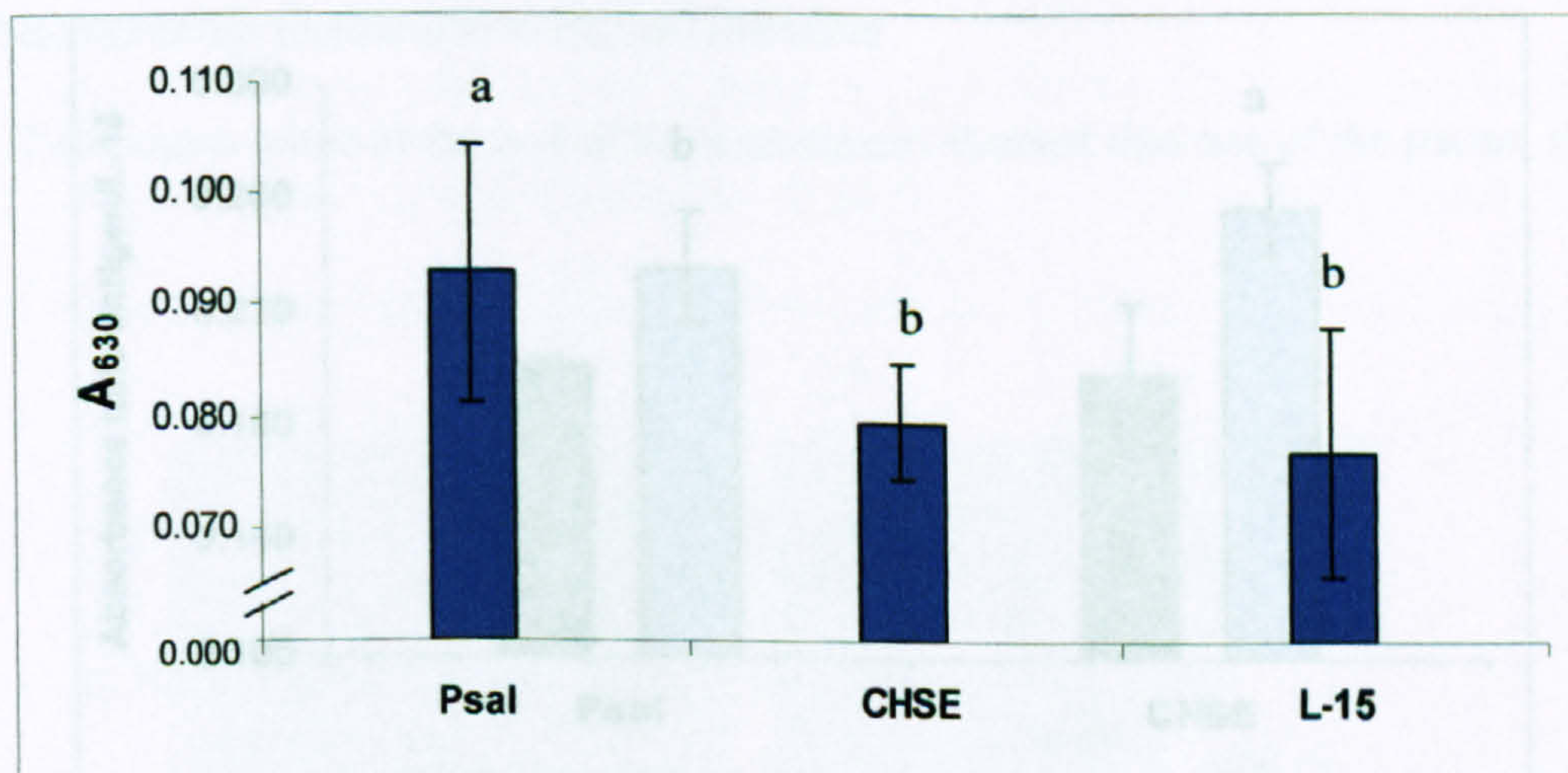


Fig. 4.3. Respiratory burst activity of rainbow trout head kidney macrophages following stimulation with *P. salmonis*-infected cell cultures, uninfected cell cultures or cell culture growth medium, measured as the reduction of NBT after 30 min incubation. Macrophages were stimulated with clarified *P. salmonis* culture supernatant (Psal), with lysed clarified CHSE-214 cell suspension (CHSE) or with L-15 culture medium (L-15). Data represent the mean absorbance values of replicate wells (n=8) at 630 nm (A_{630}) ± standard deviation (SD). Different letters denote a significant difference ($p<0.05$) between groups.

4.3.4 Optimising the methanol fixation step.

Comparison of A_{630} values in wells from which medium was decanted prior to fixing with those in wells where medium was removed by aspiration before fixing suggests that the results were affected by the method of removing medium. In decanted wells, A_{630} values were significantly higher in $CHSE_{CL}$-stimulated macrophages (n=8) than in $Psal_{CL}$-stimulated cells (n=7) (Fig 4.4). However, in aspirated wells, there was no significant difference between the two groups.

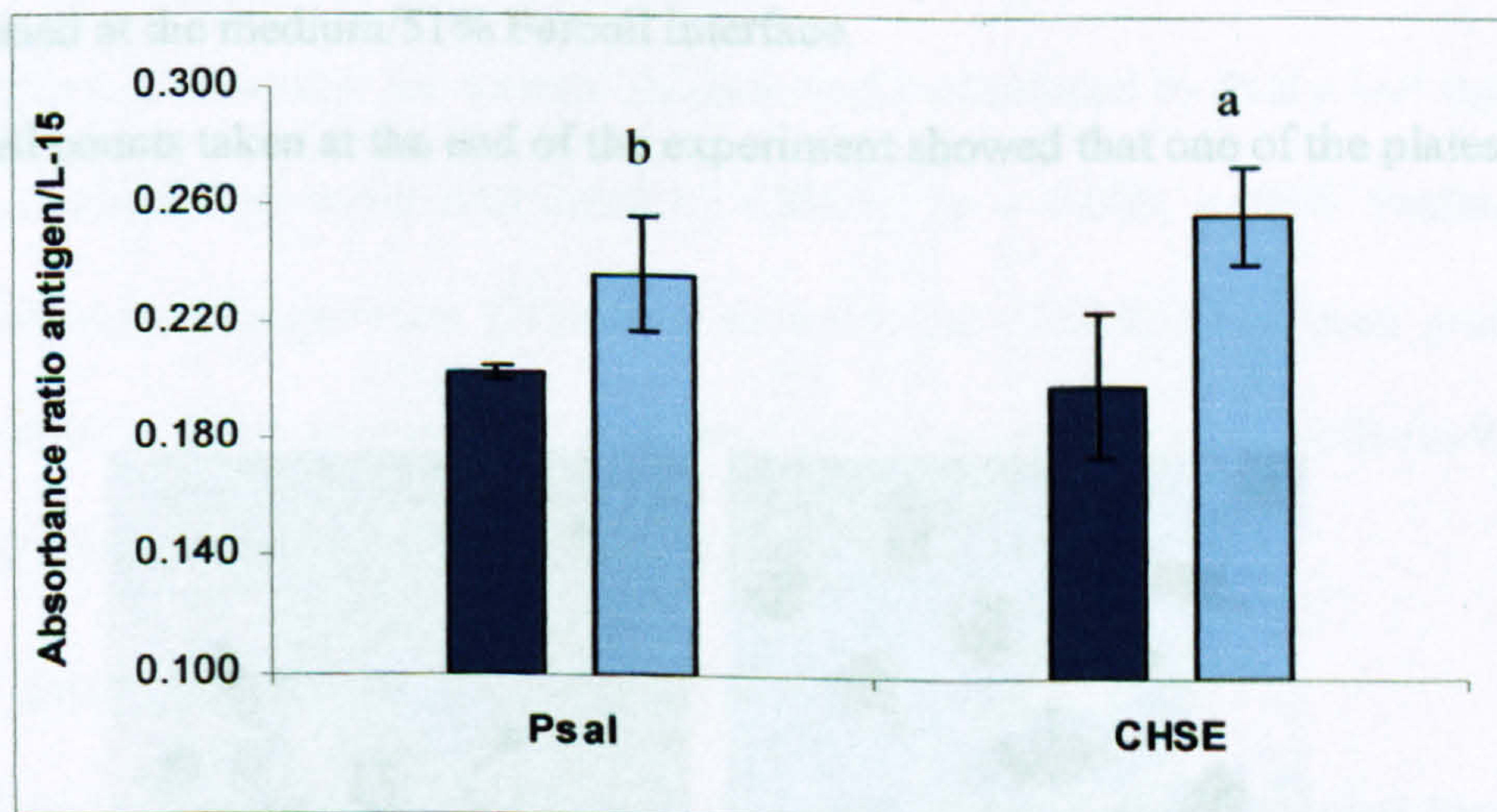


Fig. 4.4. Comparison of formazan levels remaining in rainbow trout HK macrophage monolayers following aspiration or decanting of medium prior to methanol fixation. *P. salmonis* culture supernatant (Psal, n=7 wells) or lysed, clarified CHSE-214 cell suspension (CHSE, n=8) was added to monolayers in duplicate halves of a 96-well plate to stimulate the respiratory burst. After 30 min, NBT was added to the wells and incubated for an additional 30 min prior to methanol fixing and measurement of reduced formazan. Data represent the ratio of A_{630} values ± SD from wells incubated with antigen to wells incubated with L-15. Different letters denote a significant difference ($p<0.05$) between groups.

4.3.5 Respiratory burst stimulation by washed *P. salmonis* antigen and washed CHSE antigen:

Assay 1 – delayed NBT addition

In the preparation of macrophages from HK cell suspensions, comparison of adherent cell populations obtained from the 34/51% Percoll interface with those obtained from the medium/34% Percoll interface indicated that higher numbers of adherent cells were retained at the latter interface (Fig. 4.5). Therefore, in all subsequent separations, leukocyte suspensions were prepared using a single 51% Percoll cushion for removal of red blood cells, and macrophage-enriched populations were obtained by attachment from the band of cells obtained at the medium/51% Percoll interface.

Cell counts taken at the end of the experiment showed that one of the plates, which

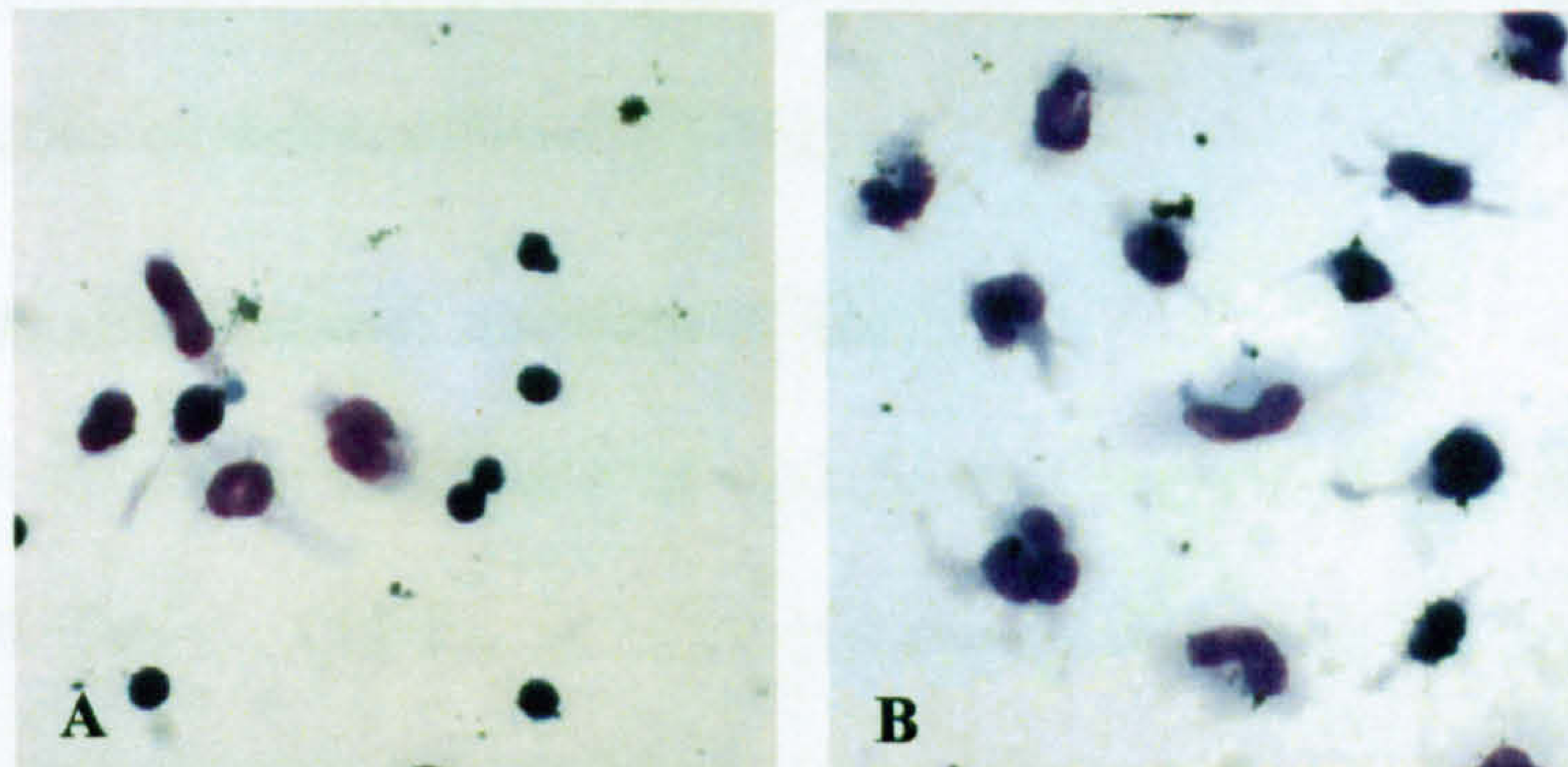


Fig. 4.5. Adherent leukocyte populations obtained following separation on 34/51% Percoll gradients. Aliquots of cells from the 34/51% Percoll interface (A) and the medium/34% Percoll interface (B) were allowed to adhere to glass slides for 3 h. Unattached cells were removed by washing and the remaining attached cells were stained using Rapi-Diff before examinination by oil-immersion lens using a Leitz Ortholux light microscope. Magnified x1000.

appeared to have the higher cell density after washing at 3 h, had the lower residual cell number at 48 h, possibly due to nutrient depletion and ensuing cell loss caused by high cell numbers. This plate (low cell density, LCD) had approximately 3.3 x 10^5 cells remaining per well compared with the second plate which had approximately 15.9 x 10^5 cells remaining per well (high cell density, HCD). Comparison of A_{630} values from the LCD plate using ANOVA revealed no difference between the antigen stimulant groups (Psal and CHSE) and/or sEMEM controls with either neat or washed antigen (n=8) (Fig. 4.6 (A)). Comparison of A_{630} values from the HCD plate using ANOVA showed that, for neat antigen, both stimulant groups were significantly lower than sEMEM controls ($p<0.001$; n=8) (Fig. 4.6 (B)). There was no significant difference between the neat Psal or CHSE stimulant groups. However, for washed antigen, wells stimulated by $Psal_W$ had significantly lower A_{630} values than wells stimulated by $CHSE_W$ ($p = 0.009$; $p<0.05$ Student's t-test; n=8). There was no significant difference between the $CHSE_W$ stimulated group and/or sEMEM controls. PMA stimulated respiratory burst in positive control wells (n=8). Results were not available for wells containing SOD.

Assay 2 – using simultaneous addition of NBT.

No significant difference was found in A_{630} between monolayers of pooled macrophages stimulated with $Psal_W$, $CHSE_W$ or L-15 controls (n=12) (Fig. 4.7 (A)) when NBT was added simultaneously with antigen. Wells stimulated with PMA (n=8) exhibited increased formazan reduction (A_{630}= 0.133) which was inhibited by SOD (n=4, A_{630}= 0.119). However, in macrophage monolayers from the fifth fish, exposure to $Psal_W$ (n=4) resulted in A_{630} values which were significantly reduced when compared with L-15 controls (n=3)($p=0.037$, $p<0.05$) (Fig. 4.7 (B)).

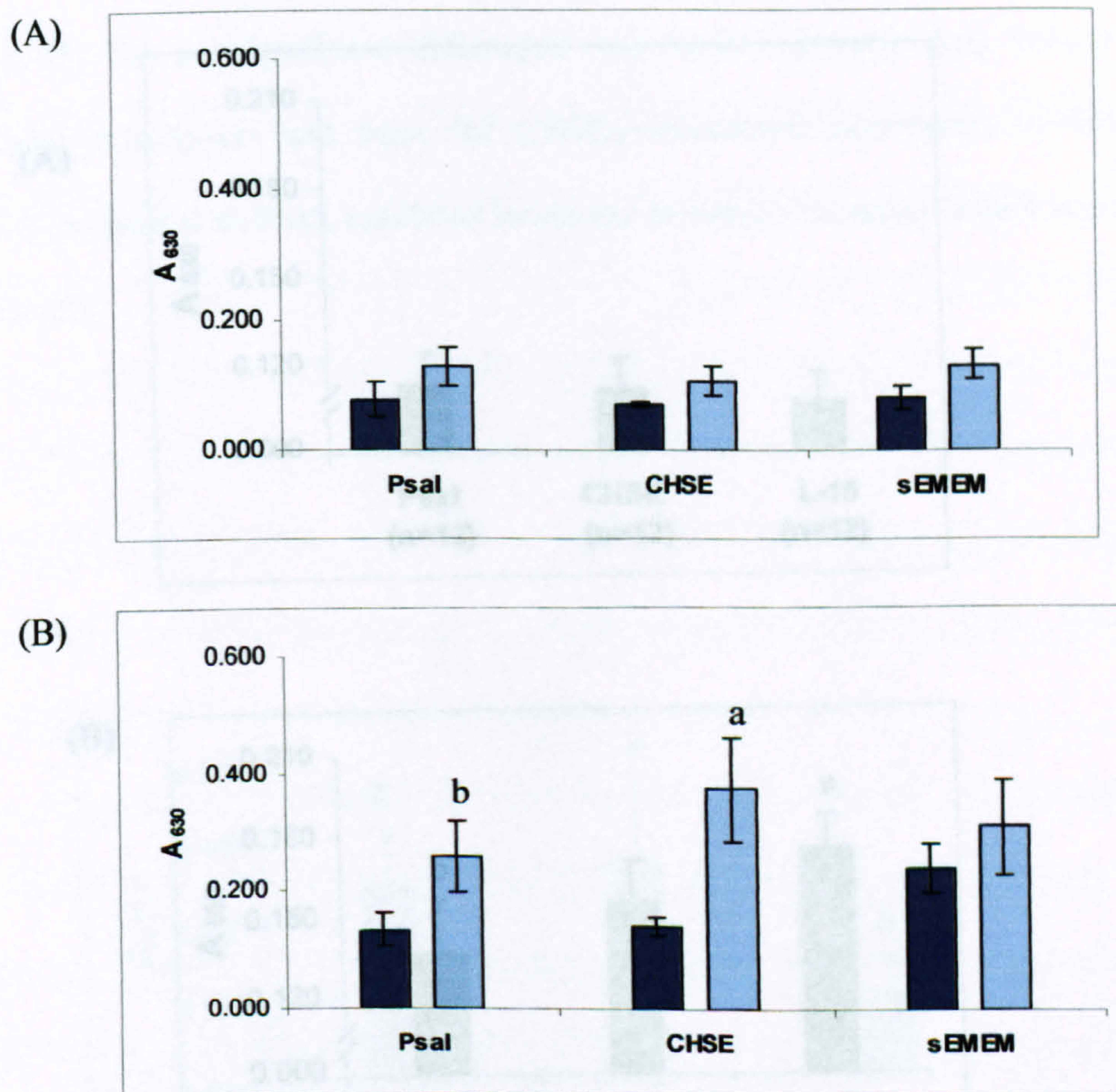
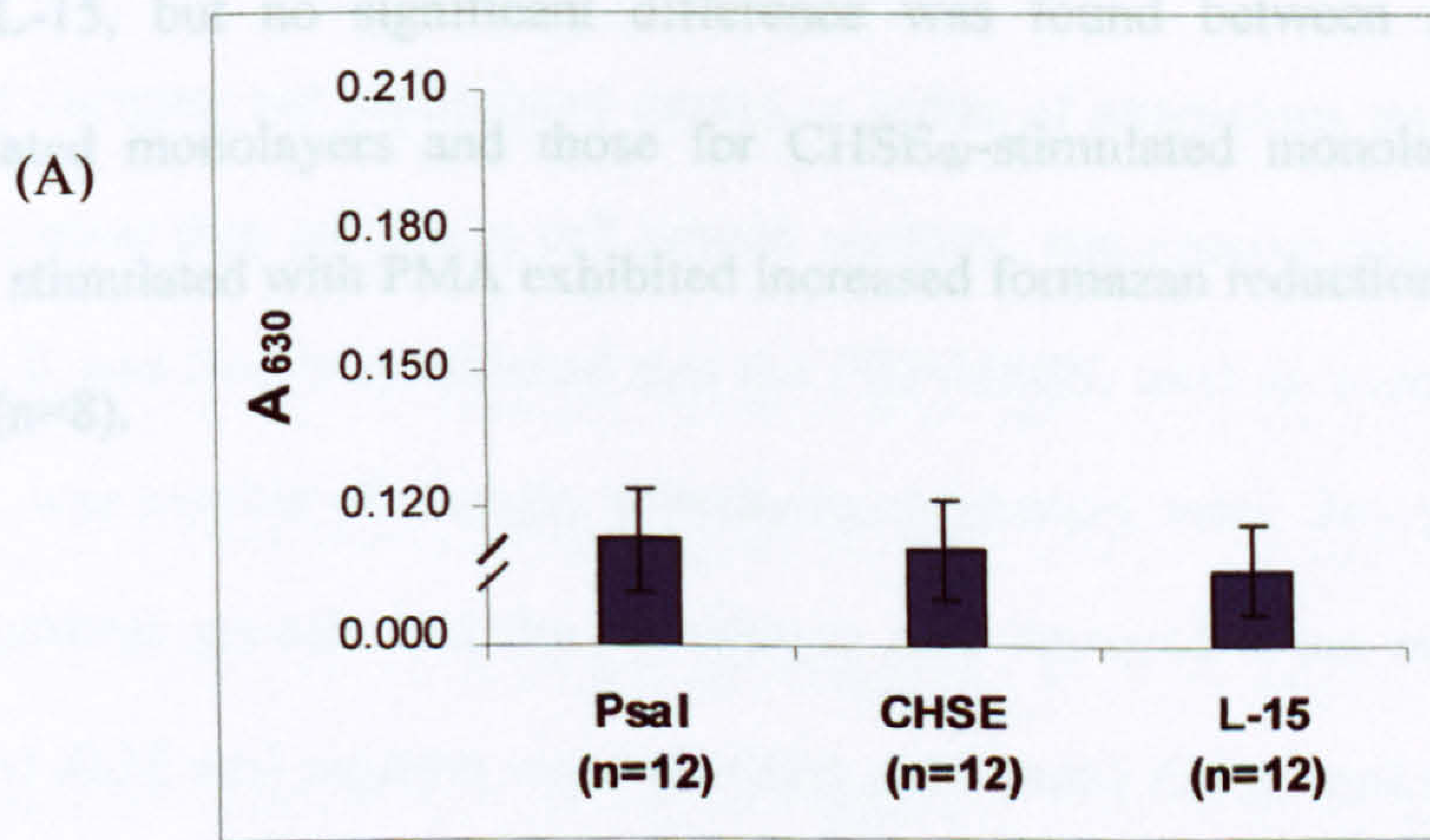


Fig. 4.6. (Assay 1) Respiratory burst activity of rainbow trout head kidney macrophages at a low cell density (A) or high cell density (B) following stimulation with *P. salmonis,* as measured by the reduction of NBT after 20 min incubation. Macrophages were stimulated with neat ■ or washed ■ *P. salmonis* culture supernatant (Psal), CHSE-214 cell suspension (CHSE) or with sEMEM culture medium (sEMEM). Data represent the mean absorbance values of replicate wells (n=8) at 630 nm (A_{630}) ± standard deviation (SD). In (B), different letters denote a significant difference ($p<0.05$) between groups.



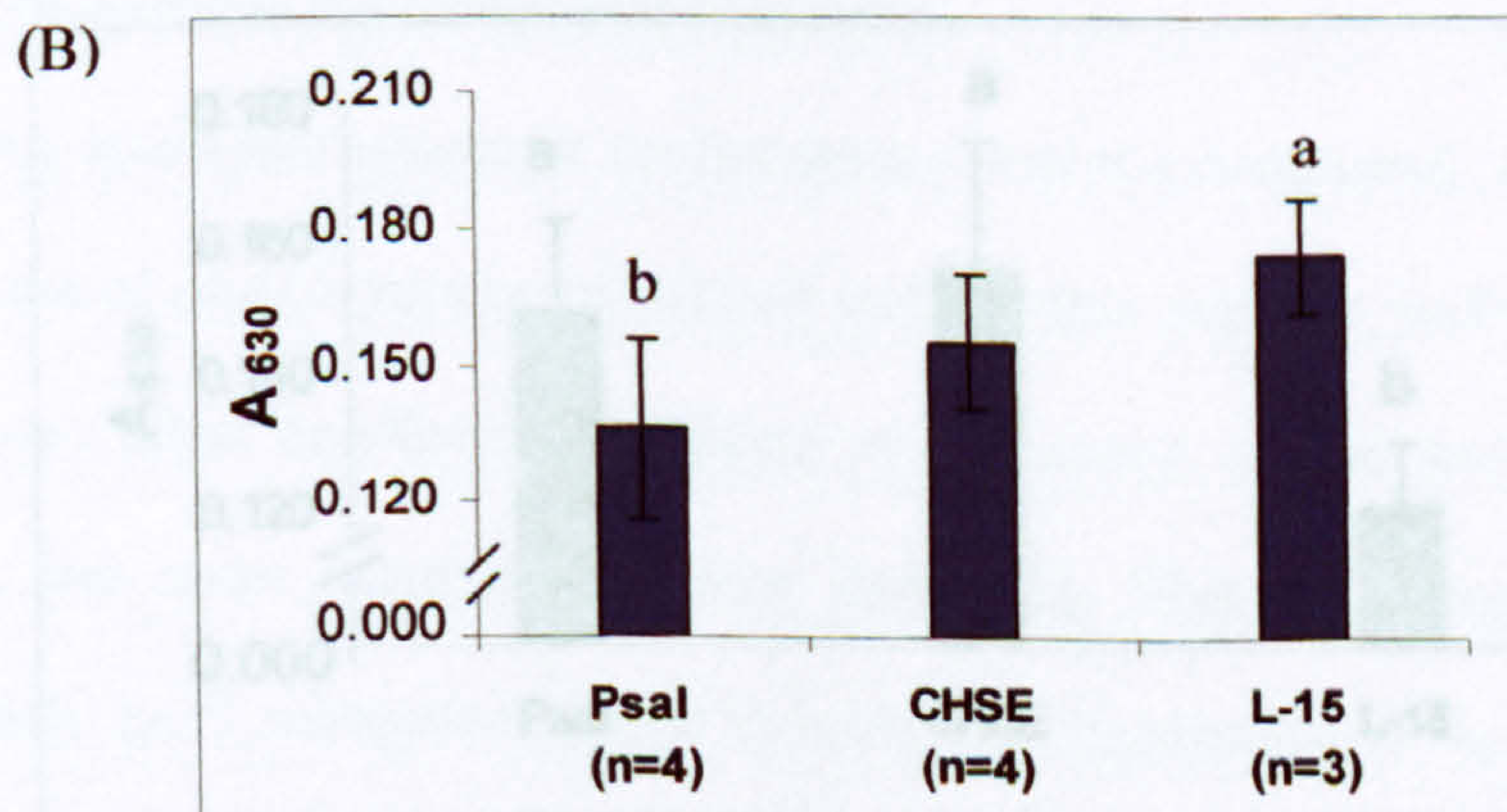


Fig. 4.7. (Assay 2) Respiratory burst activity of rainbow trout HK macrophages pooled from 4 fish (A) or from a single fish (B) following stimulation with *P. salmonis*, measured as the reduction of NBT after 20 min incubation. Macrophages were stimulated with washed *P. salmonis* culture supernatant (Psal), with washed, lysed CHSE-214 cell suspension (CHSE) or with L-15 culture medium (L-15) in the presence of NBT. Data represent the mean absorbance values of replicate wells at 630 nm (A_{630}) ± standard deviation (SD). In (B), different letters denote a significant difference ($p<0.05$) between groups

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Assay 3 – Using concentrated antigen and simultaneous addition of NBT.

Both $Psal_W$ and $CHSE_W$ concentrated antigens caused increased respiratory burst compared with L-15, but no significant difference was found between A_{630} values for $Psal_W$-stimulated monolayers and those for $CHSE_W$-stimulated monolayers (n=16) (Fig. 4.8). Wells stimulated with PMA exhibited increased formazan reduction which was inhibited by SOD (n=8).

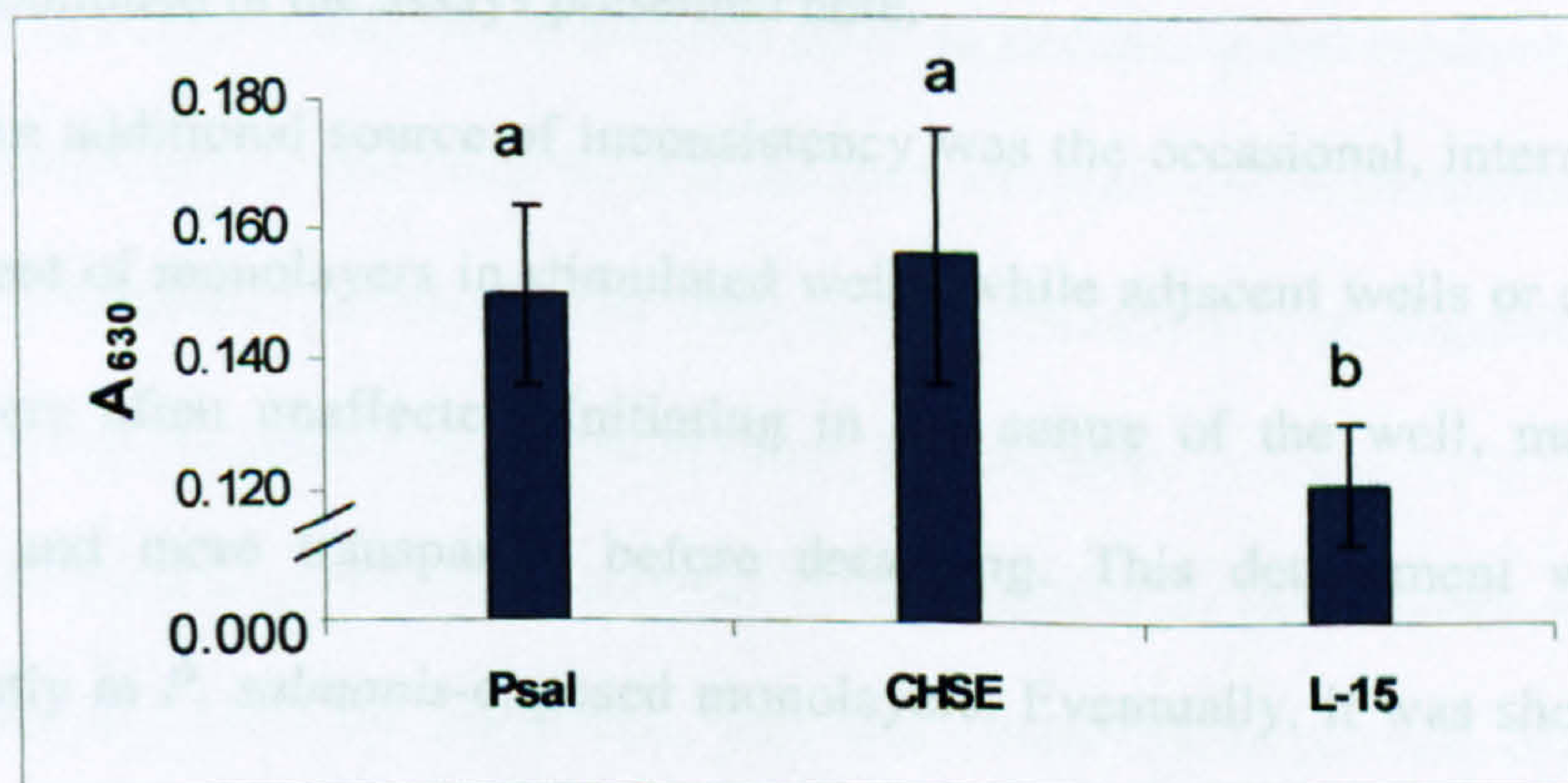


Fig. 4.8. (Assay 3) Respiratory burst activity of rainbow trout head kidney macrophages following stimulation with *P. salmonis*, measured as the reduction of NBT after 30 min incubation. Macrophages were stimulated with washed *P. salmonis* culture supernatant (Psal), with washed, lysed CHSE-214 cell suspension (CHSE) or with L-15 culture medium (L-15), in the presence of NBT (n=16 wells). Data represent the mean absorbance values of replicate wells at 630 nm (A_{630}) ± standard deviation (SD). Different letters denote a significant difference ($p<0.05$) between groups.

4.4 Discussion

While carrying out preliminary assays, a series of anomalous results suggested that some factor, other than antigen or cell growth medium, was causing stimulation of the respiratory burst. It was finally established that the PRF-HBSS, used to wash monolayers prior to the assay, was capable of strongly stimulating respiratory burst. Sterility checks were negative for bacterial growth, and the stimulation also occurred when sterile-packed and sterile-filtered (0.22 μm) medium was used (data not shown). Other workers have used PRF-HBSS for respiratory burst assays and no stimulatory effect has been reported. However, its use was discontinued in the assays presented here.

An additional source of inconsistency was the occasional, intermittent and variable detachment of monolayers in stimulated wells, while adjacent wells or consecutive 96-well plates were often unaffected. Initiating in the centre of the well, macrophages became rounded and more transparent before detaching. This detachment was observed most consistently in *P. salmonis*-exposed monolayers. Eventually, it was shown that, while cell monolayers would tolerate total aspiration of medium during multiple changes of medium (i.e. brief 'drying out'), monolayers must be completely covered by a 'buffering' liquid layer if they were to withstand the addition of *P. salmonis* preparations or MAF-containing supernatants (Chapter 6; Section 6.2.5). This effect of *P. salmonis* on HK macrophages is similar to the cytotoxic effects described by Olivier *et al.* (1992) and Leung *et al.* (1995) for *A. salmonicida* and *A. hydrophila* respectively. The partial loss of monolayer is potentially a source of false low values suggestive of respiratory burst inhibition but, with the exception

of Leung *et al.* (1995), this observation has not been reported by other investigators of respiratory burst in fish macrophages.

Another potential source of error is the method of removing supernatant prior to adding methanol fixative to the monolayers. Apparent inversions of stimulation results had been observed in preliminary assays following the methanol fixation step, and a comparative assay on a single 96-well plate was used to confirm that the method of removing supernatant could directly affect assay results (Section 4.3.4). It was thought that this may have resulted from the dislodgment, while decanting medium, of monolayers loosened by exposure to *P. salmonis*. Therefore, in subsequent assays, methanol was first applied while monolayers were covered with medium in an effort to avoid dislodging the cells and to effect a degree of fixation prior to decanting the medium. Supernatants were then decanted gently and cells were fixed fully in fresh 100 % methanol. Subsequent visual checks of formazan levels before and after methanol fixing suggested that this milder procedure was successful in retaining intact monolayers.

The results of the assay seemed to be sensitive to the number of cells remaining in the monolayers used for the assay. This was observed on several occasions (results not shown) and confirmed (Section 4.3.5, Assay 1), when a significant decrease in stimulation was observed for antigen preparations compared with sEMEM controls when the residual macrophage number was high. Using the same pool of macrophages seeded in a replicate plate and using the same antigen preparations, no significant difference was seen when the macrophage number was low. It may be that the respiratory burst in antigen-stimulated wells was stronger and became exhausted more quickly in monolayers with higher cell density. Therefore, as NBT addition was delayed for 30 min following stimulation, the

respiratory burst in antigen wells of the HCD plate may already have occurred prior to NBT addition, resulting in lower reduced formazan levels and apparently lower respiratory burst than in sEMEM control wells. It may be that the residual macrophages in the LCD plate (at 48 h) had been compromised by the initial high density of cells (at 3 h) and were no longer capable of mounting a measurable respiratory burst. Johnston *et al.* (1978) reported that macrophage density affected the level of O_2^- release and postulated that higher cell densities may have compromised the nutritional status of the cells, thereby affecting their performance. Alternatively, the apparent lack of difference between antigen-stimulated wells and controls might have been because the overall level of stimulation was low and effects of treatments were not detectable because the degree of inter-well variation masked the differences.

It is also possible, however, that the effect of cell number is caused by the percentage of cells from different individuals remaining in the monolayers. In his work, Campos-Peréz (1998) reported variation in respiratory burst between fish. Activation of neutrophils increases their 'stickiness', or ability to adhere to surfaces (Anderson *et al.* 1992) and the role of surface adherence on macrophage maturation can affect the respiratory burst (Robinson and Badwey, 1994). The fish may have undergone prior exposure to some stimulant which activated their macrophages to varying degrees, hence affecting the strength of their adhesion to the plate surface and resistance to removal by washing. Differences in adhesive strength may also simply be inherent to macrophages from different individuals, as may their level of response to stimulation by different antigens. Individual variation occurs in macrophage phagocytic ability (Braun-Nesje *et al.* 1982) and sensitivity to various antigens (Plytycz *et al.* 1989; Sarmento *et al.* 2004b) and

thus, the overall level of stimulation will reflect the proportion of macrophages from variably activated individuals remaining in the monolayer. The results of Assay 2, in Section 4.3.5, appear to lend support to this theory. While no difference was seen between responses to *P. salmonis*, CHSE or L-15 stimulants using macrophages pooled from 4 fish, macrophages from a fifth individual exhibited a statistically reduced response to *P. salmonis* compared with L-15.

One of the difficulties of performing an assay using an intracellular bacterium, such as *P. salmonis*, is separating it from the host cell or host cell material so that the assay measures the effect of the bacterium and not that of the host cell material (Weiss *et al.* 1975; Krahenbuhl and Adams, 1994). In addition, the *P. salmonis* preparation potentially will contain toxins or other substances released by the organism or by dying cells which could be capable of stimulating respiratory burst (Campos-Peréz *et al.* 1997; Dixon and Stet, 2001; Stewart and Young, 2004) and may be the cause of the higher absorbance values for $Psal_{CL}$ compared with $CHSE_{CL}$ in unwashed antigen preparations (Section 4.3.3). These factors will not be present in CHSE-214 cell controls. The *P. salmonis* organism cultured *in vitro* is closely associated with the CHSE-214 host cell and, even following several centrifugation steps, there remains a considerable proportion of cell debris in the *P. salmonis* fraction (Henriquez *et al.* 2003). Therefore, any preparation intended to contain large amounts of *P. salmonis* will also contain a considerable quantity of CHSE-214 cell material which must be controlled for in the assay. In attempting to provide adequate controls for the effect of *P. salmonis* on respiratory burst in macrophages, cell controls were obtained from CHSE-214 monolayers of the same age/batch as the infected cells. These monolayers were then subjected to freeze/thawing at –20 °C and, in later experiments,

vortexing with glass beads, in an attempt to lyse the cells so that debris could be released into the supernatant. However, from early observation of viable rickettsia counts, it appeared that glass bead lysis might severely reduce viability of *P. salmonis* to a level below that required for respiratory burst stimulation and, therefore, glass bead lysis was not used for the final assay.

In the later experiments, antigen preparations were also washed by centrifugation in an effort to remove possible toxins from the *P. salmonis* preparation and present the macrophages with *P. salmonis* antigen or CHSE-214 antigen in equivalent media. Where residual macrophage cell number was low (Section 4.3.5; Assay 1), washing the antigen preparation appeared to make no difference to its ability to stimulate respiratory burst. Conversely, where the macrophage number was high, washing the antigen appeared to remove its inhibitory effect, although washed *P. salmonis* antigen still appeared to be more inhibitory than the CHSE-214 host cell preparation. This result may have been due to timing and level of respiratory burst in monolayers of different densities, and delayed addition of NBT, as previously discussed. In the final two assays, simultaneous addition of NBT with antigen preparations ensured detection of early stimulation of respiratory burst. Again, it was suspected that viability of *P. salmonis* might be reduced greatly during two centrifugation steps so, in the final assay, a single centrifugation wash step was used and washed *P. salmonis* culture supernatant was concentrated 3-fold to increase the number of viable organisms in the stimulatory dose. These final assays suggested that *P. salmonis* did not have an overall significant stimulatory effect on the macrophage respiratory burst.

Using killed *P. salmonis* antigen, Trevors *et al.* (1997) reported inhibition of superoxide anion production in Atlantic salmon macrophages, while carp macrophages

were unaffected. However, no information is given about the method of antigen preparation or the constituents of the stimulant, and the affect of viable organisms was not studied. Respiratory burst assays, including those in fish, frequently use bacteria at a multiplicity of infection (MOI) of 1 – 100 relative to macrophage numbers (Stave *et al.* 1985; Waterstrat *et al.* 1991; Bandin *et al.* 1993; Rao *et al.* 2001). From $TCID_{50}$ calculations, it is estimated that the MOIs used in this work were closer to 0.01 – 0.1 (5×10^3 - 5×10^4 viable *P. salmonis*.ml^{-1}: 3-4 x 10^5 macrophages.ml^{-1}) and, therefore, the level of challenge by *P. salmonis* was perhaps too low for any effect on respiratory burst to be measurable. To achieve MOI levels of 1.0 - 10 would require the cultivation and concentration of 150-1500 ml *P. salmonis* which was not possible given the time constraints of this project. It might be possible to produce large numbers of piscirickettsiae in insect tissue culture (Birkbeck *et al.* 2004a) but the cytotoxic effect of *P. salmonis* on macrophages, described above, may be exacerbated at higher MOIs (Garduno and Kay, 1992; Leung *et al.* 1995; Boesen *et al.* 2001).

While *R. salmoninarum* has been shown to exert a time and dose-dependent inhibitory effect on respiratory burst triggered by PMA in macrophages, in the period immediately following infection the bacterium itself causes low level stimulation of the respiratory burst (Bandin *et al.* 1993; Campos-Peréz *et al.* 1997). Therefore, it appears that *R. salmoninarum* requires time to express, or otherwise exert, its inhibitory effect. An alternative means of avoiding the killing effect of ROIs is to express enzymes, such as SOD and catalase which convert toxic molecules to non-toxic products (Barnes *et al.* 1999a; Barnes *et al.* 1999b; Campos-Peréz *et al.* 2000a; Boesen *et al.* 2001), or which affect the signalling pathway for the respiratory burst (Baca and Mallavia, 1997). However, a more

efficient and immediate method for the pathogen to avoid oxidative killing may be to enter the macrophage without triggering the respiratory burst. Binding of Fc receptors initiates production of the respiratory burst (Fenton and Vermeulen, 1996): mannose and Fc receptors are proficient at directing particles to phagolysosomes and triggering the burst (Mosser, 1994). In contrast, complement-mediated uptake does not trigger the respiratory burst and bacteria such as *Bordetella pertussis* utilise complement receptors to achieve 'silent' uptake (Sandstrom *et al.* 1988; Kaufmann, 1993; Sandros and Tuomanen, 1993). Nevertheless, circumvention of the respiratory burst by a pathogen does not ensure its subsequent survival in the macrophage (Winkler and Turco, 1994) as the phagocyte possesses other killing methods such as NO, and its defence mechanisms can be greatly enhanced by vaccination. These defences are discussed in the ensuing Chapters 5 and 6.

It has been demonstrated that a number of factors can affect the outcome of a respiratory burst assay using reduction of NBT. The assay is inherently subject to considerable variation from the number of cells in the monolayer and the tendency of cells to detach during stimulation and fixation steps. In addition, with respect to *P. salmonis*, the quantity of host cell debris remaining in piscirickettsial and cell control preparations is difficult to equalise and the quantity or potency of toxins is an unknown factor. The early work reported in this chapter was undertaken to develop a robust assay through minimising sources of variation and controlling for host cell effects. However, despite having finally designed a reliable assay (Sections 4.2.6.4; Assays 2 & 3), it was still not possible to distinguish between the effects of *P. salmonis* and CHSE-214 antigens, and establish whether or not *P. salmonis* has the ability to stimulate or inhibit the respiratory burst.

Chapter 5 - iNOS expression and nitric oxide production in rainbow trout challenged with *P. salmonis*

5.1 Introduction

In addition to ROIs, macrophages produce reactive nitrogen intermediates (RNIs) which can combine with products of the oxygen pathway to produce highly effective bactericidal species/components (Neumann *et al.* 2001; Acosta *et al.* 2003). The existence of this nitrogen-dependent killing mechanism was first intimated by the detection of nitrates excreted in the urine of febrile patients. Eventually, the activated macrophage was identified as the cell responsible for producing this nitrate and subsequent work has identified the enzyme and pathway involved (MacMicking *et al.* 1997).

Nitric oxide (NO) is produced by most nucleated cells in the body and has many functions (Michel and Feron, 1997; Nathan, 1997; Laing *et al.* 1999; Saeij *et al.* 2000). It is an inorganic gas which reacts in water with oxygen and its reactive intermediates to yield other radicals eg. NO_2, NO_2^-, NO_3^-, N_2O_3, $ONOO^-$ (Martin and Edwards, 1993; MacMicking *et al.* 1997). A product of normal metabolic processes, it is involved in smooth muscle relaxation, intercellular signalling and neurotransmission, and takes part in the antimicrobial and antitumour defences of macrophages (Weinberg *et al.* 1995). Due to its high affinity for iron, NO can affect iron-containing enzymes which are employed in such essential cellular processes as oxidative phosphorylation, iron storage and DNA replication (Woods *et al.* 1994; Laing *et al.* 1999; Neumann *et al.* 2001; Acosta *et al.* 2003).

The enzyme responsible for NO production, nitric oxide synthase (NOS) has three isoforms, each one coded by a different gene (MacMicking *et al.* 1997). Two forms are relatively constitutively expressed (Michel and Feron, 1997) and are Ca^{2+}/ calmodulin dependent. These forms are named neuronal NOS (nNOS) and endothelial NOS (eNOS), according to the cell type from which they were first isolated (MacMicking *et al.* 1997; Laing *et al.* 1999). The third form is expressed mainly in activated macrophages, but is also found in cells such as hepatocytes and chondrocytes, and is Ca^{2+}/ calmodulin independent (Laing *et al.* 1996; Saeij *et al.* 2000). This form is expressed after exposure of tissues to cytokines or bacterial LPS and is often referred to as inducible NOS (iNOS). In two sequential monooxygenase reactions, the substrate, L-arginine, is oxidised at a guanadino nitrogen to produce NO and L-citrulline (MacMicking *et al.* 1997). Flavine adenine dinucleotide (FAD), flavine mononucleotide, tetrahydrobiopterin and calmodulin act as cofactors (Saeij *et al.* 2000).

The primary product, NO, is itself capable of exerting bactericidal effects. However, peroxynitrite ($ONOO^-$), formed through reaction of NO with superoxide anion (O_2^-), a product of the respiratory burst, has even greater bactericidal ability (Acosta *et al.* 2003). While its impact is non-specific, NO is a small, amphipathic molecule and is capable of exerting bactericidal effects even in the host cell cytoplasm (Ohya *et al.* 1998). This makes it an important defence against bacteria such as *L. monocytogenes*, *M. tuberculosis* and *Rickettsia* spp. which can escape from the phagosome into the macrophage cytoplasm (Feng *et al.* 1994; Ohya *et al.* 1998; Alvarez-Dominguez *et al.* 2000; Raupach and Kaufmann, 2001). Reactive nitrogen intermediates can also act sequentially with ROIs to exert bactericidal effects (Ohya *et al.* 1998; Raupach and Kaufmann, 2001).

While the expression of iNOS and production of NO by murine macrophages has been well established, for many years there has been controversy about the ability of human macrophages to produce NO (MacMicking *et al.* 1997; Eremeeva *et al.* 2000). In teleosts, the existence of an iNOS gene has been confirmed and iNOS sequences of goldfish (*Carassius auratus*), carp and rainbow trout have been obtained (Saeij *et al.* 2000; Wang *et al.* 2001). Various groups have demonstrated the activity of an inducible NOS by fish following exposure to bacterial products or MAF-containing supernatants (Schoor and Plumb, 1994; Neumann *et al.* 1995; Yin *et al.* 1997; Campos-Peréz *et al.* 2000b; Tafalla and Novoa, 2000). However, there are differences between fish species in their requirements for induction of NO production. In carp, it was possible to stimulate NO production in adherent phagocytes *in vitro* using LPS alone (Saeij *et al.* 2000) while, in goldfish, enhanced induction of NO required synergy between MAF and LPS (Neumann *et al.* 1995). In addition, while head kidney (HK) macrophages isolated from immunised gilthead seabream (*Sparus aurata*) were capable of NO production for several days following challenge *in vitro* with *Ph. damselae* subsp. *piscicida* (Acosta *et al.* 2005), the ability to express iNOS in rainbow trout HK macrophages appears to be down-regulated rapidly *in vitro*, as no iNOS signal was detected by reverse transcription polymerase chain reaction (RT-PCR) in macrophages cultured *in vitro* for 24 h prior to challenge (Campos-Peréz, 1998).

This limited expression of iNOS *in vitro* by rainbow trout kidney macrophages echoes the pattern of expression found *in vivo*. While iNOS expression could be shown in gill tissues of rainbow trout between 6 h and 5 d following intraperitoneal injection of *R. salmoninarum*, expression in kidney tissue was found only at 24 h post-challenge, with a

limited expression still detectable at 3 d. Again, while iNOS expression could be found in the gill tissues of all trout 24 h following immersion challenge with *R. salmoninarum*, expression was only detected in the kidneys of 4 out of 6 fish (Campos-Peréz *et al.* 2000b).

Studies in catfish (*Clarias gariepinus*) (Yin *et al.* 1997), carp (Saeij *et al.* 2000) and gilthead seabream (Acosta *et al.* 2003) have established an association between inducible NO production in adherent macrophages and their ability to kill bacteria or parasites. Furthermore, Yin *et al.* (1997), Campos-Pérez *et al.* (2000b) and Acosta *et al.* (2005) demonstrated that it was possible to upregulate NO production by prior immunisation, suggesting that it might be possible to increase this non-specific response through vaccination, presumably through the production of cytokines by primed lymphocytes (Feng *et al.* 1994; Ohya *et al.* 1998; Feng and Walker, 2000).

The aim of the present chapter was to investigate if iNOS expression was induced in rainbow trout in response to challenge with *P. salmonis* and to determine if this expression could be up-regulated by vaccination with a recombinant vaccine. The collapse of a previous trial due to technical failures limited the resources available for sample analysis. *Piscirickettsia salmonis* produces marked pathology in the kidneys of affected fish (Chapter 1; Section 1.1.4) and can readily be re-isolated from the HK of artificially challenged rainbow trout (personal observation). Furthermore, the HK is central to immune responses against invading pathogens (Press and Evensen, 1999) and iNOS expression in this tissue may be of relevance in the mounting of an immune response following vaccination. Therefore, while it was likely that iNOS expression would be detected in gill tissue from infected fish, given the limited resources, it was decided to focus the present investigation on HK tissue. In order to test for expression of iNOS, the presence of iNOS mRNA was

assayed by RT-PCR of kidney tissue. As an additional test for iNOS expression, serum from vaccinated and challenged trout was tested for elevated levels of nitrates/nitrites, the end-product of NO metabolism.

5.2 Materials and Methods

5.2.1 Fish

Rainbow trout, obtained from local fish farms were held in tanks supplied with aerated flow-through dechlorinated water at the Aquatic Research Facility, Institute of Aquaculture, Stirling. The fish were fed daily on a dry pelleted commercial diet.

5.2.2 Piscirickettsiae

Piscirickettsia salmonis was propagated in CHSE-214 cell monolayers as previously described (Section 3.2.1). Titres of *P. salmonis* were calculated by end-point dilution using the method of Reed and Muench (1938).

5.2.3 Vaccination and challenge

Four weeks prior to the start of the experiment, fish (200-250 g) were acclimatised to a water temperature of 15-17°C. Following anaesthetisation in 4-ethyl aminobenzocaine, as described in Section 3.2.2, fish were immunised by i.p. injection of 0.1 ml SRS/Exp 4 vaccine (Schering-Plough Aquaculture). This is an experimental recombinant vaccine currently being field tested by Schering-Plough Aquaculture. The control fish received 0.1 ml sterile PBS by i.p. injection. Six weeks after vaccination, pre-challenge samples were

obtained from 4 vaccinated fish and 2 controls. Half of the remaining vaccinated fish were challenged by i.p. injection with 0.1 ml *P. salmonis* culture supernatant (18 fish – designated 'VR') while the other half were injected i.p.with 0.1 ml suspended CHSE-214 cell monolayer as challenge controls (17 fish – 'VC'). The sham-vaccinated fish were treated likewise, with half the number receiving *P. salmonis* challenge (18 fish – 'CR') and half receiving CHSE-214 cell suspension (18 fish – 'CC'). Each group was placed in a separate tank. On Days 1, 3 and 8 following challenge, 6 fish were sampled from each group. Fish were euthanised using 4-ethyl-aminobenzocaine, as described in Section 3.2.2, and ex-sanguinated by withdrawing blood from the caudal vein. This blood was retained to prepare serum for nitrate/nitrite measurements. To reduce the level of red blood cell contamination in kidney tissue samples, gill arteries were severed. Head kidney tissue (approx 1.0 g) was excised aseptically, using a sterile scalpel, placed immediately in 5 volumes RNAlater® and samples were stored at –20 °C.

5.2.4 Measurement of iNOS mRNA expression

5.2.4.1 Isolation of total RNA from head kidney

Total RNA was extracted from head kidney tissue samples (0.01 g) using TRI Reagent® following the manufacturer's instructions, and RNA pellets were stored overnight under 70 % (v/v) ethanol (RNase-free) at –20 °C. Ethanol was aspirated off, pellets were dried and resuspended in 50 µl diethyl-pyrocarbonate (DEPC)-treated water (RNase-free H_2O). Aliquots (1 µl) were diluted in 59 µl RNase-free Tris-EDTA (TE) buffer and concentration and purity of the RNA were quantified using a GeneQuant II (Pharmacia Biotech)

spectrophotometer. The RNA concentration was adjusted to 5.0 $\mu g.\mu l^{-1}$ with RNase-free H_2O.

5.2.4.2 cDNA synthesis

mRNA was reverse transcribed from 5 μg total RNA using the ThermoScript™ RT-PCR System and following the manufacturer's instructions for oligo (dT) priming. Reactions containing RNase-free H_2O instead of total RNA were used as negative controls for possible aerosol contamination.

5.2.4.3 Optimisation of PCR conditions

PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

PCR amplifications were performed with primers for GAPDH as a positive control for RT-PCR, as this gene is constitutively expressed. Primers were: GAPDH Forward (5'-ATGTC AGACC TCTGT GTTGG-3') and GAPDH Reverse (5'-TCCTC GATGC CGAAG TTGTC G-3'). Reactions were carried out using 0.2 ml Ready-to-Go PCR beads, following the manufacturer's instructions, with a final concentration of 0.5 μM of each primer and 1 μl template cDNA. The mixture was denatured at 95 °C for 5 min followed by cycling at 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 7 min. Initially, amplification was carried out with a range of cycles from 21-30 to determine the linear range of amplification. All amplifications were performed using a Biometra T Gradient thermocycler and 5 μl of each amplification reaction was examined for specificity by electrophoresis on a 1 % agarose gel containing 0.5 $mg.ml^{-1}$ ethidium bromide. Molecular weight markers (PCR 100bp ladder) were added to the gel as a reference.

Positive control for iNOS PCR

Gill tissue, obtained previously from two non-vaccinated pre-challenge and two *P. salmonis*-challenged (Day 4 post-challenge) rainbow trout, was subjected to the same total RNA extraction and RT procedures as described above for kidney tissue. Reaction products were pooled and used to optimise the iNOS PCR cycling conditions. In addition, cDNA template obtained from gill tissue of un-vaccinated, un-challenged fish was also amplified by iNOS PCR. Amplifications were carried out using iNOS primers F4 (positions 2394 – 2417 in the iNOS cDNA sequence) and R5 (positions 3139 - 3116 in the iNOS cDNA sequence), designed by Wang *et al.* (2001). These primers were: INOS F4 (5'-CATAC GCCCC CAACA AACCA GTGC-3') and INOS R5 (5'-CCTCG CCTTC TCATC TCCAG TGTC-3'). Reactions were performed using 0.2 ml Ready-to-Go PCR beads, following the manufacturer's instructions, with a final concentration of 1.0 μM of each primer and 2 μl template cDNA. All amplifications were performed using a Biometra T Gradient thermocycler. The mixture was denatured at 95 °C for 5 min. Cycling parameters were 95 °C for 1 min, 61-67 °C annealing gradient for 40 sec, 72 °C for 40 sec <u>or</u> 95 °C for 1 min, 61-67 °C annealing gradient for 1 min, 72 °C for 40 sec. After 40 cycles of amplification followed by an extension step at 72 °C for 7 min, 5 μl of each amplification reaction was examined by electrophoresis on a 1 % agarose gel, as above.

To confirm that this PCR product was part of the iNOS gene, the amplified band was excised from the gel and cleaned using a GFX™ PCR DNA and Gel Band Purification Kit to obtain template DNA for sequencing. Sequencing reactions were carried out using a DYEnamic™ ET terminator kit Cycle Sequencing Kit following the manufacturer's instructions. Primers used for the PCR reaction were diluted to 5 pmol and used as

sequencing primers and the DNA was sequenced in the forward and reverse directions. Sequencing reactions were run on the ABI PRISM™ 377 DNA Sequencer, were viewed using BioEdit software (Hall, 1999) and aligned using Clustal X (Thompson *et al.* 1994) with manual editing.

5.2.4.4 PCR amplification of iNOS cDNA

Amplifications were carried out on cDNA prepared from kidney samples using iNOS primers F4 and R5, as above, and amplified by denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 66.4 °C for 40 sec, 72 °C for 40 sec with an extension at 72 °C for 7 min. Reactions contained 1.0 μM of each primer and 2 μl template cDNA. Positive control reactions contained 2 μl cDNA obtained from gill tissue, as described above, and negative control reactions contained 2 μl RNase-free H_2O instead of cDNA template.

5.2.4.5 Densitometric comparison of amplification products

Gel images were captured using a UVP Gel Doc System (UVP, Cambridge, UK) and images were analysed using TotalLab software (UVP). Amplified GAPDH bands from each gel were normalised to a band from the molecular weight marker (MWM) on the same gel which was within the linear range of fluorescence detected by the camera. Relative concentrations of amplified GAPDH DNA could then be calculated from the known quantity of DNA in the band of the MWM. Where possible, the same band from the MWM was used as the reference band on every gel, in an attempt to minimise inter-gel variation. By calibrating concentrations of amplified iNOS bands in a similar manner, relative concentrations of iNOS and GAPDH PCR products could then be calculated for each fish. By establishing the expression level of iNOS mRNA relative to the constitutive expression

level of GAPDH, it could be established if iNOS expression in the individual fish was up- or downregulated, or unchanged.

5.2.5 Measurement of serum nitrate/nitrite levels

Blood samples were allowed to clot for 2 h at room temperature (RT, approx 21 °C) or at 4 °C overnight. Following centrifugation at 9,300 x g, serum was removed by aspiration and stored at –20 °C until required. Serum (100 µl) from each fish was dispensed into a 96-well microtitre plate, in triplicate. Nitrates were converted to nitrites by adding 3 µl nitrate reductase mix (1.5 $U.ml^{-1}$ nitrate reductase, 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 5 µM FAD) and incubating at 27 °C for 1 h. Serum nitrate/nitrite levels were measured using the method of Campos-Pérez (1998), with modifications. To prevent interference with subsequent nitrite determinations, NADPH was removed by oxidation with 1 µl lactate dehydrogenase (2.5 U) in 10 µl 100 mM sodium pyruvate at 37°C for 1 h. Nitrite quantities were then determined using the Greiss reaction (Campos-Peréz, 1998). To each 100 µl volume of serum was added first 50 µl 1.0 % (w/v) sulphanilamide/ 5 % (v/v) phosphoric acid and then 50 µl 0.1 % (w/v) napthylethylene diamine dihydrochloride and, after mixing, samples were held at RT for 10 min prior to reading the absorbance at 540nm using a Dynex MRX II ELISA reader. Serial doubling dilutions of 0.1 mM sodium nitrite, subjected to the Greiss reaction, were used to generate a standard curve.

5.3 Results

5.3.1 Measurement of iNOS mRNA expression

5.3.1.1 Optimisation of PCR conditions.

PCR amplification of GAPDH

PCR using 25 cycles of amplification was within the linear phase of signal amplification for GAPDH (Fig. 5.1). Subsequent GAPDH PCR amplifications using 25 cycles allowed titration of the amount of template used for PCR reactions so that the products of each sample amplification could be compared.

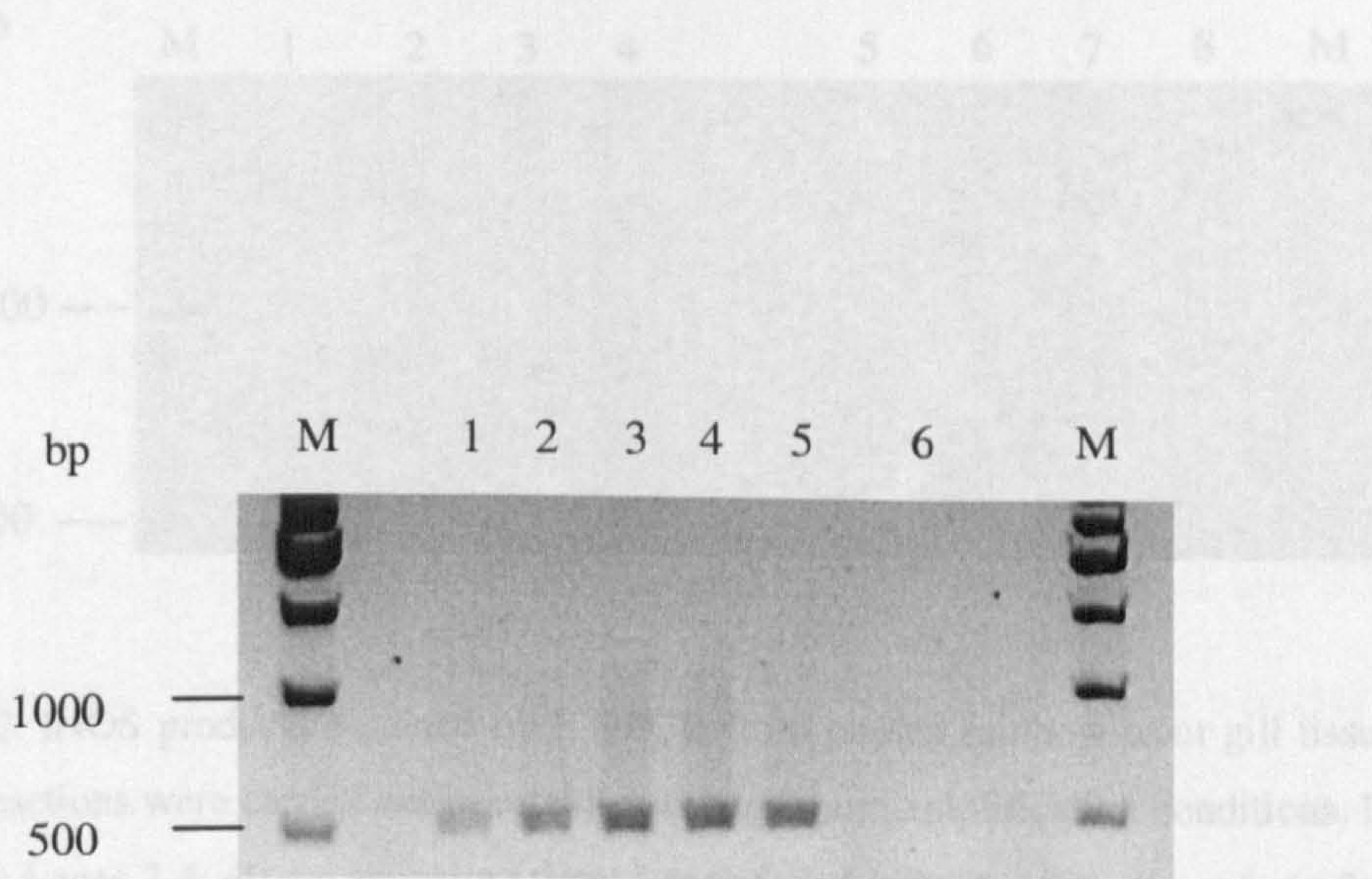


Fig. 5.1. GAPDH products from RT-PCR amplifications conducted to establish the linear phase of signal amplification. Lane 1: 23 PCR amplification cycles; Lane 2: 25 cycles; Lane 3: 27 amplifications; Lane 4: 29 amplifications; Lane 5: 30 amplifications; Lane 6: negative control; M: molecular weight marker.

Positive control for iNOS PCR.

PCR amplifications were carried out using pooled cDNA template obtained from gill tissues as described in Section 5.2.4.3. A 746 bp product, confirmed by sequencing as iNOS DNA, was successfully amplified indicating that PCR conditions were suitable for DNA amplification with iNOS primers, F4 and R5. Optimum amplification was obtained with an annealing temperature of 66.4 °C and an annealing time of 40 sec (Fig. 5.2).

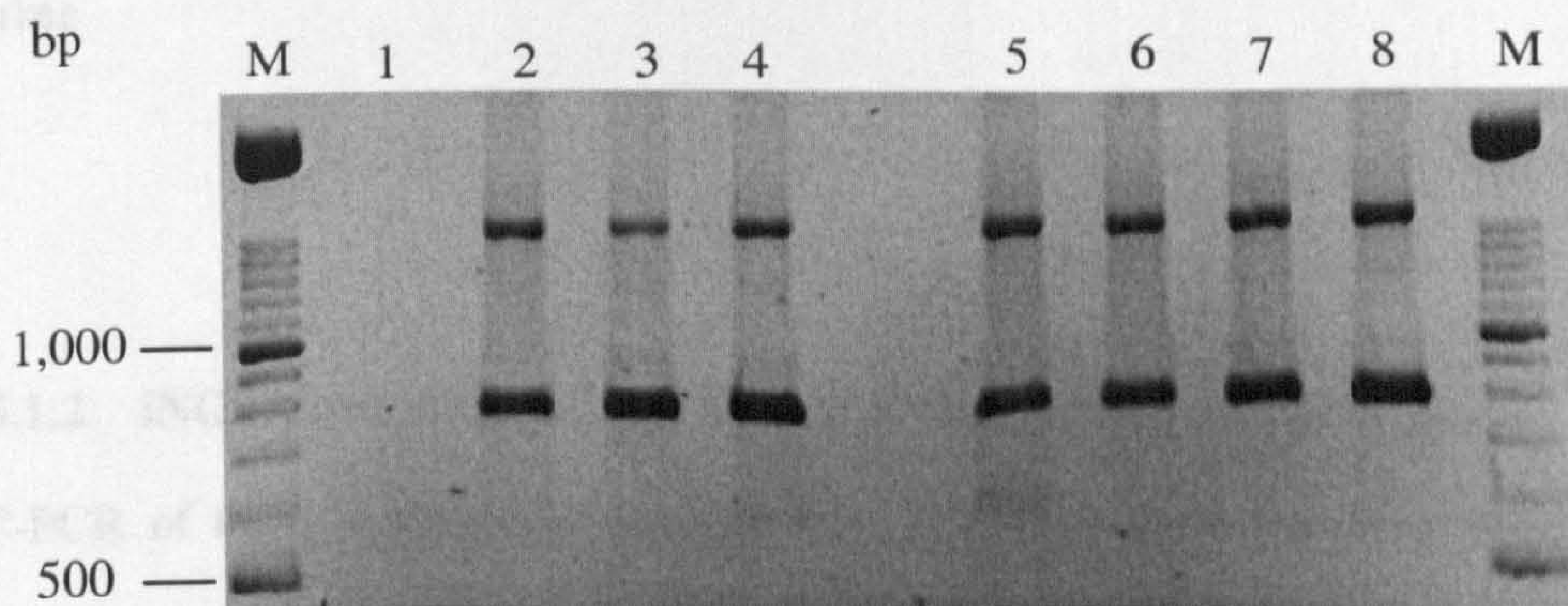


Fig. 5.2. iNOS products obtained by RT-PCR from pooled rainbow trout gill tissue. A number of PCR reactions were carried out to establish the optimum amplification conditions. Lane 1: negative control; Lanes 2-4: 40 sec annealing time; Lanes 5-8: 1 min annealing time; Lane 2: 61°C annealing temperature; Lane 3: 66.4°C; Lane 4: 67°C; Lane 5: 61°C; Lane 6: 64°C; Lane 7: 66.4°C; Lane 8: 67°C; M: molecular weight marker.

iNOS expression in gill tissue from non-vaccinated fish

An iNOS product was amplified from gill tissue of un-vaccinated, un-challenged fish (Fig. 5.3).

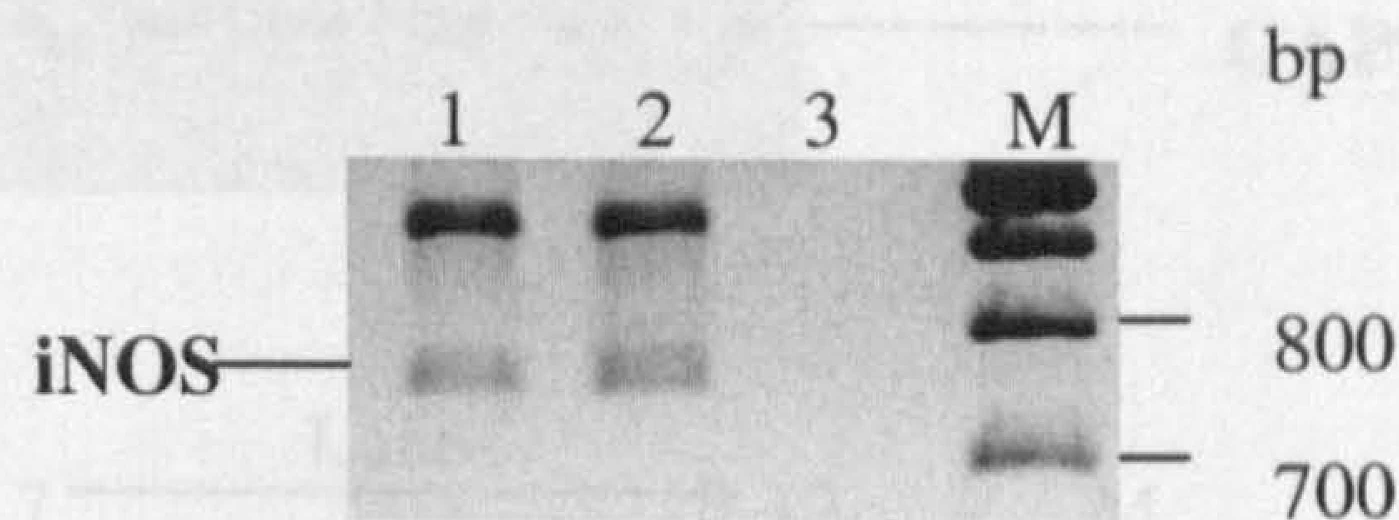
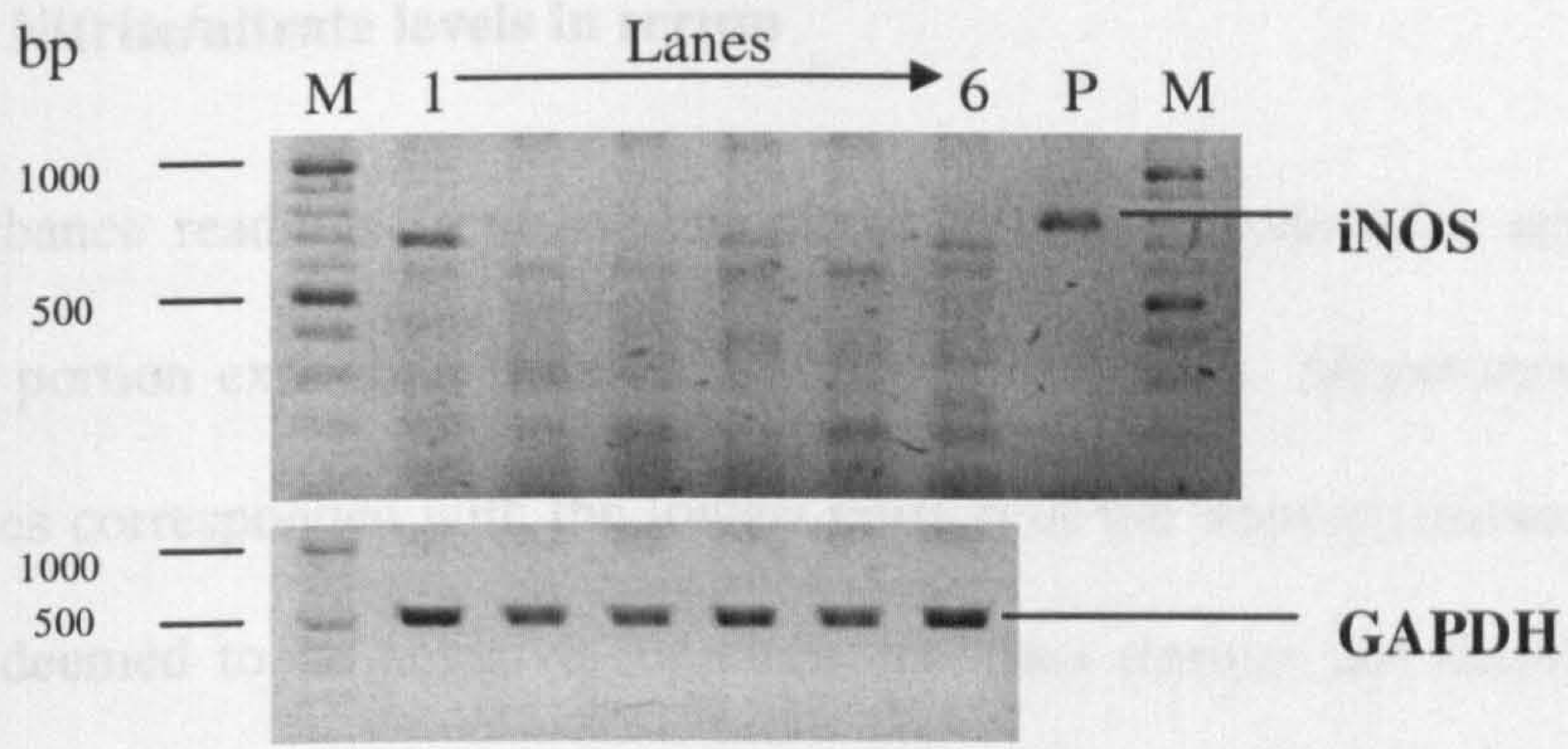


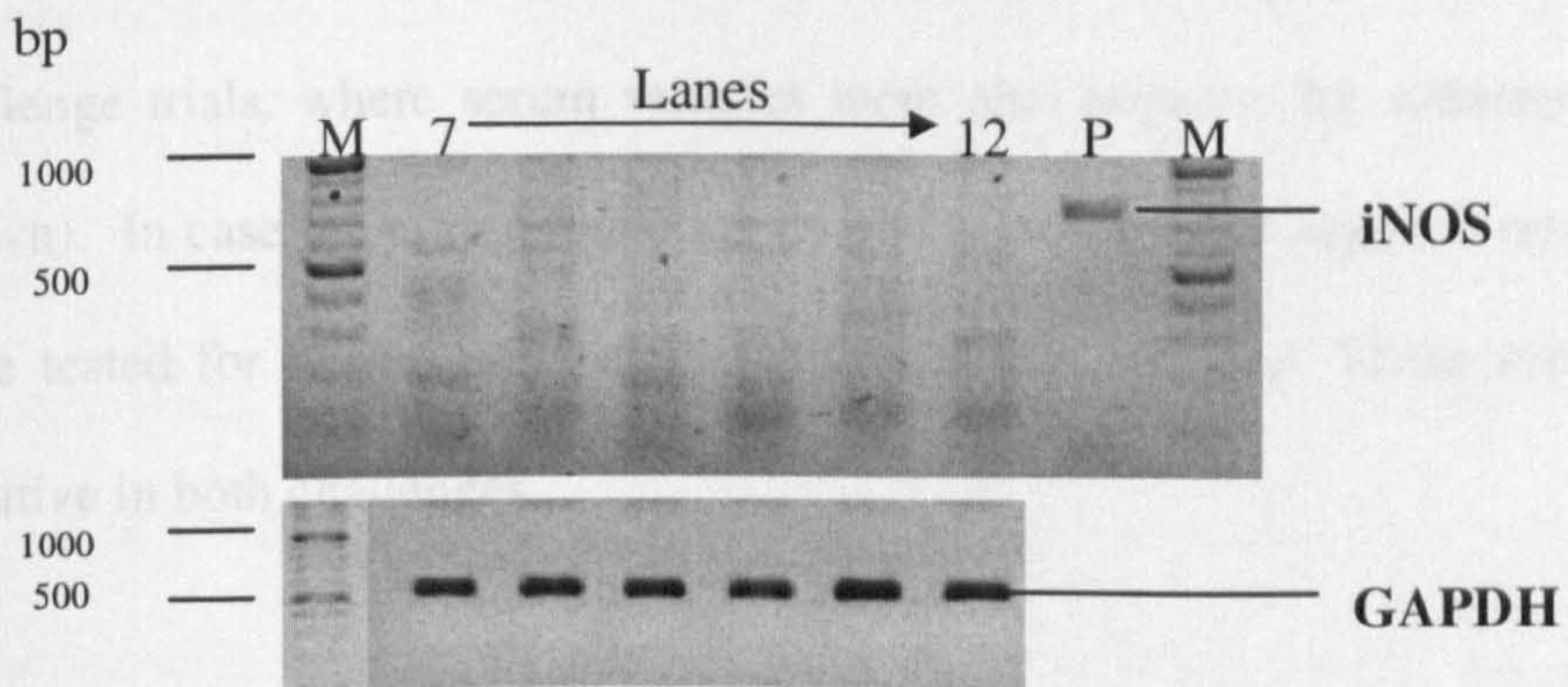
Fig. 5.3. iNOS products amplified by RT-PCR from gill tissue of non-vaccinated, pre-challenge rainbow trout. Lane 1: Fish 1; Lane 2: Fish 2; Lane 3: negative control; M: molecular weight marker.

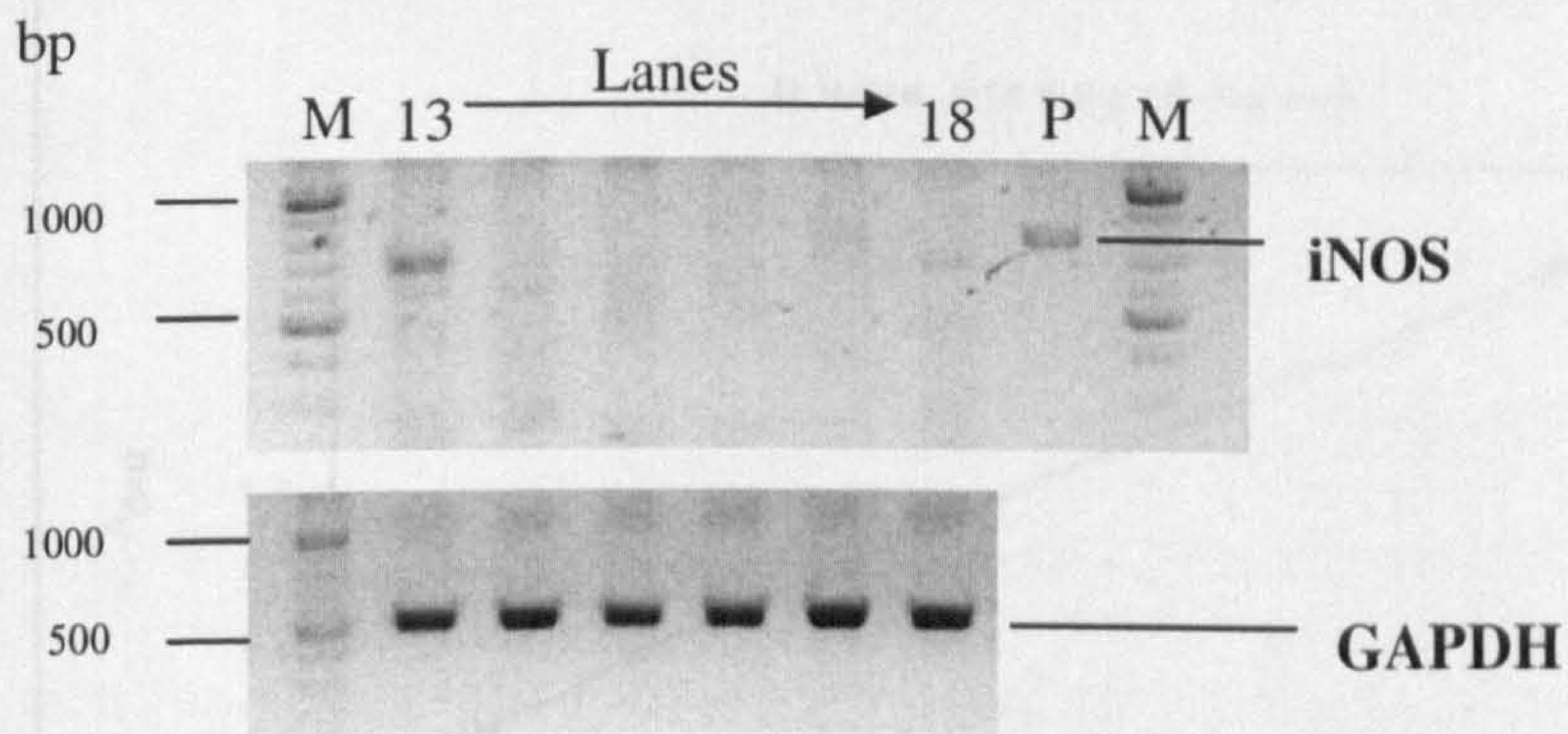
5.3.1.2 iNOS expression in head kidney tissue

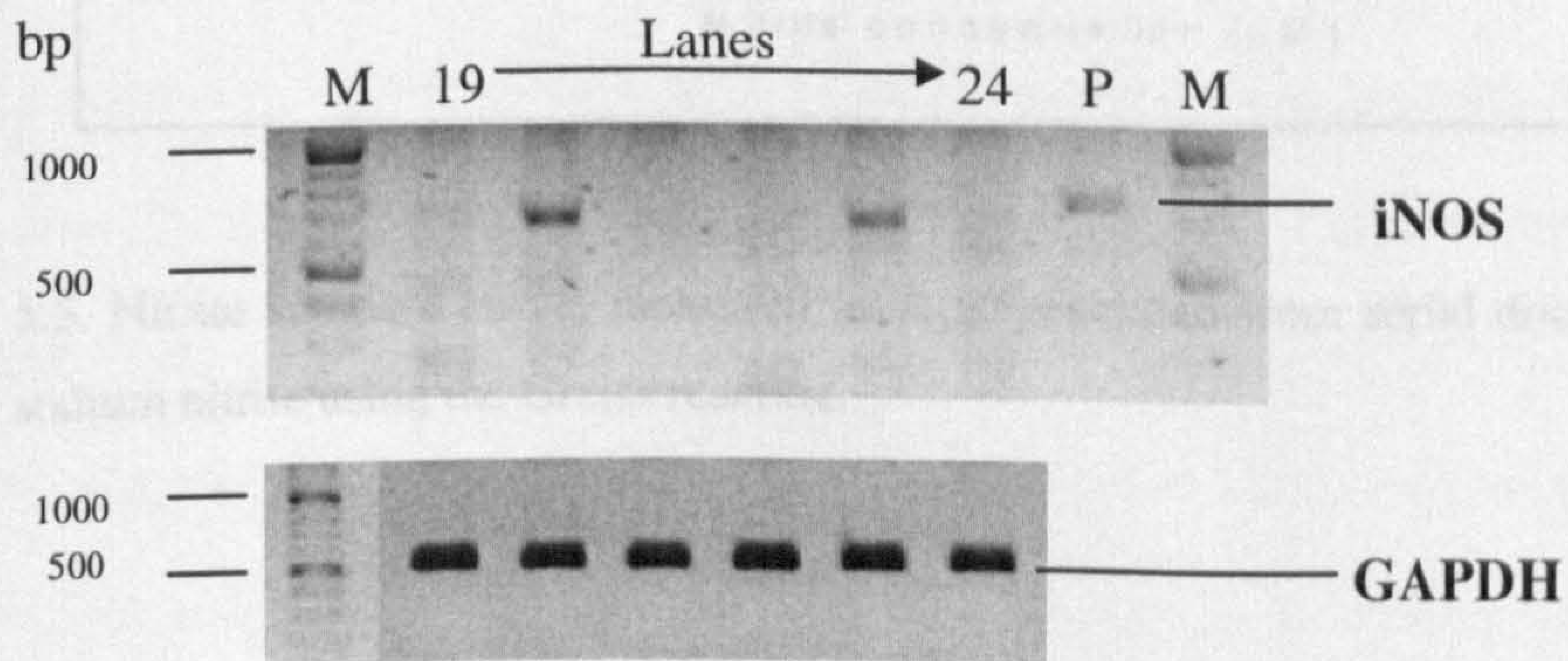
RT-PCR of head kidney cDNA using GAPDH primers successfully amplified a 515 bp product from most kidney samples, indicating that RNA extraction and RT reactions had produced amplifiable cDNA from these fish. However, all kidney samples were negative for iNOS DNA amplification. Representative results, from RT-PCRs carried out on kidney samples collected 24 h following challenge with *P. salmonis*, are shown in Fig. 5.4. Similar results were obtained with samples taken pre-challenge and on days 3 and 8 following challenge (not shown). Prominent bands in the region of 700 bp were confirmed not to be iNOS products by excising them from the gel and sequencing.

Fig. 5.4. Results from GAPDH and iNOS RT-PCR of kidney samples taken from rainbow trout 24 h following challenge with *P. salmonis*. Lanes 1-6: sham-vaccinated fish challenged with CHSE-214 cell suspension (CC); Lanes 7-12: sham-vaccinated fish challenged with *P. salmonis* (CR); Lanes 13-18: vaccinated fish challenged with CHSE-214 cell suspension (VC); Lanes 19-24: vaccinated fish challenged with *P. salmonis* (VR); P: positive control gill sample; M: molecular weight marker.


bp
M 1 Lanes 6 P M
1000
500
iNOS
1000
500
GAPDH


bp
M 7 Lanes 12 P M
1000
500
iNOS
1000
500
GAPDH


bp
M 13 Lanes 18 P M
1000
500
iNOS
1000
500
GAPDH


bp
M 19 Lanes 24 P M
1000
500
iNOS
1000
500
GAPDH

5.3.2 Nitrite/nitrate levels in serum

Absorbance readings from sodium nitrite dilutions produced a standard curve, with the linear portion extending from 25.0-0.78 μM (Fig. 5.5). Absorbance readings from serum samples corresponded with the lowest portion of the standard curve so that serum samples were deemed to be negative for nitrates/nitrites (results not shown). Statistical analyses confirmed the absence of significance in the results. This agreed with results of preliminary challenge trials, where serum samples were also negative for nitrates/nitrites (results not shown). In case the nitrate reduction step was the cause of negative results, serum samples were tested for nitrites using only the Greiss reaction step. These results, likewise, were negative in both challenges.

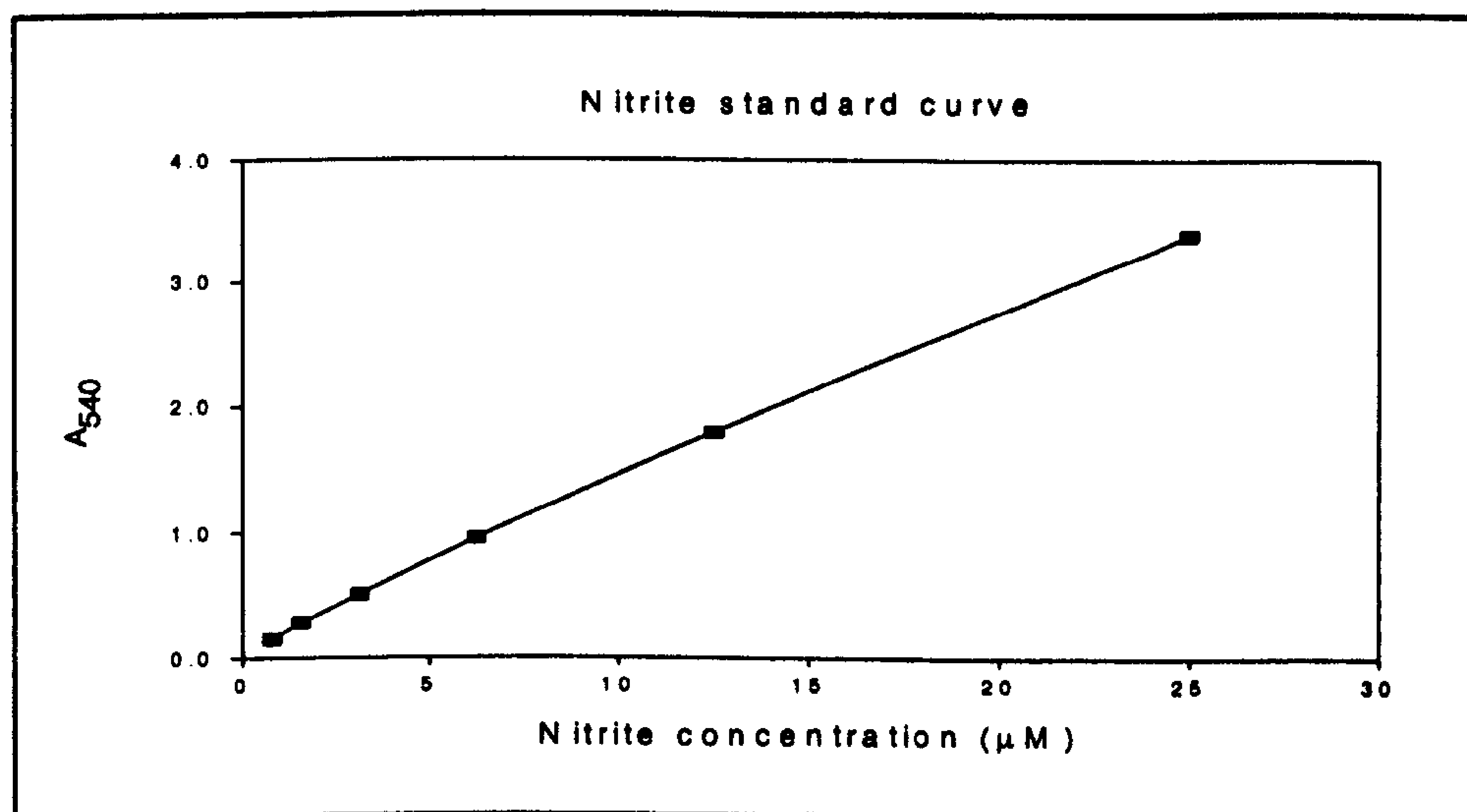


Fig. 5.5. Nitrite standard curve, measured as A_{540}, generated from serial doubling dilutions of 0.1 mM sodium nitrite using the Greiss reaction.

serum nitrites/nitrates, Campos-Peréz (1998) demonstrated that elevated levels occurred later following challenge with a virulent *R. salmoninarum* strain than with attenuated strains. From this, it appeared that a virulent organism might produce a delayed but more prolonged expression of iNOS. Therefore, in the present work, where fish were challenged with virulent *P. salmonis*, samples were taken at 24 h, but also at 3d and 8d following challenge in order to detect delayed expression. Nevertheless, all kidney samples tested negative by RT-PCR for iNOS expression.

There are several possible reasons for the failure to detect iNOS expression in kidney tissues. Firstly, the challenge dose of *P. salmonis* may have been suboptimal. It was not possible to directly quantify the number of viable organisms in the challenge dose as the CHSE-214 cells, used to culture the *P. salmonis* in the $TCID_{50}$ assay, failed to produce a monolayer on which *P. salmonis* plaques could be detected. However, *P. salmonis* culture supernatant from the same batch rapidly produced 100 % CPE in CHSE-214 cell monolayers during routine passage, indicating that the supernatant contained a large number of viable, infective organisms. Previous challenges, using cultures with similar growth dynamics, were successful in inducing mortality of rainbow trout (personal observation). Furthermore, Acosta *et al.* (2005) found elevated nitrite levels, indicative of iNOS expression, in seabream inoculated with a sub-lethal dose of *Ph. damselae* subsp. *piscicida*. Therefore, the challenge dose was deemed to be capable of inducing an immune response.

Secondly, lack of signal from the kidney may have resulted from the presence of some inhibitory substance present in the tissue which carried over into RT-PCR reactions. Constitutively expressed GAPDH mRNA is likely to be present in such quantities that such an inhibitor has no apparent affect on its amplification. However, the presence of secondary

5.4 Discussion

In the present study, iNOS expression was detected in the gills of unvaccinated, unchallenged fish. Campos-Peréz (1998) argued that the finding of early and prolonged iNOS expression in rainbow trout gills following challenge with *R. salmoninarum* might be due to physical containment of the pathogen in the gills, and the presence in this tissue of immune-responsive cells. Davidson *et al.* (1997), reporting the presence of considerable numbers of 'constitutive' antibody secreting cells in the gills of dab (*Limanda limanda*), suggested that this might relate to the role of the gill as a portal of entry for pathogens. In humans, iNOS is expressed constitutively in all airway epithelial cell types but this expression of the same isoform is not observed in human peripheral or resident lung macrophages (Guo *et al.* 1995; Michel and Feron, 1997). Given that the gill and the lung are exposed to pathogens in the environment and fulfil similar roles, it would not be surprising to find that iNOS is also constitutively expressed in the gill tissue of rainbow trout.

In two previous studies of iNOS expression in rainbow trout, iNOS mRNA was detected by RT-PCR in kidney tissues following bacterial challenge. Laing *et al.* (1999) successfully amplified iNOS product from HK macrophages, 48 h after i.p. challenge with an *Aro*A$^-$ mutant of *A. salmonicida*. Campos-Peréz (1998) found iNOS mRNA in kidney at 24 h, following i.p. challenge with a non-autoagglutinating strain of *R. salmoninarum*. However, by three days after challenge only one of three fish showed a faint iNOS band in kidney tissue and, after five days, kidney tissue was negative for iNOS mRNA. Thus, in both cases, iNOS mRNA expression in kidney occurred within 48 h of challenge. However, both of these groups used attenuated strains for challenge. In a timecourse assay measuring

structure in iNOS mRNA can interfere with reverse transcription (Wang *et al.* 2001) and, at low levels of expression, the presence of an inhibitor might be sufficient to prevent iNOS amplification. The presence of such an inhibitor might be detected by spiking positive gill samples with kidney samples and checking for reduction in amplification signal. Ten-fold dilution of total RNA prior to RT-PCR amplification, or use of purer mRNA isolated from kidney tissue using oligo dT capture methods, might eliminate such an inhibitor so that low-level iNOS expression could be detected.

In mammalian tissues, iNOS expression has been identified in such diverse cells as cardiac myocytes and vascular smooth muscle cells (Michel and Feron, 1997). In nNOS, the neuronal isoform, differential tissue splicing has been shown and, given the level of conservation between the isoforms (55% amino acid sequence identity between human isoforms (Michel and Feron, 1997) and 68% amino acid identity between the trout iNOS isoform and mammalian nNOS (Laing *et al.* 1999)) it is not inconceivable that differential splicing might also occur in the iNOS isoform. The iNOS cDNA sequenced by Wang and from which the PCR primers in the present investigation (F4 and R5) were designed (Wang *et al.* 2001), was isolated from fibroblast (rainbow trout gonad, RTG-2) cells and not from kidney tissue. In fact, the published sequence of the cDNA from kidney tissue differs at its 3' end from the fibroblast sequence (Laing *et al.* 1999). If the cDNA expressed in trout kidney tissue is a splice variant of the fibroblast cDNA, then it is possible that the binding site for the reverse primer, R5, is not present in the kidney sequence. Obviously, the absence of this binding site would prevent PCR amplification of the iNOS fragment. Alternative primers, such as those described by Laing *et al.* (1999) or Campos-Peréz *et al.*

(2000b) could be used to establish whether or not inappropriate primers were responsible for the absence of signal from the kidney tissue.

Finally, it is also possible that iNOS mRNA was not detected in kidney samples because it was not being expressed, or its expression was down-regulated, at the times that samples were taken. This argument is apparently strengthened by the inability to detect elevated levels of nitrates/nitrites in the serum, particularly when detection of these NO metabolites has been possible in the absence of positive RT-PCR signal (Campos-Peréz, 1998) and has been used successfully in a range of fish species (Neumann *et al.* 1995; Yin *et al.* 1997; Saeij *et al.* 2000; Tafalla and Novoa, 2000; Acosta *et al.* 2003). Optimal induction of iNOS expression requires priming of macrophages with cytokines, in particular IFN-γ (Iyengar *et al.* 1987; Jerrells, 1997; Campos-Peréz *et al.* 2000b; Secombes *et al.* 2001; Acosta *et al.* 2005). In mammalian rickettsial infections, the two cytokines, IFN-γ and TNF-α, act synergistically in the clearance of organisms through induction of NO synthesis, and depletion of IFN-γ or TNF-α was found to reduce the levels of urinary nitrate in animal models of infection (Feng and Walker, 1993; Feng *et al.* 1994; Feng and Walker, 2000). The role of IFN-γ as an inducer of iNOS has been underlined further by the difficulty with which iNOS is expressed in the macrophages of IFN-γ-deficient mice (MacMicking *et al.* 1997). However, differences in iNOS induction have been reported for different cell types. While IFN-γ, TNF-α, LPS and IL-1 are required for induction of iNOS in human hepatocytes, IL-1 alone is sufficient for induction in human chondrocytes (Guo *et al.* 1995). Likewise, while stimulation with LPS seems sufficient to induce the production *in vitro* of RNIs in carp macrophages, a combination of LPS and MAF was required to enhance induction in goldfish macrophages (Saeij *et al.* 2000; Neumann *et al.* 2001).

Working with rainbow trout head kidney leukocytes *in vitro* however, Stafford *et al.* (2001a) failed to induce iNOS using LPS and MAF stimulation and suggested that this may be because rainbow trout macrophages required additional developmental signals to allow production of iNOS. Consistent with this is the observation that, in contrast to goldfish, carp, catfish and seabream leukocytes, nested PCR was required to detect iNOS transcript in trout (Wang *et al.* 2001).

Piscirickettsia salmonis produces marked pathology in the liver of affected fish (Branson and Diaz-Munoz, 1991; Cvitanich *et al.* 1991) and is found within cytoplasmic vacuoles of hepatocytes (Almendras *et al.* 2000). Mammalian rickettsiae are also found within hepatocytes and, in a study of rickettsiacidal mechanisms by human cells, it was found that a hepatocyte cell line appeared to produce NO which had anti-rickettsial activity (Feng and Walker, 2000). Therefore, it may be of interest to examine iNOS expression in the liver tissue of susceptible fish. Alternatively, the role of NO and RNIs in the pathology of *P. salmonis* could be assessed by treating fish with the iNOS inhibitor, N^G-monomethyl-L-arginine (N^G-MMLA) and monitoring survival in challenge experiments.

In conclusion, iNOS expression was readily detected in rainbow trout gill tissue using RT-PCR, but the detection of iNOS mRNA in the gills of unchallenged fish suggests that, in this tissue, iNOS may be constitutively expressed. On the other hand, iNOS expression was not detected in rainbow trout HK tissue at any time pre- or post-challenge with *P. salmonis*, nor was there any indication of up-regulated iNOS expression following vaccination. This finding was supported by the failure to detect increased nitrate/nitrite levels, indicative of up-regulated NO metabolism, in the serum of challenged fish.

Chapter 6 - MAF production in response to *P. salmonis* vaccination

6.1 Introduction

Through studies of the diseases caused by intracellular bacteria, it has become increasingly clear that the cell-mediated immune system plays a crucial role in fighting and containing the infections (Johnston *et al.* 1978; Nacy and Osterman, 1979; Kokorin *et al.* 1980; Popov *et al.* 1987; Harty and Bevan, 1999; Ouadrhiri *et al.* 1999; Raupach and Kaufmann, 2001; Walker *et al.* 2001; Masopust *et al.* 2004). From the expansion of CTL populations to the activation of macrophages, T-lymphocytes are an intrinsic and essential element of this system, providing the defence cells capable of destroying and removing infected host cells (Harty and Bevan, 1999; Esser *et al.* 2003). In addition to tackling the primary infection, T-lymphocytes are required for protection against secondary and chronic infections, as the development of a competent anamnestic cell-mediated response is dependent on the establishment of an enduring population of memory cells from T-lymphocytes following clearance of the initial infection (Esser *et al.* 2003; Ottenhoff and Bevan, 2004). Therefore, an effective long-lasting defence against intracellular pathogens requires that a significant primary immune response is mounted by the host animal's T-lymphocytes. In fish, while the response of B-lymphocytes could be determined relatively easily by measuring the levels of pathogen-specific antibody in serum, the absence of specific markers for T-lymphocytes made measurement of their responses more difficult (Smith and Braun-Nesje, 1982). However, several cytokines have now been identified in fish and a number of groups have used cytokine activity to measure putative T-lymphocyte responses following

exposure to various pathogens or environmental toxins (Francis and Ellis, 1994; Marsden *et al.* 1994; Hardie *et al.* 1996; Sarmento *et al.* 2004a).

Cytokines are polypeptides or glycoproteins produced as signalling molecules by cells involved in immune responses and used in the co-ordination of these responses (discussed in more detail in Section 1.2.2.2). They can act in a synergistic or antagonistic manner and are capable of inducing different responses in different target cells (Jang *et al.* 1994; Novoa *et al.* 1996; Secombes *et al.* 1996; De Assis *et al.* 2000). Furthermore, cytokines can influence the expression of other cytokines or their receptors (Secombes *et al.* 1996; Jerrells, 1997). One of the cytokines produced by activated T-lymphocytes is IFN-γ (Nathan, 1997). This cytokine is involved, among other things, in the priming of macrophages to produce ROIs and RNIs, and its expression by activated T-lymphocytes can be measured by the ability of supernatants from these cells to prime elevated respiratory burst in naïve macrophages (Graham and Secombes, 1988; Mulero and Meseguer, 1998; Ohya *et al.* 1998).

In fish, the presence of a factor capable of priming elevated respiratory burst in macrophages has been demonstrated in the supernatants of leukocytes stimulated with mitogens (Graham and Secombes, 1988). This macrophage-activating factor (MAF) has been identified as IFN-γ because of its inactivation by heat and low pH and its production by surface immunoglobulin negative (sIg$^-$) lymphocytes (presumed to be T-lymphocytes) (Graham and Secombes, 1990a, 1990b). Because it is a product of activated T-lymphocytes, several groups have utilised its effect on macrophage respiratory burst to ascertain if prior immunisation can be used to up-regulate its expression and, more importantly, if this up-regulation can be correlated with improved disease resistance following vaccination (Francis and Ellis, 1994; Marsden *et al.* 1994; Yin *et al.* 1997; Chen *et al.* 2001).

As *P. salmonis* is an intracellular parasite, it is possible that specific MAF production could be primed by immunisation with recombinant *P. salmonis* vaccine. If subsequent exposure to *P. salmonis* antigen *in vitro* resulted in higher MAF activity of immune leukocytes, this would suggest that the recombinant vaccine was capable of stimulating the cell-mediated arm of the adaptive immune response and producing a reservoir of T-lymphocytes able to mount a secondary immune response against the disease. Therefore, supernatants from vaccinated and non-vaccinated, pathogen-stimulated rainbow trout leukocytes were tested for the presence of MAF by measuring their ability to prime respiratory burst in non-activated macrophages. In addition, macrophages among the stimulated leukocytes used to produce the MAF supernatants were also tested by respiratory burst assay to determine if vaccination could prime these cells for a more active response on second exposure to the pathogen.

6.2 Materials and methods

6.2.1 Piscirickettsiae

Piscirickettsia salmonis was propagated in CHSE-214 cell monolayers, as previously described (Section 3.2.1). Titres of *P. salmonis* were calculated by end-point dilution using the method of Reed and Muench (1938).

6.2.2 Fish

Rainbow trout, obtained from local fish farms, were held in tanks supplied with aerated flow-through dechlorinated water at the Aquatic Research Facility, University of Stirling, Stirling, UK. The fish were fed daily on a dry pelleted commercial diet.

6.2.3 Production of MAF-containing supernatants

Four weeks prior to the start of the experiment, fish (200-250 g) were acclimatised to 15 °C water temperature. Following anaesthetisation in 4-ethyl aminobenzocaine, fish were immunised by i.p. injection of 0.1 ml SRS/Exp 4 vaccine (Schering-Plough Aquaculture, see Section 5.2.3). The control fish received 0.1 ml sterile PBS by i.p. injection. After 45 d, 7 fish from each group were euthanised using 4-ethyl-aminobenzocaine and ex-sanguinated by withdrawing blood from the caudal vein. To reduce the red blood cell contamination of kidney tissue samples, gill arteries were severed. Head kidney was removed aseptically and a cell suspension prepared by pushing the tissue through sterile 100 μm nylon mesh into L-15 containing 10 i.u. heparin.ml^{-1} and 2 % FBS. Suspensions (10 ml) prepared from each fish were layered onto 2 x 10 ml 51% (v/v) Percoll gradients and centrifuged at 400 x g for 30 min at 4 °C. The bands of cells at the medium/Percoll interface were collected and washed in L-15 with 5 x 10^{-5} M 2-mercaptoethanol (2ME) by centrifugation at 800 x g for 10 min. The pellets were resuspended to 5 x 10^{6} viable cells.ml^{-1} in L-15 with 2ME. Cell suspensions were aliquoted, in 1 ml volumes, into the wells of a sterile 24-well flat-bottomed plate and allowed to attach or settle for 1 h at 18 °C. The *P. salmonis* antigen used to stimulate MAF release was obtained from fully-lysed (100 % CPE; approx. $10^{5.7}$ $TCID_{50}$) infected cell culture, subjected to one freeze-thaw cycle at –20°C to inactivate the piscirickettsiae. Control CHSE antigen was uninfected CHSE-214 cell monolayer from the same passage as the *P. salmonis*-infected cells, also subjected to freeze-thaw to effect a degree of cell lysis. Antigen was added, in 500 μl volumes, to leukocyte monolayers in triplicate wells and incubated for 3.5 h at 18 °C. Supernatants were aspirated gently, monolayers were washed carefully with PBS and then incubated in L-

15 with 10 % FBS and 2ME for a total of 62 h at 18 °C. Control supernatants were prepared by adding antigen to wells without monolayers (MAF control supernatants). Supernatants from triplicate wells were then pooled, centrifuged, filtered through 0.2 μm and stored at -20°C until required.

6.2.4 Respiratory burst assay of macrophages in leukocyte monolayers.

Following removal of putative MAF-containing supernatants, leukocyte monolayers were washed gently with PBS and 400 μl NBT (1 $mg.ml^{-1}$) containing PMA (1 $\mu g.ml^{-1}$) was added to each well, as previously described in Chapter 4; Section 4.2.4. After 30 min, 100 % methanol was added gently to each well and allowed to fix the monolayers for 5 min. Then supernatants were aspirated off and the monolayers fixed in fresh 100 % methanol for 5 min before washing in two changes of 70 % methanol to remove any unreduced formazan. Reduced formazan was solubilised in 480 μl KOH/ 560 μl DMSO and 100 μl aliquots taken in triplicate to be read in a 96-well plate at 630 nm.

6.2.5 Assay of MAF activity.

Target macrophage cell monolayers were prepared from head kidney tissue as described previously (Section 3.2.4) and seeded in a 96-well plate. Non-adherent cells were removed after 4 h, by washing x 4 with PBS, and monolayers were incubated overnight at 18 °C. The MAF-containing supernatants, diluted 1/8, 1/16 and 1/32 in L-15 supplemented with 10 % FBS and 2ME, were added carefully to triplicate wells, without washing or otherwise disturbing macrophage monolayers. The MAF supernatants were not used at a 1/4 dilution as this caused detachment of monolayers, as described in Chapter 4; Section 4.3.1. Monolayers were also incubated in MAF control supernatants. After incubation for 48 h at 18 °C, supernatants were removed and respiratory burst

activity of the macrophages was measured as described in Section 6.2.3, using volumes appropriate to a 96-well plate. For statistical analysis, the absorbance values were converted to a stimulation index by dividing the A_{630} values from monolayers stimulated with 'MAF-containing supernatants' with the values from monolayers stimulated with 'MAF-control supernatants'.

6.2.6 Statistical analysis.

The data were analysed using Student's t-test or one-way ANOVA where appropriate and significance was set at $p<0.05$.

6.3 Results

6.3.1 Respiratory burst assay of macrophages in leukocyte monolayers.

Absorbance values at 630 nm (A_{630}) are shown in Fig. 6.1. Macrophages exposed to *P. salmonis* antigen did not exhibit significantly higher respiratory burst than the corresponding control macrophages exposed to CHSE antigen, whether obtained from *P. salmonis* immunised (p=0.053) or control fish (p=0.255). Likewise, the response to stimulation with CHSE antigen was not significantly higher in vaccinated fish compared with control fish (p=0.164). However, macrophages from fish which had been immunised against *P. salmonis*, *in vivo*, and stimulated with *P. salmonis*, *in vitro*, did exhibit a significantly higher respiratory burst than non-immunised fish stimulated with *P. salmonis* antigen ($p=0.016$, $p<0.05$) or with CHSE antigen ($p=0.003$, $p<0.05$).

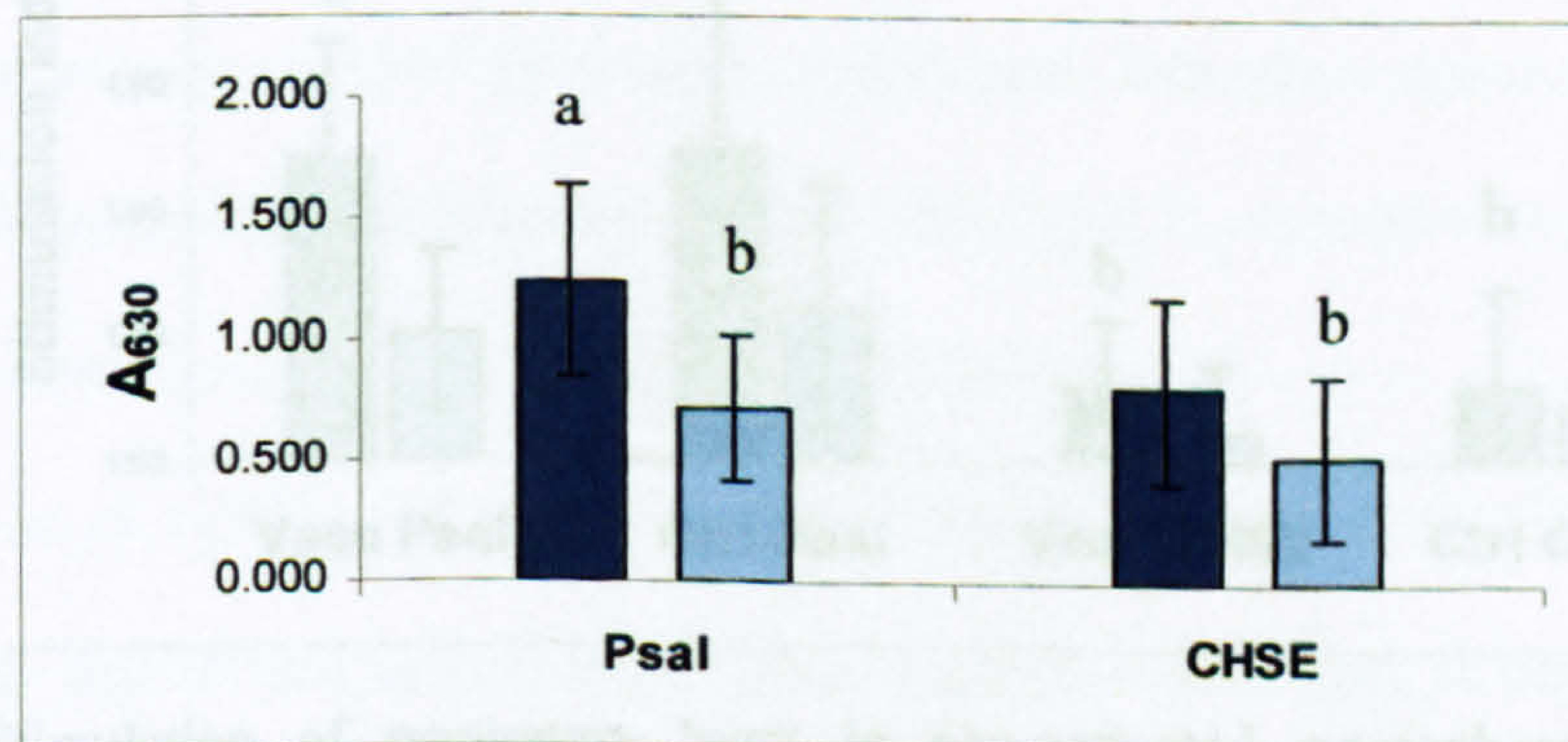


Fig. 6.1. Respiratory burst of macrophages in leukocyte monolayers from vaccinated ■ and sham-vaccinated ■ rainbow trout, stimulated for 62 h *in vitro* with *P. salmonis* antigen (Psal) or CHSE-214 cell antigen (CHSE). The production of O_2^- was measured by the reduction of NBT and expressed as the absorbance at 630 nm (A_{630}) of solubilised formazan. Data are expressed as means of 7 fish, each in triplicate wells ± standard deviation (SD) and different letters denote a significant difference ($p<0.05$) between groups.

6.3.2 Assay of MAF activity.

Supernatants from 5 vaccinated and 5 control fish were tested separately and the results compared. At a 1/8 dilution, there was no significant difference in the ability of MAF supernatants from vaccinated fish to stimulate respiratory burst, compared with supernatants from control fish. However, MAF supernatants from leukocytes exposed to *P. salmonis* stimulated significantly higher respiratory burst than those from leukocytes exposed to CHSE ($p=0.005$, $p=0.045$, for supernatants from immunised and control fish, respectively). At 1/16 and higher dilutions, there was no significant difference in stimulatory ability between any of the MAF supernatant groups (Fig. 6.2).

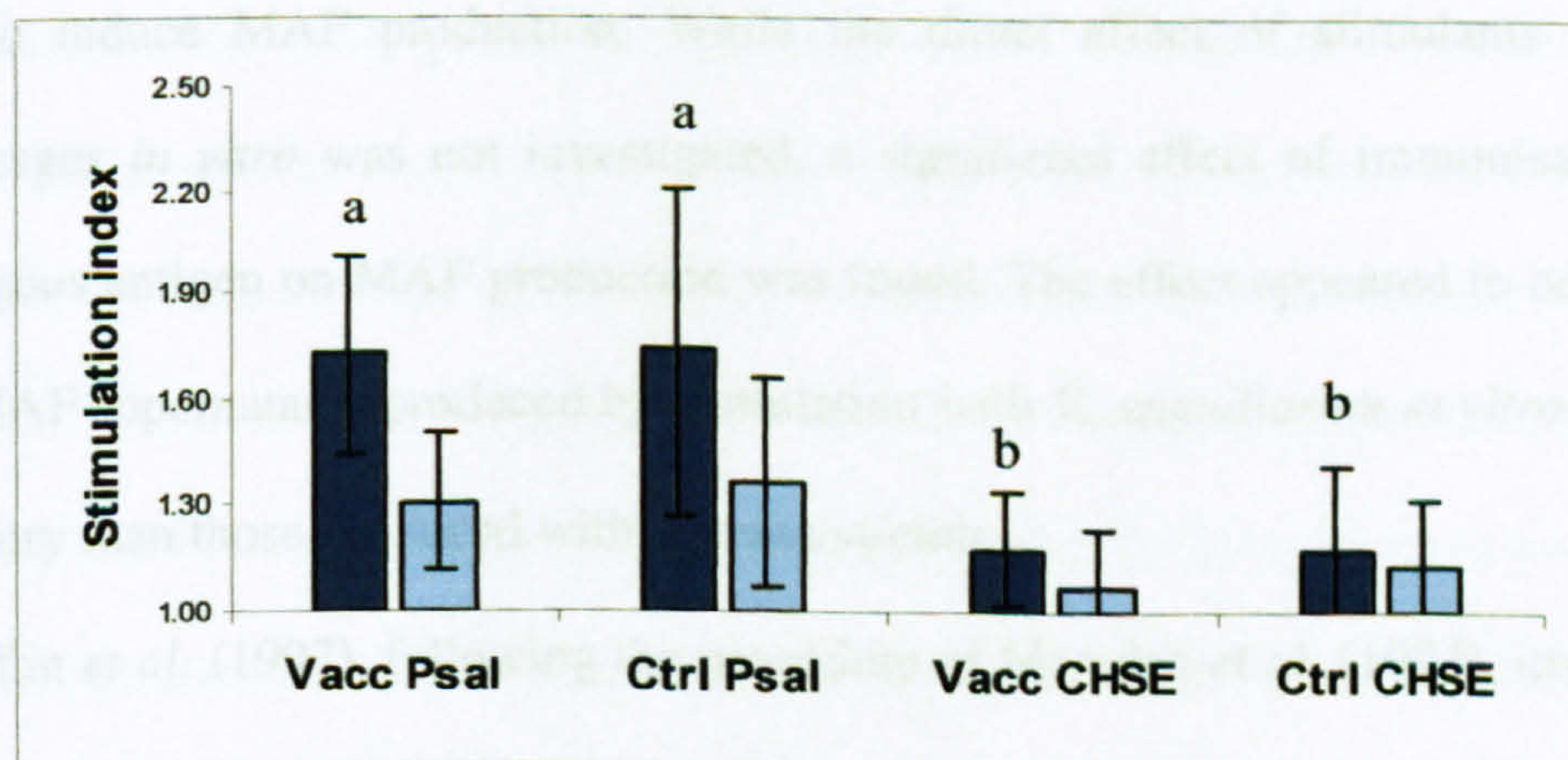


Fig. 6.2. Stimulation of respiratory burst in non-activated macrophages by macrophage activating factor (MAF)-containing supernatants of leukocyte monolayers from vaccinated (Vacc) or sham-vaccinated (Ctrl) trout, stimulated *in vitro* with *P. salmonis* (Psal) or CHSE-214 cell (CHSE) antigen. MAF supernatants were diluted 1/8 ■ or 1/16 ■. Data are represented as mean ± SD stimulation indices (n=5 fish), obtained by dividing the A_{630} values of macrophages incubated with test supernatants by the A_{630} of macrophages incubated with control supernatants. Different letters denote significant differences ($p<0.05$) between groups.

6.4 Discussion

In the study which first indicated the ability of rainbow trout lymphocytes to produce a MAF, leukocyte cultures were stimulated with the mitogens Con A and PMA (Graham and Secombes, 1988). However, this method of stimulating MAF production is quite artificial and gives no information about the ability of a particular pathogen to elicit an immune response following vaccination. In a study designed to examine MAF release in response to specific antigens, Marsden *et al.* (1994) measured MAF production in cultures of rainbow trout leukocytes during a 5 week period following vaccination against *A. salmonicida*. Having immunised fish using formalin-killed *A. salmonicida* in Freund's incomplete adjuvant (FIA), HK leukocytes from immunised and non-immunised fish were exposed *in vitro* to killed *A. salmonicida* or to extracellular product (ECP) to induce MAF production. While the direct effect of stimulants on target macrophages *in vitro* was not investigated, a significant effect of immunisation with homologous antigen on MAF production was found. The effect appeared to be specific, as the MAF supernatants produced by stimulation with *V. anguillarum in vitro* were less stimulatory than those produced with *A. salmonicida*.

Yin *et al.* (1997), following the procedure of Marsden *et al.* (1994), investigated the ability of immunised catfish leukocytes to produce cytokines in response to *in vitro* stimulation with *Aeromonas hydrophila*. In this work, the supernatants derived from immunised fish were compared directly with those from control fish and the ability to prime respiratory burst was shown to be significantly higher in vaccinated fish. In addition, supernatants from immune leukocytes, stimulated *in vitro* with homologous antigen, induced significantly higher NO production than non-immune leukocytes. The

demonstration of a significant vaccination effect in this work may have been due to the fact that fish were immunised with antigen (formalin-killed *A. hydrophila*) in Freund's complete adjuvant (FCA) and also received a booster vaccination. Secombes (1994), in his review of fish phagocyte activity, suggests that either multiple injections or the combination of antigen with FCA are required to increase phagocyte bactericidal activity (a marker of macrophage activation) when using killed bacterial pathogens as immunogens. However, working with both whole cells and ECP of *Mycobacterium marinum*, Chen *et al.* (2001) demonstrated antigen-induced MAF production by HK leucocytes from rainbow trout immunised using a single injection administered in FIA. Furthermore, while exposure of immune leukocytes to antigen preparations *in vitro* increased the ability of leukocytes to prime macrophages, vaccination alone was sufficient to increase MAF activity.

In this latter study (Chen *et al.* 2001), while no direct statistical comparison was made between the MAF produced following *in vitro*-stimulation of immunised and control fish in order to confirm the effect of vaccination, the results presented suggest that such a vaccination effect might indeed have been present. In the study by Yin *et al.* (1997), the effect of vaccination on MAF activity is unequivocal. In this regard, it is interesting that Francis and Ellis (1994) reported MAF activity in supernatants from HK leucocytes exposed *in vitro* to *A. salmonicida* iron-regulated outer membrane protein (IROMP) antigens although there was no statistical difference between the priming activity of supernatants from fish vaccinated against furunculosis and those from non-vaccinated fish. As it was shown that the macrophage respiratory burst was not due to the direct effect of the stimulating antigen, it would appear that the priming factor(s) was, in fact, MAF produced by stimulated lymphocytes. Although, in this case, MAF production was not increased following vaccination, it was considered that the ability of

small quantities of antigen to stimulate potent MAF production non-specifically might contribute towards protection against furunculosis.

The results presented in this chapter mirror those of Francis and Ellis (1994) in that no statistical difference was found between vaccinated fish and sham-vaccinated fish in the MAF activity of supernatants from leukocytes stimulated *in vitro*. At a 1/8 dilution, the supernatants of leukocytes stimulated *in vitro* with *P. salmonis* appeared to possess the same priming activity whether the cells were obtained from vaccinated fish or control fish. However, stimulation *in vitro* with *P. salmonis* produced supernatants with statistically greater priming activity than stimulation with CHSE-214 cells. Thus, it appears that *in vitro* stimulation of leukocytes for 62 h with *P. salmonis* can induce the expression of a MAF which is capable of priming respiratory burst activity in naïve macrophages. In constrast, examination of the respiratory burst activity in the leukocyte monolayers used to produce MAF supernatants does point towards a priming affect of vaccination. Following *in vitro* incubation with *P. salmonis* or CHSE-214 cell supernatants, the respiratory burst of immune macrophages exposed to *P. salmonis* was statistically greater than the respiratory burst of non-immune macrophages. It is possible that if antigen-specific T-lymphocytes were present among the leukocytes from vaccinated fish, early MAF production by these cells in response to *P. salmonis* stimulation *in vitro* may have primed the macrophage population by the time they were assayed for respiratory burst. However, as non-specific T-lymphocytes would be in great majority, MAF produced by them would exceed that produced by specific T-lymphocytes and the eventual titre of MAF would be similar in the leukocyte supernatants from vaccinated and non-vaccinated fish.

The current methods of measuring MAF activity in fish, while they may reveal possible differences between the effects of different immunisation protocols and

pathogens, are really not very satisfactory in terms of elucidating immune mechanisms. The crude MAF supernatants produced by stimulation of mixed leukocyte populations may contain more than one type of cytokine depending on the nature and length of the stimulus (Nacy *et al.* 1981; Crawford *et al.* 1994; Paulnock, 1994; Novoa *et al.* 1996; Neumann *et al.* 2000). In addition, the types and ratios of cells used to produce so-called 'MAF' supernatants will vary between different groups and different experiments, resulting in supernatants with different cytokine profiles. For example, Neumann and Belosevic (1996) report the activity of a MAF produced in leukocyte monolayers from which non-adherent cells had been removed. Presumably, if most lymphocytes had been removed by washing, the levels of IFN-γ (a T-lymphocyte product) would have been reduced in the resulting supernatants (Graham and Secombes, 1990a). Consequently, as the cell-type was not optimal for expression of IFN-γ, it is probable that some other cytokine(s) was predominant. This, rather than the type of target cells used to assay the MAF activity, is likely to be the reason for the discrepancy in priming kinetics which were found to differ greatly from those described by Graham and Secombes (1988). Thus, this approach to measuring cytokine expression as an indicator of T-lymphocyte immune response is highly prone to error because the cytokines induced by different stimuli cannot be accurately identified and quantitated.

As more cytokine genes are identified in fish, the level, timing and relative sequence of their expression will be accessible to measurement by northern blotting or quantitative real-time RT-PCR (Brubacher *et al.* 2000; Purcell *et al.* 2004). Alternatively, the generation of mAbs against different cytokines will allow the development of enzyme immunoassays, similar to those which are available for mammalian cytokines. These assays, which can be adapted for use in multiplex assays (Reddy *et al.* 2004), will also help to elucidate the patterns of cytokine expression

following immune stimulation. Using these methodologies, it may be possible to obtain more robust measurements of the effects of vaccination against different fish pathogens and to optimise immunisation protocols so as to obtain better immune stimulation and protection.

Chapter 7 - Measurement of the antibody response of rainbow trout to *P. salmonis* using a double ELISA system

7.1 Introduction

Piscirickettsia salmonis, the causative agent of SRS (Cvitanich *et al.* 1991; Fryer *et al.* 1992), is an obligate intracellular bacterium which has resulted in up to 90% mortality amongst farmed salmonids in Chile (Fryer *et al.* 1990). The organism has also been responsible for disease outbreaks among farmed salmonids in Canada (Brocklebank *et al.* 1992), Ireland (Rodger and Drinan, 1993; Palmer *et al.* 1996), Norway (Olsen *et al.* 1997) and Scotland (Birrell *et al.* 2003) and, recently, *P. salmonis* has been confirmed as a pathogen of juvenile European sea bass in the Mediterranean (McCarthy *et al.* (2005) and Chapter 2). The cost of antibiotic treatments and their ineffectiveness against this intracellular disease have emphasised the need to develop an effective vaccine (Smith *et al.* 1999; Kuzyk *et al.* 2001a). Schering-Plough Aquaculture is currently developing a recombinant vaccine, which incorporates two antigens of *P. salmonis* expressed in a carrier organism. This vaccine has shown promising results in challenge trials (Patrick Smith personal communication). However, a method for routine testing of vaccine efficacy is required. One way of doing this is to use an ELISA to examine the antibody responses elicited by vaccinated fish to the vaccine (Reitan and Secombes, 1997). This method is widely used in clinical medicine (Weiss *et al.* 1975; Chou *et al.* 2004; Ngeh *et al.* 2004; Lopez *et al.* 2004; Zsivanovits *et al.* 2004; Leung *et al.* 2005).

In order to assess whether the antibody response generated against the recombinant antigens is also capable of recognising *P. salmonis*, it is first necessary to purify whole-cell

P. salmonis from the fish cell line used to culture the organism so it can be used as antigen to screen serum from vaccinated fish. Previous workers (Kuzyk *et al.* 1996; Barnes *et al.* 1998; Jamett *et al.* 2001; Yuksel *et al.* 2001) have used Renografin or Percoll gradients to obtain pure rickettsial fractions from cell culture. However, this method of purification is impractical for screening the large numbers of serum samples necessary for efficacy testing vaccines, simply because of the quantity of *P. salmonis* required to coat the ELISA plates. Therefore, a method for crudely purifying milligram quantities of *P. salmonis* was developed and an ELISA devised to measure the antibody response of vaccinated fish. A double ELISA system was used to measure this response in rainbow trout vaccinated with the recombinant vaccine, whereby antisera were screened against the crudely purified *P. salmonis* as well as the fish cell line used to culture the bacterium. This allowed control for the component of the antibody response directed against the unknown proportion of CHSE cell-derived antigen remaining in the crudely purified *P. salmonis* culture supernatant.

7.2 Materials and Methods

7.2.1 Fish

Rainbow trout (approximately 60 g), obtained from Buckieburn Fish Farm, Stirling, UK, were held in 250 l tanks supplied with flow-through dechlorinated water in the Aquatic Research Facility, University of Stirling, and acclimatised to a water temperature of 15°C. One week before vaccination, fish were allocated to tanks as a single group of controls (60 fish) and two groups of vaccinated fish (50 fish per group). Vaccinated fish received 0.1 ml SRS-3 vaccine (Schering-Plough Aquaculture) injected i.p. while control fish received 0.1

ml sterile PBS (0.01 M, pH 7.4). Fish were anaesthetised using 4-ethyl aminobenzocaine before vaccinating or taking blood samples, and returned to the tanks after sampling. Prior to vaccination, blood samples (0.2-0.4 ml per fish) were taken by caudal puncture of 20 fish in order to examine pre-immune levels of antibodies to *P. salmonis*. At fortnightly intervals, from 2 - 12 weeks post-vaccination, blood samples were collected from 10 fish per group (10 controls, 2 x 10 vaccinated fish) as described above. Blood was allowed to clot at room temperature (RT, 21 °C) for 3 h and then overnight at 4 °C before centrifuging at 10,000 x g for 10 min and aspirating off the serum. Serum samples were stored at –80 °C until analysed in the ELISA.

7.2.2 Piscirickettsiae

Piscirickettsia salmonis was propagated routinely in CHSE-214 cells as described in Section 3.2.1. Titres of *P. salmonis* were calculated by end-point dilution using the method of Reed and Muench (1938).

7.2.3 ELISA antigen preparation.

7.2.3.1 Rickettsial antigen.

For the rickettsial antigen preparation, sealed tissue 175 cm^2 culture flasks (T-175) were seeded with 45 ml CHSE-214 cells (1.5-3 x 10^5 $cells.ml^{-1}$) in EMEM, as for routine culture, except 2 % FBS was used, rather than 5 %, to prevent overgrowth of the monolayer, and flasks were incubated for 24 – 48 h at 20 °C until 60-80 % confluency was reached. Cells were then cooled to 15 °C, medium was decanted and monolayers were inoculated with approximately 15 ml rickettsial culture supernatant. After incubating for 2 h at 15°C, the

inoculum was decanted, monolayers were rinsed with PBS and the culture medium was replaced. Cultures were incubated at 15 °C until CPE had reached 90 % - 100 %. Flask surfaces were scraped to dislodge any remaining attached cells and the contents were centrifuged at approximately 20,000 x g for 35 min in a Beckman OptimaTM L preparative ultracentrifuge, using Beckman Ultra-ClearTM centrifuge tubes. The supernatants were decanted and the pellets resuspended in PBS adding approximately 10 ml PBS per 200 ml original culture supernatant. Suspensions were centrifuged at 200 x g for 10 min to remove host cell debris and supernatants were retained. To improve the yield of rickettsiae, pellets from this low-speed centrifugation were again resuspended in approximately 2 ml PBS, 20 % volume 0.1 mm glass beads was added and the pellets vortexed at a high speed for 30 s to rupture rickettsia-containing vacuoles in the cells. PBS was added to make a final volume of 10 ml and the suspension was again centrifuged at 200 x g for 10 min. Supernatant suspensions were pooled and centrifuged at 20,000 x g for 35 min. Supernatants were decanted, pellets were resuspended in PBS and pooled (final volume approx. 35 ml) and a second high-speed centrifugation used to wash the pellet. The final pellet, referred to as Crude Purified LF-89 (rickettsial antigen) in the ELISA procedure, was finally resuspended in 0.5-1.0 ml PBS, aliquoted and stored at –20 °C until assayed for protein concentration.

The proportion of contaminating CHSE-214 host cell protein likely to remain in the Crude Purified LF-89 preparation was estimated by subjecting uninfected, lysed CHSE-214 cell monolayer to the preparation procedure described above and comparing the protein yield with that from a similar volume of *P. salmonis*-infected monolayer.

7.2.3.2 CHSE antigen.

For preparation of host cell antigen, uninfected monolayers of CHSE-214 cells, cultured as above, were washed twice with PBS to remove culture medium, harvested by scraping and pelleted by centrifugation at 2000 x g for 10 min at 4 °C. The pellets were resuspended in 2 ml PBS, vortexed vigorously with glass beads to break open the cells, adjusted to 10 ml volume with PBS and washed by centrifuging twice at 2000 x g for 10 min. The final pellet, referred to as CHSE Antigen, was resuspended in 1.5 ml PBS and stored frozen at – 20 °C until assayed for protein concentration.

7.2.3.3 *A. salmonicida* antigen.

In order to assess the specificity of the antibody response, *A. salmonicida* antigen was prepared by culturing *A. salmonicida* (NCIMB1102), in Tryptic Soya Broth at 22 °C for 48 h, pelleting by centrifugation (1,500 x g for 10 min), and the bacterial pellet was washed several times in PBS. The optical density of the final suspension was adjusted to 1.04 at 610 nm with PBS. Aliquots of the bacterium were then stored frozen at –20 °C.

7.2.3.4 Protein assays.

The protein concentration of each antigen preparation was determined using the Bicinchoninic Acid (BCA) Protein Assay. In addition, a protein assay was carried out to estimate the concentration of residual CHSE-214 cell protein remaining in the Crude Purified LF-89 antigen preparation. Bovine serum albumin (BSA) was used as the assay standard (25 $\mu g.ml^{-1}$ – 1.0 $mg.ml^{-1}$).

7.2.4 ELISA procedure

7.2.4.1 Optimisation of serum dilutions and concentrations of plate-coating antigens

In order to determine the optimal serum dilution for use in the ELISA, preliminary assays were carried out to compare the results from pre-immune (negative) serum with results from immune (positive) serum. The preliminary assays were also used to determine the optimal concentrations of rickettsial antigen and CHSE antigen which should be used to coat the ELISA plates. Pre-immune serum was obtained from two naïve rainbow trout. Immune serum was obtained from a fish of the same batch, 7 weeks following vaccination with the recombinant *P. salmonis* vaccine, SRS-3. The optimal serum dilution was the highest dilution at which a difference in reactivity between the positive and negative serum was clearly detectable. The optimal antigen concentration was the lowest protein concentration at which a difference in reactivity between the positive and negative serum was clearly detectable, and was dependent on the optimal serum dilution.

7.2.4.2 Double ELISA

The antibody responses of the fish were quantified using ELISA to measure serum absorbances. In order to control for the component of the antibody response directed against the unknown proportion of CHSE cells/debris remaining in the Crude Purified LF-89, half of the wells in an ELISA plate were coated with Crude Purified LF-89 and the other half with CHSE Antigen adjusted to the same protein level as the rickettsial antigen (approx. 66 $\mu g.ml^{-1}$). Serum antibody levels to both antigens were then measured simultaneously, as an absorbance at 450 nm (A_{450}). Pre-immune sera (Week 0) and sera from Weeks 4, 6, 8 and 10 were tested against Crude Purified LF-89 and CHSE Antigen. Sera from 10 individuals were tested at each time point from both vaccinated and control

groups. In addition, the responses of sera sampled at Weeks 6 – 12 were compared using 20 vaccinated fish and 10 control fish at each time point, screening against rickettsial antigen alone. To confirm the specificity of the response, antibody levels were screened and compared between equivalent protein concentrations (approx. 66 $\mu g.ml^{-1}$) of Crude Purified LF-89 and *A. salmonicida* antigen using sera from both vaccinated fish (n = 11) and control fish (n=8) collected at Week 6.

The ELISA method was as follows: Greiner 96-well ELISA plates were activated with 0.05 % (w/v) poly-L-lysine in coating buffer (50 $\mu l.well^{-1}$). Following incubation for 60 min at RT, plates were washed twice with low salt wash buffer (LSWB, 0.2 M Tris, 3.8 M NaCl, 0.5 % (v/v) Tween-20, pH 7.3). Antigen preparations were dispensed at 100 $\mu l.well^{-1}$ and incubated overnight at 4°C. To crosslink the antigen to the plate, 50 µl 0.05 % (v/v) glutaraldehyde in PBS was dispensed into each well and incubated for a further 20 min at RT. Plates were washed 3 times with LSWB and blocked with 1 % (w/v) BSA (250 $\mu l.well^{-1}$) for 2 h at RT. After a further 3 washes with LSWB, 100 µl aliquots of fish serum (diluted to 1/64 in PBS) and PBS as controls were added to wells and incubated overnight at 4 °C. Following overnight incubation, plates were washed 5 times with high salt wash buffer (HSWB, 0.2 M Tris, 5 M NaCl, 1 % (v/v) Tween-20, pH 7.7) incubating for 5 min on the last wash. Reconstituted mouse anti-trout/salmon IgM mAb was added (100 $\mu l\ well^{-1}$) and incubated for 60 min at RT. As before, plates were washed 5 times with HSWB, 100 $\mu l.well^{-1}$ sheep anti-mouse IgG horseradish peroxidase (HRP)-labelled antibody, was added, diluted 1/1000 in conjugate buffer, consisting of 1 % (w/v) BSA in LSWB, and incubated for 60 min at RT. Plates were washed again 5 times with HSWB before incubating with 100 $\mu l.well^{-1}$ chromogen [150 µl 42mM 3'3'5'5'- tetramethylbenzidine dihydrochloride

(TMB) in 15 ml 33.3 % (v/v) glacial acetic acid and 5 µl H_2O_2 in substrate buffer (1 M citric acid, 1 M sodium acetate, pH 5.4)] for approximately 10 min at RT. The reaction was stopped with 50 $\mu l.well^{-1}$ of stop solution (2 M sulphuric acid (H_2SO_4)) and the absorbance measured at 450 nm (A_{450}) in a Dynex MRX II ELISA reader.

7.2.4.3 Statistical analysis of ELISA results

Data were analysed by the Mann Whitney U Test. Results are expressed as median absorbance ± standard error of the mean (SEM). Differences were considered statistically significant at $p<0.05$.

7.2.5 Western blotting

Antibody responses of vaccinated and non-vaccinated fish were also examined by western blot analysis. Aliquots of rickettsial antigen and CHSE-214 antigen, prepared as above, were adjusted to 4 $mg.ml^{-1}$ protein for Crude Purified LF-89, and 3 $mg.ml^{-1}$ protein for CHSE Antigen. These were diluted 1:1 in 2X sample buffer (100 mM Tris, pH 6.8, 4 % (w/v) sodium lauryl sulphate, 200 mM dithiothreitol, 20% (v/v) glycerol, 0.01 % (w/v) bromophenol blue) and heated to 98 °C for 4 min. In addition, an aliquot of one of the recombinant antigen preparations used in the vaccine (provided by Schering-Plough Aquaculture) was adjusted to 2 $mg.ml^{-1}$ protein and heated in the same way. For electrophoresis, 20 µl volumes of sample were run on a 12 % SDS-PAGE gel, prepared according to the method of Laemmli (1970), and electrophoresis was carried out at 200 V until the dye front reached the bottom of the gel. Coomassie staining (0.25 % (w/v) Coomassie Brilliant Blue, 50 % (v/v) methanol, 10% (v/v) acetic acid) was carried out on representative lanes of the gel to visualise protein bands and gels were destained with 40 %

(v/v) methanol/10 % (v/v) acetic acid until the bands were clearly visible. Western blotting was by transfer onto nitrocellulose membrane, as described by Wiens *et al.* (1990) by applying 60 V for 70 min. Full Range Rainbow™ Molecular Weight Markers were used to calculate the molecular weight of proteins. Membranes were blocked overnight in 1 % (w/v) BSA in Tris-buffered saline (TBS), pH 7.5, at RT. Following this incubation, membranes were washed three times for 5 min in TBS plus 0.1 % (v/v) Tween-20 (TTBS) and cut into strips of approximately 0.5 cm. Strips were incubated for 5 h in 1/10 and 1/20 dilutions of pooled serum taken from 5 vaccinated and 5 naïve, non-vaccinated rainbow trout. After the incubation, the membranes were washed as above and incubated overnight at 4°C in reconstituted mouse anti-trout/salmon IgM mAb. Strips were washed again as above, and incubated for 60 min at RT, with sheep anti-mouse IgG HRP-labelled antibody, diluted 1/100 in TBS. The strips were washed again, as above, together with a fourth wash in TBS without Tween-20. The reaction was developed by placing the membrane strip into chromogen (0.5 ml stock solution of 10 mg DAB dissolved in 6.67 ml TBS, and added to 4.5 ml TBS and 0.1 ml 1 % (v/v) H_2O_2). The reaction was stopped by rinsing the membranes with tap water.

7.3 Results

7.3.1 Optimisation of assay

The total yield of rickettsial antigen (Crude Purified LF-89) from 14 x T-175 flasks (approx. 600 ml *P. salmonis*-infected supernatant) was estimated at 5.7 mg protein. Contaminating CHSE-214 host cell antigen was estimated to be 20 % of this Crude Purified LF-89 protein concentration. The total yield of CHSE Antigen from uninfected control supernatants was estimated at 5.25 mg protein. The results of preliminary assays indicated that the differences in reactivity against rickettsial and CHSE antigens between immune and pre-immune serum could be detected clearly with a serum dilution of 1/64 and using the plate coating antigens at a concentration of approx. 70 $\mu g.ml^{-1}$ (Fig. 7.1).

7.3.2 Double ELISA

The antibody response measured by ELISA is shown in Table 7.1 as median absorbance values (A_{450}) for each of the vaccination groups at each sampling time point. Examination of the A_{450} data at Weeks 0, 4 (not shown), 6, 8 and 10 suggests that the antibody response to rickettsial antigen increased following vaccination (Fig. 7.2). In pre-immune and control (sham-vaccinated fish) sera, median A_{450} values were ≤ 0.4. However, by Week 6 post-vaccination, 6 out of the 10 individual samples had an A_{450} over twice that seen in control sera (median A_{450} = 1.016 and 0.375 for the vaccinated and control groups, respectively). Of the remainder, 2 individuals exhibited a slightly raised response ($A_{450} > 0.7$) while 2 fish exhibited no increased response and may be deemed 'non-responders'. At Week 8, the data indicate a reduction in antibody levels. Only 2 samples from vaccinated fish exhibited an

A_{450} twice that of control sera while, in 6 out of 10 samples, absorbance values were less than or equal to control sera. However, at Week 10, 5 out of 10 samples showed an A_{450} value twice that of control sera and 2 samples exhibited a slightly raised response (A_{450} >0.6). At Week 6 and at Week 10, there was a significantly higher response to rickettsial

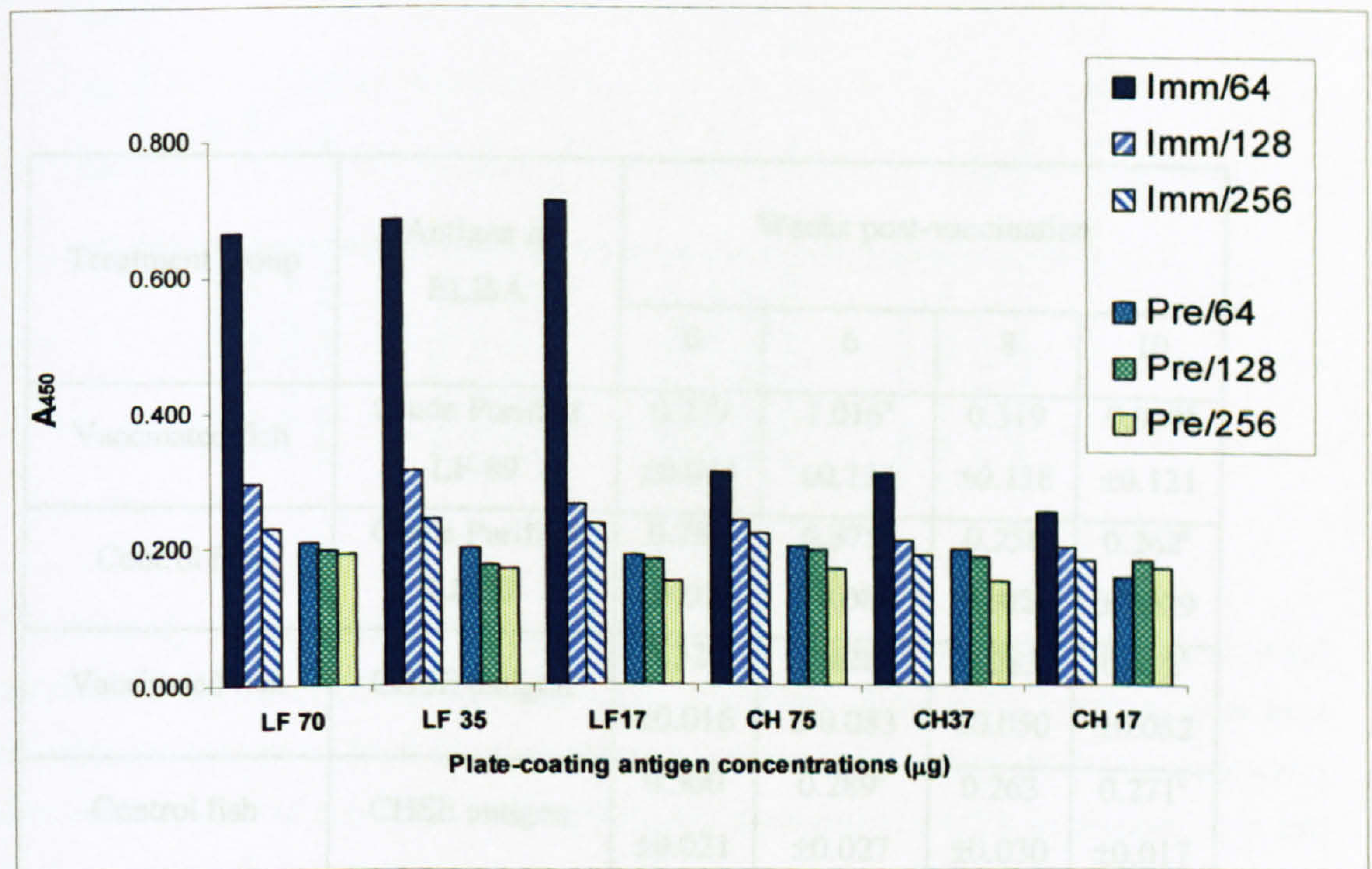


Fig. 7.1. Absorbance values at 450nm obtained for the preliminary ELISA to determine optimum dilutions of test sera and optimum concentrations of plate-coating antigens. Microtitre plates were coated with Crude Purified LF-89 at protein concentrations of approx. 70, 35 or 17 µg.ml-1 (LF 70, LF 35, LF 17) or CHSE antigen at protein concentrations of approx. 75, 37 or 17 µg.ml-1 (CH 75, CH 37, CH 17). A double ELISA was then carried out using serum from a fish vaccinated against *P. salmonis* with recombinant vaccine SRS-3 (Imm) diluted 1/64, 1/128 or 1/256 or pre-immune serum (Pre) diluted 1/64, 1/128 or 1/256.

Table 7.1. A comparison of the antibody response of experimental fish when screened against rickettsial and CHSE antigens in the ELISA. Median antibody levels ± SEM, as measured by absorbance (A450), for sera from each fish group at Weeks 0, 6, 8 and 10 post-vaccination: 1) vaccinated fish serum tested against Crude Purified LF-89 (rickettsial antigen); 2) control (sham-vaccinated) fish tested against rickettsial antigen; 3) vaccinated fish tested against CHSE antigen; 4) control fish tested against CHSE antigen. Medians are calculated using data from all individuals in each group, including non-responders.

Treatment group	Antigen in ELISA	Weeks post-vaccination			
		0	6	8	10
Vaccinated fish	Crude Purified LF-89	0.279 ±0.014	1.016[a] ±0.134	0.319 ±0.118	0.939[a] ±0.121
Control fish	Crude Purified LF-89	0.263 ±0.035	0.375[c] ±0.080	0.258 ±0.024	0.262[c] ±0.029
Vaccinated fish	CHSE antigen	0.326 ±0.016	0.428[b] ± 0.083	0.283 ±0.050	0.452[b] ±0.052
Control fish	CHSE antigen	0.300 ±0.021	0.289[c] ±0.027	0.263 ±0.030	0.271[c] ±0.017

Statistical differences, as calculated by the Mann Whitney U Test, are: (a) vs (b) $p<0.05$; (a) vs (c) $p<0.01$.

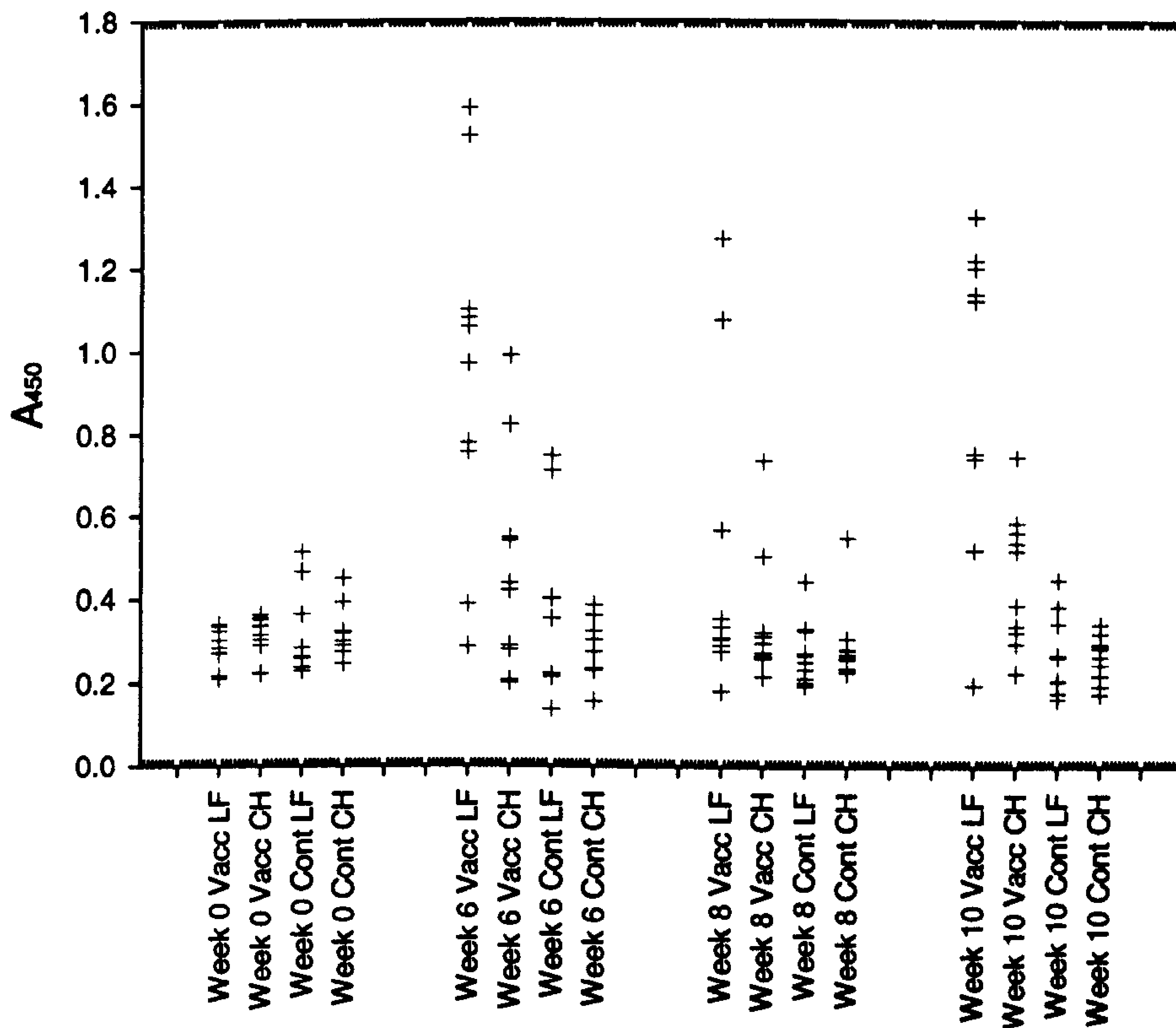


Fig. 7.2. Antibody responses in individual vaccinated (Vacc) and control (Cont) fish screened against Crude Purified LF-89 (rickettsial antigen, LF) and CHSE antigen (CH). Serum samples were collected prior to vaccination (Week 0) and at Weeks 6 – 10 post-vaccination. Each cross represents the serum antibody level of individual fish, diluted 1/64, measured by ELISA as absorbance at 450 nm (A_{450}). At Week 0, the serum represents pre-immune serum, and 'control' and 'vaccinated' serum samples were taken from the same respective group of fish at the times indicated. At Week 6, data are only available from 8 control fish; in all other cases, data are from 10 individuals.

antigen in vaccinated fish compared with sham-vaccinated controls (Week 6 $p=0.004$, Week 10 $p=0.001$, $p<0.05$). However, at Week 8, comparing 10 vaccinated with 10 control fish, the difference in response between vaccinated and control fish was not significant ($p=0.052$). When the antibody levels of sera from a greater number of vaccinated individuals were measured ($n = 20$), the antibody response to rickettsial antigen (Crude Purified LF-89) was found to peak at Week 6, with a significantly increased response in some individuals through Weeks 8-12 post-vaccination (Fig. 7.3). The antibody response is shown as median absorbance for both groups in Table 7.2.

In an effort to examine specificity of the antibody response, sera from Week 6 post-vaccination were tested against rickettsial antigen and *A. salmonicida* antigen. Again, the antibody response to rickettsial antigen was found to be significantly higher than the response to *A. salmonicida* antigen ($0.003=p<0.05$) (Fig. 7.4). Furthermore, sera which were positive on LF-89 coated wells gave similar low A_{450} values to LF-89 negative sera on *A. salmonicida* coated wells ($0.1=p>0.05$).

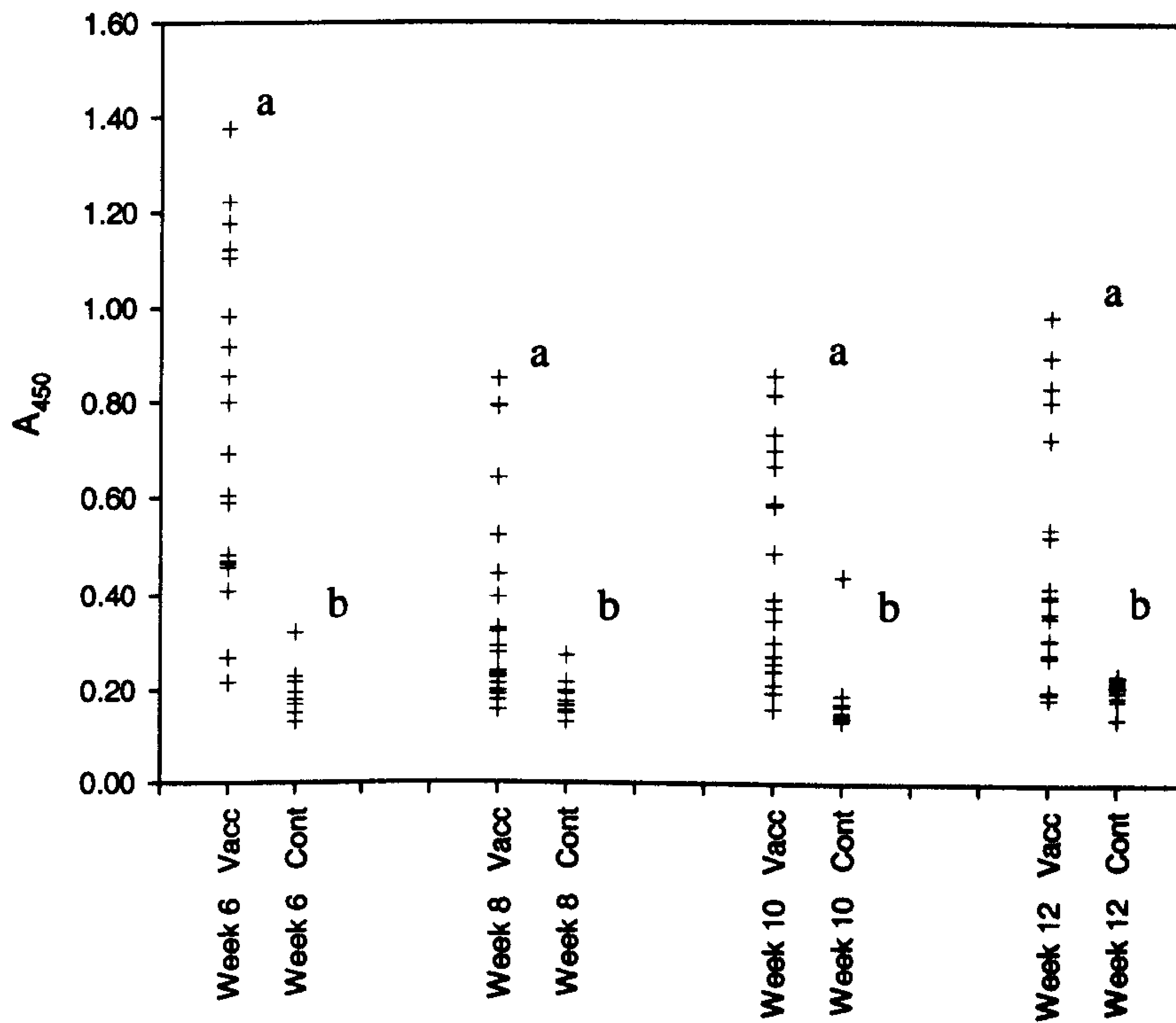


Fig. 7.3. Antibody responses of individual vaccinated (Vacc) and control (Cont) fish against rickettsial antigen, measured at Weeks 6 – 12 post-vaccination. Each cross represents the serum antibody level of individual fish, diluted 1/64, measured by ELISA as absorbance at 450 nm (A450). At Week 6, data are only available from 8 control fish; in all other cases, data are from 10 control fish and 20 vaccinated fish. Using the Mann Whitney U Test, differences between (a) and (b) were significant ($p<0.01$).

Table 7.2. Comparison of antibody responses in vaccinated and control fish when screened against rickettsial antigen by ELISA. Median antibody levels ± SEM, as measured by absorbance (A450), for sera from each fish group at Weeks 6, 8, 10 and 12 post-vaccination: 1) vaccinated fish serum tested against Crude Purified LF-89 (rickettsial antigen); 2) control (sham-vaccinated) fish tested against rickettsial antigen. Medians are calculated using data from all individuals in each group, including non-responders.

Treatment group	Antigen in ELISA	Weeks post-vaccination			
		6	8	10	12
Vaccinated fish	Crude Purified LF-89	0.648 ±0.079	0.286 ±0.050	0.380 ±0.051	0.375 ±0.057
Control fish	Crude Purified LF-89	0.188 ±0.021	0.172 ±0.011	0.157 ±0.028	0.203 ±0.010

Vaccinated fish in every week differ from Control fish $p<0.01$ by Mann Whitney U Test

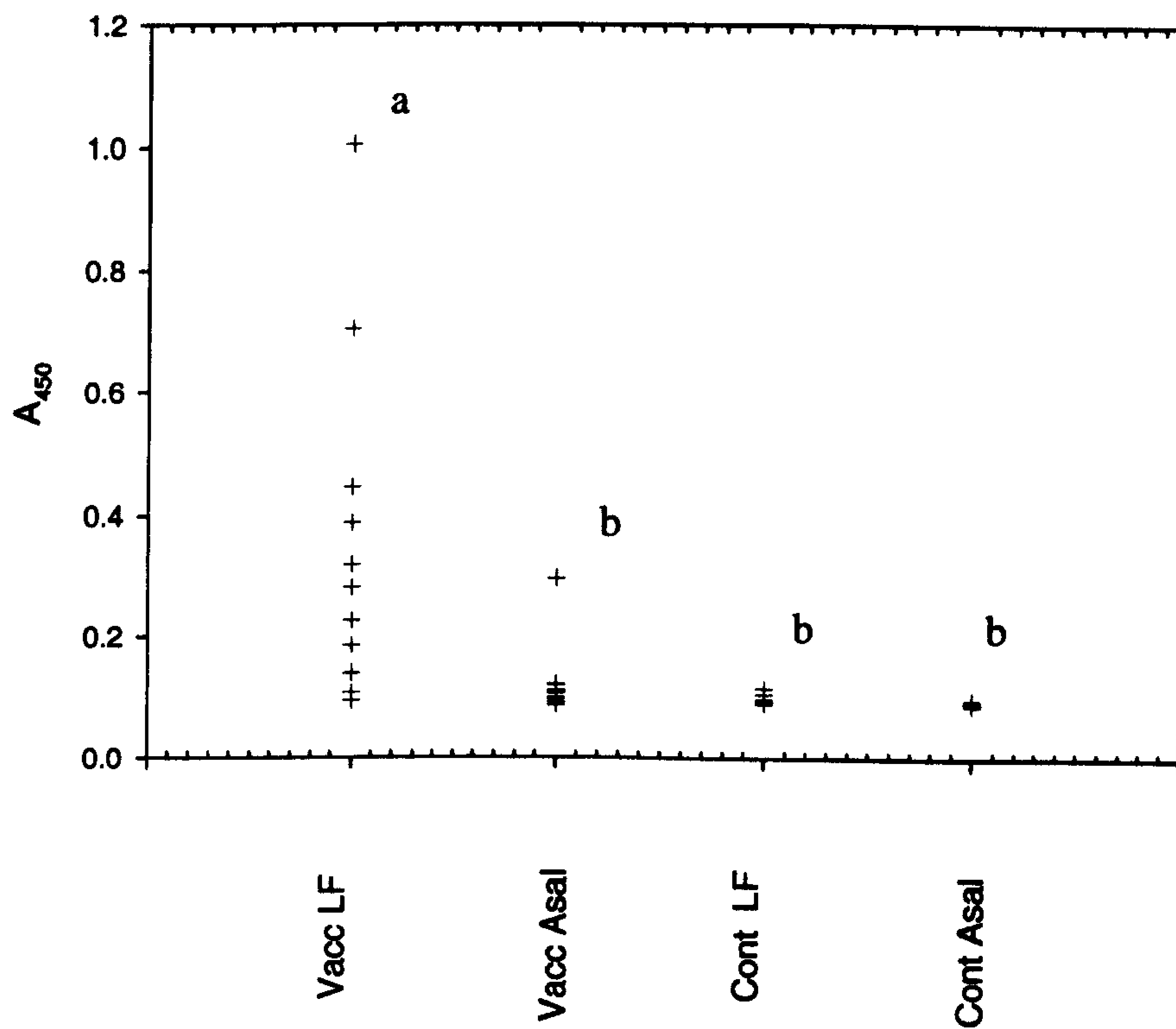


Fig. 7.4. Antibody responses of individual vaccinated (Vacc) and control (Cont) fish against rickettsial antigen (LF) and *Aeromonas salmonicida* antigen (Asal). Each cross represents the serum antibody level of individual fish, diluted 1/64, measured by ELISA as absorbance at 450 nm (A450). Data were from serum obtained at Week 6 post-vaccination from 11 vaccinated and 8 control fish. Using the Mann Whitney U Test, differences between (a) and (b) were significant ($p<0.01$).

7.3.3 Western blotting

Serum from vaccinated fish not only recognised antigens from the recombinant vaccine but also recognised many antigen bands which were present in both Crude Purified LF-89 and CHSE Antigen preparations (Fig. 7.5 (A) and (B)). Furthermore, it appeared that some of these antigens were also recognised by serum from unvaccinated fish, albeit to a much lesser degree than found in immune serum (Fig. 7.5 (C) and (D)).

7.4 Discussion

The difficulty of obtaining rickettsial antigen from cell cultures has been discussed previously by other workers (Yuksel *et al.* 2001; Henriquez *et al.* 2003). Many of the organisms remain closely associated with host cell debris with the result that the yield of pure *P. salmonis* from infected cell cultures is low. Other workers have used density gradient centrifugation, employing Renografin and Percoll, to separate *P. salmonis* from the host cells but the aim has been to obtain pure fractions of *P. salmonis* rather than to obtain high yields of the organism (Kuzyk *et al.* 1996; Barnes *et al.* 1998). The aim of the current study was to screen the antibody response against whole-cell *P. salmonis* of fish vaccinated with a recombinant vaccine. As density gradient centrifugation was unlikely to produce high yields of *P. salmonis*, a method of harvesting piscirickettsial antigen was developed, without recourse to laborious and expensive density gradients. Using this method, a crude rickettsial antigen preparation was obtained. Preliminary ELISAs, using culture supernatants as the plate-coating antigen, had shown that background absorbance from

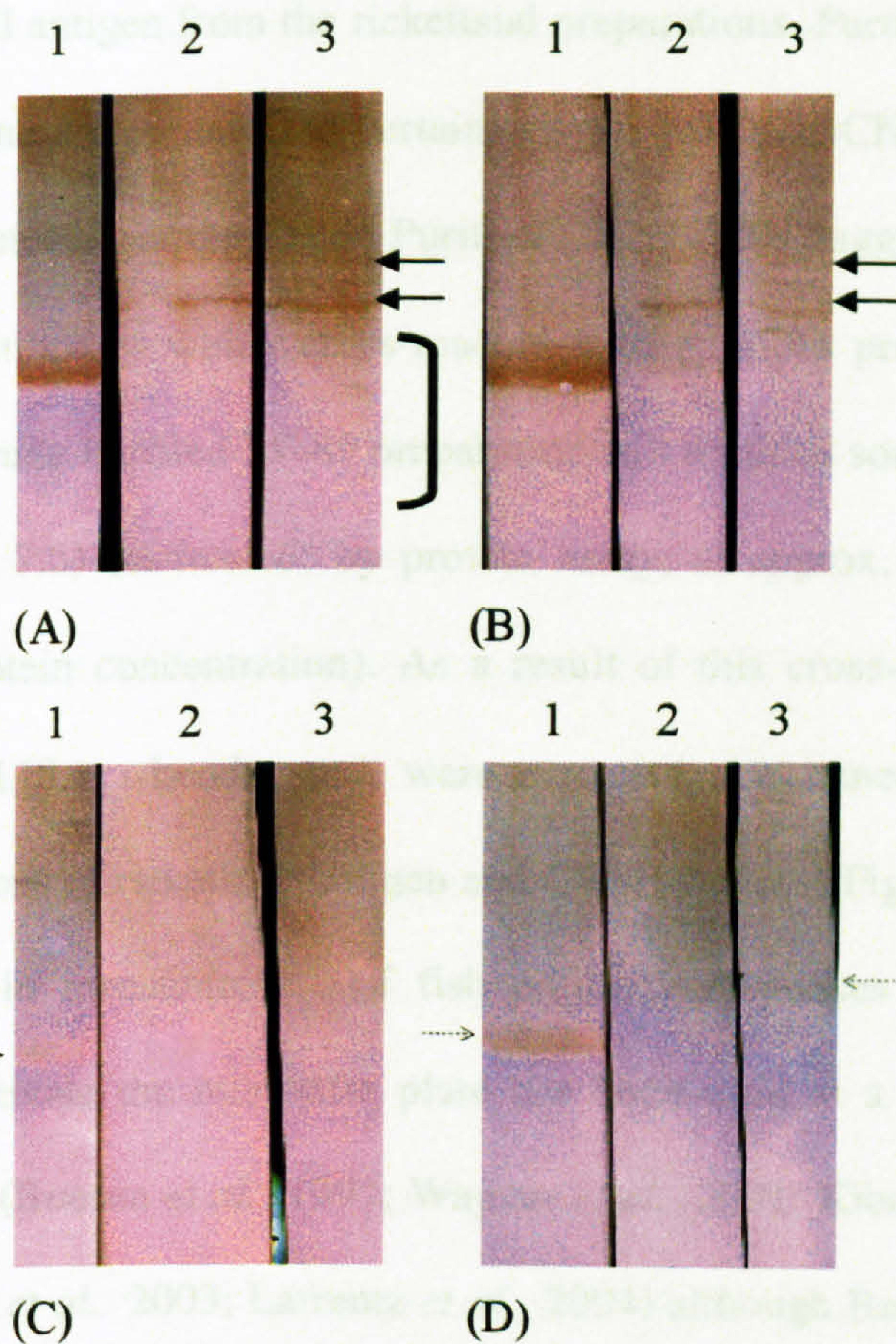


Fig. 7.5. Examination of the antibody profiles of sera from vaccinated (diluted (A) 1/10 or (B) 1/20) and non-vaccinated fish (diluted (C) 1/10 and (D) 1/20) against (1) Recombinant protein preparation; (2) CHSE antigen; (3) and Crude Purified LF-89 in western blot analysis. Solid arrows indicate antigen bands apparently shared between the Crude Purified LF-89 and CHSE preparations, while antigen bands apparently exclusive to the rickettsial preparation are indicated by a bracket. Dashed arrows indicate bands detected also by serum from non-vaccinated fish.

CHSE-214 cell monolayers was capable of masking the response to the rickettsial antigen in an ELISA. Therefore it was imperative to remove as much as possible of the contaminating CHSE-214 host cell antigen from the rickettsial preparations. Furthermore, western blotting showed that immune serum detected certain protein bands in CHSE Antigen preparations which were also detected in the Crude Purified LF-89. This suggests that the recombinant vaccine elicited antibodies which cross-reacted with epitopes present in CHSE-214 cells and also that the Crude Purified LF-89 preparation still retained some contaminating CHSE-214 antigens (Fig. 7.5) (estimated, by protein assay, at approx. 20% of the total Crude Purified LF-89 protein concentration). As a result of this cross-reactivity, a control was included in the ELISA whereby sera were screened simultaneously against equivalent protein concentrations of rickettsial antigen and CHSE antigen (Fig. 7.2).

Frequently in measurements of fish antibody responses by ELISA, the coating antigen used to activate the microtitre plate has been used at a protein concentration of approx. 10 $\mu g.ml^{-1}$ (Boesen *et al.* 1997; Wagner *et al.* 2001; Kibenge *et al.* 2002; Miquel *et al.* 2003; Grove *et al.* 2003; Lafrentz *et al.* 2004) although Bricknell *et al.* (1999) used 200 $\mu g.ml^{-1}$ IROMP protein and 2 $mg.ml^{-1}$ polysaccharide antigen in their study of antibody responses to *A. salmonicida*. Working with *P. salmonis*, Birkbeck *et al.* (2004b) used a 1/20 dilution of infected cell culture supernatant as the coating antigen but gave no indication of the protein concentration. In the current work, the concentration of the antigens used for coating the ELISA plates (66 μg $protein.ml^{-1}$) was a compromise between diluting the Crude Purified LF-89 antigen sufficiently to analyse all of the samples and providing the control CHSE Antigen in sufficient excess to bind and detect all of the antibodies cross-reacting with the contaminating CHSE-214 cell antigens.

Of the responding fish in the vaccinated groups, the A_{450} value for Crude Purified LF-89 was always higher than for CHSE antigen. At Weeks 6 and 10, the antibody response to rickettsial antigen was found to be significantly higher than the response to an equivalent concentration of CHSE antigen. As the concentration of CHSE-214 antigen remaining in the Crude Purified LF-89 must have been lower than the concentration in the CHSE antigen preparation (20 % compared with 100 %), it can be deduced that the observed antibody response was directed against the rickettsial antigen and, therefore, that the magnitude of the antibody response in vaccinated fish to the rickettsial antigen was indeed greater than the response to the CHSE-214 host cell antigen. It could be argued that, as vaccination resulted in increased antibody levels to the unrelated CHSE antigen, it was possible that the ELISA data reflected a generalised non-specific increase in antibody levels. However, the antibody response to rickettsial antigen was found to be significantly higher than the response to *A. salmonicida* antigen.

As the available *P. salmonis* protein was limited, it was not possible to measure antibody titres in the conventional manner, using serial dilutions of serum. However, for routine testing of vaccine efficiency, an ELISA is required in which antibody titres of vaccinated fish can be measured accurately and compared with control fish. Furthermore, it is necessary to correlate antibody titres with protection against challenge, as not all antibodies produced in response to vaccination will be protective (Reitan and Secombes, 1997). In the present study, it was not possible to conduct a challenge test on the vaccinated fish due to technical difficulties. Therefore, any correlation between production of antibodies against *P. salmonis* and the protective effect of vaccination could not be determined. A correlation has been shown between the development of high antibody titres

and protection against virulent *A. salmonicida* (Bricknell *et al.* 1999) and the demonstration of a similar correlation may prove useful in the further development of vaccines against *P. salmonis*. Given the difficulty of obtaining sufficient yields of *P. salmonis* antigen from cell cultures for use in coating the ELISA plates, the possibility of utilizing the recombinant antigens used in the *P. salmonis* vaccine should be investigated. This approach of using a recombinant fragment of an immunogenic viral protein has been used successfully in measurement of antibody responses to viral haemorrhagic septicaemia virus (VHSV) (Rocha *et al.* 2002).

While there appeared to be a large number of non-responders, it was not possible to determine if this was due to repeated sampling of non-responding individuals at subsequent time-points or was an accurate reflection of the proportion of non-responders in the population, as individual fish were not marked. A more accurate indication of the proportion of non-responders would require that every individual in the population be sampled at each time point.

In conclusion, it has been possible, using a crudely purified preparation of cultured piscirickettsiae, to detect by ELISA an immune response to the complete *P. salmonis* LF-89 organism in fish which have been vaccinated using recombinant rickettsial antigens and which appear to produce antibodies cross-reactive with CHSE-214 host cell antigens. By measuring the response against equivalent protein concentrations of CHSE-214 antigen and *A. salmonicida*, it was possible to demonstrate a significant and specific antibody response against *P. salmonis*.

Chapter 8 - Final discussion

8.1 Discussion

The aim of the presented work was to study the effects of *P. salmonis*, an intracellular pathogen of salmonid fish, on various immune responses in rainbow trout, and to ascertain if certain immune responses could be upregulated by immunisation using a recombinant vaccine. The confirmation of *P. salmonis* as a pathogenic organism in European seabass from the Mediterranean, and its close relationship to salmonid pathogens from Western Europe, Canada and Chile, points to the ubiquitous nature of this organism and underlines its potential to cause disease in many of the species used for aquaculture. Indeed, pathogens found in white seabass in California (Arkush *et al.* 2005) and in Atlantic salmon in Tasmania (Corbeil *et al.* 2005) have recently been identified as *P. salmonis* from DNA sequences of rRNA and ITS regions. Furthermore, from comparison of the available 16S rRNA and ITS sequences, it seems that the Tasmanian salmon isolate is more closely related to SBPLO, the European seabass isolate described in the present study, than to the previously reported salmonid strains. This raises interesting questions about the historical spread of the *P. salmonis* pathogen.

By combining phylogenetic analysis of 16S rRNA sequences with the more detailed information available from the ITS regions, it will be possible to elucidate the relationships between fish pathogenic strains of piscirickettsiae and environmental strains. This may provide some clues about their means of dissemination and the identity of reservoirs and vectors. In this regard, it is interesting that outbreaks in Chile and Norway both followed a period of algal bloom (Branson and Diaz-Munoz, 1991; Olsen *et al.* 1997) and that Mauel

and Fryer (2001) identified a *P. salmonis*-like 16S rDNA sequence in bacterioplankton from the coastal waters of Oregon, USA. Whether or not the algae acted as disease vectors in these outbreaks or caused immunosuppression in the fish which rendered them more susceptible to *P. salmonis* in the environment has not been established. However, as it is clear that the *P. salmonis* organism has the potential to cause disease in a wide range of species, it poses a considerable threat to the Aquaculture Industry worldwide.

Antibiotic therapy of piscirickettsiosis, or SRS, has proven ineffective and expensive (Smith *et al.* 1997; Miquel *et al.* 2003) and, therefore, attention has been focused on the development of vaccines as a means of preventing the disease (Smith *et al.* 1999; Kuzyk *et al.* 2001b; Miquel *et al.* 2003). However, in order to develop an effective vaccine strategy, more information is needed about the interaction between *P. salmonis* and the host, and about possible virulence factors which may be used by *P. salmonis* for attachment/invasion or for evading the host immune response. *Piscirickettsia salmonis* is defined as an obligate intracellular parasite (Fryer *et al.* 1992) and, in natural disease, can be observed within cytoplasmic vacuoles in hepatocytes and macrophages (Cvitanich *et al.* 1991). The present work has indicated that the presence of *P. salmonis* within macrophages is not simply a result of scavenging by these phagocytes but that *P. salmonis* is capable of long-term survival within macrophages (Chapter 3) and therefore, that a proportion of the invading organisms are capable of evading the macrophage bactericidal mechanisms. While it is unclear whether or not *P. salmonis* avoids phagolysosomal destruction by escaping into the cytoplasm, there is no evidence to suggest that the pathogen utilises ABM as a means of intracellular dissemination, as do some of the mammalian rickettsiae (Heinzen *et al.* 1993; Gouin *et al.* 1999).

Although there is evidence from the presented study that the organism can be destroyed rapidly by the phagocyte, it appears that *P. salmonis* is capable of concurrent multiplication in the same cell. The observation of apparently intact and replicating organisms within macrophages suggests that these organisms have been able to enter the phagocyte without being exposed to the toxic products of the respiratory burst. However, experiments to determine if *P. salmonis* stimulates or inhibits the respiratory burst in rainbow trout macrophages were not conclusive, possibly because a low ratio of *P. salmonis* organisms to macrophages meant that the effect was undetectable (Chapter 4). Other intracellular pathogens are capable of entering macrophages without triggering the respiratory burst by using receptor-mediated pathways which are not coupled to the activation of antimicrobial mechanisms such as the production of ROIs. These pathways include opsonic or non-opsonic ingestion via complement, mannose or fibronectin receptors (Bermudez *et al.* 1991; Ernst, 1998; Le Cabec *et al.* 2000). As uptake of *P. salmonis* may be mediated via several pathways, the subsequent cell trafficking of different organisms may not be the same, which could account for the differential survival rates of organisms within the same cell (Ernst, 1998).

Alternatively, *P. salmonis* may avoid triggering the respiratory burst by releasing an inhibitor of the NADPH oxidase. *Renibacterium salmoninarum*, another fish pathogen which is capable of surviving within macrophages, produces p57, a soluble protein antigen, which has been suggested to have an inhibitory effect on the respiratory burst (Densmore *et al.* 1998). Campos-Peréz *et al.* (1997) demonstrated that heat-killed *R. salmoninarum* elicited a higher respiratory burst in rainbow trout macrophages than did live bacteria, which supports the involvement of a protein inhibitor. From their study of gene expression

during the early phase of macrophage infection by *R. salmoninarum*, Grayson *et al.* (2002) proposed that p57 reduces the expression of IL-1β, a pro-inflammatory cytokine. However, they suggested that initial survival of *R. salmoninarum* may depend on interference with TNF-α-dependent killing mechanisms. As TNF-α synergises with other mediators to up-regulate both ROIs and RNIs, reduction of TNF-α expression may explain the inability, in the present investigation, to detect iNOS expression or the by-products of NO metabolism in rainbow trout which had been challenged with *P. salmonis*. Another link between respiratory burst and iNOS expression has been shown in work by Han *et al.* (2001) which suggests that H_2O_2-dependent oxidative stress is involved in the induction of iNOS via nuclear factor (NF)-kB activation in LPS-stimulated macrophages. Thus, it is possible that the failure to detect respiratory burst or iNOS expression following exposure to *P. salmonis* are connected and were due to the signalling pathways triggered by this pathogen (Pieters, 2001).

Another observation which may have relevance to pathogenic mechanisms of *P. salmonis* is the apparent ability of *P. salmonis* culture supernatants to cause rapid lysis of macrophages in *in vitro* cultures (Chapters 4 & 5). Cytotoxic effects of *A. salmonicida*, *A. hydrophila* and *V. anguillarum* on macrophages have been described (Olivier *et al.* 1992; Leung *et al.* 1995; Boesen *et al.* 2001). All of these pathogens are bacteria which can be cultivated in defined media and therefore, toxins can be more easily detected and characterised (Gunnlaugsdottir and Gudmundsdottir, 1997; Hussain *et al.* 2000). In the case of *P. salmonis*, however, it was not verified if the toxic factor was a direct product of the organism or was produced by CHSE-214 cells as a result of *P. salmonis* infection. The

production of such a toxin may be intrinsic to the development of necrosis found in affected fish and could be of interest from the perspective of vaccine development.

One of the aims of the present work was to investigate the ability of a recombinant *P. salmonis* vaccine to induce an immune response in salmonids. It appeared that macrophages from vaccinated fish may be capable of clearing *in vitro P. salmonis* infection more rapidly than macrophages from non-vaccinated fish (Chapter 2). This increased activation has been reported for mammalian rickettsiae where macrophages derived from convalescent guinea pigs and mice were capable of more rapid clearance of *R. conorii* following infection *in vitro* than macrophages from naïve animals (Kokorin *et al.* 1980). Activation of trout macrophages to increase their bactericidal capacity was found to require stimulation *in vivo* (Chung and Secombes, 1987; Graham *et al.* 1988) and has been shown to be mediated by lymphokines secreted by surface Ig^- lymphocytes (Graham and Secombes, 1990b). Therefore, it is possible that macrophages isolated from fish vaccinated against *P. salmonis* were primed for increased bactericidal activity by immune lymphocytes *in vivo*. Alternatively, it is possible that *in vitro* macrophage cultures contained a small number of immune lymphocytes which were re-stimulated *in vitro* and, because of this secondary stimulus, were sufficient to cause macrophage activation. This ability of vaccination against *P. salmonis* to induce an elevated cell-mediated response was also found in leukocyte monolayers from vaccinated fish (Chapter 6) where *in vitro* exposure of immune leukocytes to *P. salmonis* resulted in greater respiratory burst activity of stimulated macrophages.

In addition to examining the effect of vaccination on the cell-mediated response, the humoral response of immunised fish was evaluated by using ELISA to measure serum

antibody levels (Chapter 7). To ensure that the humoral response elicited by the recombinant vaccine would be capable of recognising the complete organism, a method was developed whereby serum samples could be screened against *P. salmonis* isolated from cell culture. Significantly raised levels of anti-*P. salmonis* antibodies were observed by 6 weeks following vaccination of rainbow trout and elevated levels were still present 12 weeks after vaccination. Unlike previously reported studies, where serum from naturally infected fish was shown to recognise the recombinant antigens (Kuzyk *et al.* 2001b; Wilhelm *et al.* 2003; Wilhelm *et al.* 2004), the present investigation showed that a humoral response generated using recombinant proteins was capable of recognising whole-cell *P. salmonis*. In conclusion, the ability of the recombinant vaccine to stimulate a humoral response against whole-cell *P. salmonis*, together with its capacity to prime an elevated cell mediated response, indicate that it is a promising candidate for use in combatting piscirickettsiosis.

8.2 Future work

One of the main difficulties faced by investigators of interactions between the intracellular *P. salmonis* and the fish is achieving adequate separation of the organism from its host cell, usually CHSE-214, when cultured *in vitro* (Kuzyk *et al.* 1996; Barnes *et al.* 1998; Henriquez *et al.* 2003). As many of the organisms remain closely associated with cellular material, even after lysis of *P. salmonis*-containing vacuoles, methods used to remove host-cell material also remove a large number of the piscirickettsiae (personal observation). Therefore, the relative yield of *P. salmonis* can be quite low compared with the amount of contaminating host-cell debris. This not only makes detection of responses to *P. salmonis* quite difficult but also requires the use of extra resources to ensure that experiments include

adequate controls for host-cell contamination. The recent report of high yields of *P. salmonis* using insect cell culture (Birkbeck *et al.* 2004a) suggests that more efficient cultivation methods may exist and, with the increasing availability of new culture techniques, alternative means of production *in vitro* should be investigated.

The mechanism by which *P. salmonis* achieves uptake to its host cell deserves further study. While the organism can achieve entry to macrophages simply by being taken up as part of the cell debris, it is possible that attachment to cell surface receptors is utilised for entry by free organisms. The pathogen is also capable of entering cells other than macrophages (hepatocytes *in vivo* and various fish cell lines *in vitro*) which again is likely to require attachment to a receptor. While Larenas *et al.* (2003) have demonstrated that a piscirickettsial attachment complex is involved in the entry of *P. salmonis* to salmonid ova, no similar structure was observed by TEM during the present investigation. However, observation of initial contact between *P. salmonis* and CHSE-214 cells or macrophages, using a synchronised infection, may yield some information about the physical mechanism of uptake. In addition, studies using inactivated *P. salmonis* or inhibitors of actin polymerisation e.g. cytochalasins, may indicate if *P. salmonis* plays an active or passive role in uptake. The identification of cell-surface ligands used by *P. salmonis* in attachment and uptake to host cells would be of benefit for immunisation, as these could be incorporated into vaccines and used to prime neutralising Ab production (Anacker *et al.* 1987; Díaz-Montero *et al.* 2001).

The apparent ability of macrophages from immunised fish to achieve more rapid clearance of *P. salmonis* is of interest and further *in vitro* studies are required to compare survival in immune and naïve macrophages. By treating macrophages with SOD and

catalase, or with the iNOS inhibitor, N^G-MMLA, it may be possible to determine if up-regulation of ROIs or RNIs plays a role in this protection (Hong *et al.* 1998; Feng and Walker, 2000). As an adjunct to this, the contribution of delayed apoptosis to *P. salmonis* survival in naïve macrophages is worthy of investigation, as prevention of apoptosis by activation of NF-kB has been proposed as a mechanism for prolonging intracellular survival of mammalian rickettsiae (Clifton *et al.* 1998; Radulovic *et al.* 2002).

As more cytokine sequences are identified in fish species, gene expression studies and the production of anti-cytokine mAbs will facilitate their quantitation at the transcriptional and translational levels (Grayson *et al.* 2002; Lindenstrom *et al.* 2004; Sigh *et al.* 2004). This will not only help to elucidate the cytokine pathways triggered in response to *P. salmonis* infection but may also reveal differences in responses between immune and naïve individuals. Furthermore, by generating specific cytokine-depleted individuals using mAbs administered *in vivo*, it may be possible to identify those cytokines which are crucial to the fish defence against the pathogen (Li *et al.* 1987; Feng *et al.* 1994).

As we learn more about the interaction between *P. salmonis* and the fish immune system, it may be possible to further refine vaccines so as to optimise protection. One area of vaccine research which is attracting considerable attention is the potential to manipulate the immune response by the co-administration of cytokines or T-cell epitopes with the antigen (Esser *et al.* 2003; Frauenschuh *et al.* 2004; Franssila and Hedman, 2004; Northrop and Shen, 2004). Already, Kuzyk *et al.* (2001a) have demonstrated improved protection against *P. salmonis* in coho salmon using a recombinant vaccine which incorporated T-cell epitopes. By using appropriate chemokines and cytokines as vaccine adjuvants it may be

possible to elicit the desired effector and memory cell populations so as to ensure the most effective and long-lasting immunity to *P. salmonis*.

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List of chemical suppliers

- **0.2 ml Ready-to-Go PCR beads** - *Amersham Biosciences UK Ltd., Buckinghamshire, UK*
- **2 mM L-glutamine** - *(Gibco) Invitrogen , Paisley, Scotland, UK*
- **Alexa Fluor 633® goat anti-rabbit IgG** – *(Molecular Probes) Invitrogen*
- **Alexa Fluor® 488 phalloidin** – *(Molecular Probes) Invitrogen*
- **Beckman Ultra-ClearTM centrifuge tubes** – *Beckman Coulter (UK) Ltd., High Wycombe, Buckinghamshire, UK*
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- **cacodylate buffer** - *Agar Scientific Ltd.*
- **3,3-diaminobenzidine tetrahydrochloride (DAB)** - *Sigma-Aldrich Company Ltd., Dorset, UK*
- **diethyl-pyrocarbonate (DEPC)** - *Sigma-Aldrich Company Ltd*
- **dimethylsulphoxide** - *Sigma-Aldrich Company Ltd.*
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- **DYEnamic™ ET terminator kit Cycle Sequencing Kit** - *Amersham Biosciences UK Ltd.*
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Vol. 64: 107–119, 2005 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published April 18

Confirmation of *Piscirickettsia salmonis* as a pathogen in European sea bass *Dicentrarchus labrax* and phylogenetic comparison with salmonid strains

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ABSTRACT: European sea bass *Dicentrarchus labrax* from the Mediterranean were diagnosed with a severe encephalitis. Rickettsia-like organisms (RLOs) were associated with brain lesions in routine paraffin sections. These were found to share common antigens with the *Piscirickettsia salmonis* type-strain, LF-89, by indirect fluorescent antibody test (IFAT) and by immunohistochemistry (IHC). In addition, we compared the DNA sequences of the 16S rDNA and 16S-23S internal transcribed spacer region (ITS) with those published for *P. salmonis* strains and found that the sea bass piscirickettsia-like organism (SBPLO) was another strain of *P. salmonis*, closely related to the salmonid pathogens. Furthermore, we showed that the SBPLO possessed at least 2 ITS regions, 1 of which contained tRNA genes.

KEY WORDS: *Piscirickettsia salmonis* · European sea bass *Dicentrarchus labrax* · IFAT · Immunohistochemistry · rDNA · Phylogeny

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INTRODUCTION

Piscirickettsia salmonis, the causative agent of Piscirickettsiosis or Salmonid Rickettsial Syndrome, was first identified as a pathogenic agent in disease outbreaks amongst farmed coho salmon *Oncorhynchus kisutch* in Chile during 1989 (Fryer et al. 1990, Branson & Diaz-Munoz 1991, Cvitanich et al. 1991). Subsequently, the organism was confirmed as the agent responsible for clinical and chronic disease amongst farmed salmonids from both the Pacific and Atlantic coasts of Canada, Ireland, Norway and Scotland (Brocklebank et al. 1992, Palmer et al. 1996, Olsen et al. 1997, Birrell et al. 2003). The disease has appeared primarily to affect salmonids; chinook salmon *O. tshawytscha*, sakura salmon *O. masou*, rainbow trout *O. mykiss*, pink salmon *O. gorbuscha* and Atlantic salmon *Salmo salar* are all susceptible, albeit to differing degrees (House et al. 1999). While rickettsia-like organisms (RLOs) have been observed in non-salmonid hosts such as the blue-eyed plecostomus *Panaque suttoni* (Khoo et al. 1995), 5 species of cultured tilapia in Taiwan (Chern & Chao 1994, Chen et al. 1994) and in Hawaiian tilapia (Mauel 2003), to date the only non-salmonids in which a piscirickettsia-like organism (PLO) has been confirmed using serological methods are the white seabass *Atractoscion nobilis* in California, USA (M. F. Chen et al. 2000) and the grouper *Epinephelus melanostigma* in Taiwan (S. C. Chen et al. 2000). RLOs have also been identified in European sea bass (Comps et al. 1996) but, while antigenic similarities have been confirmed (Steiropoulos et al. 2002), the relatedness of these organisms to *P. salmonis* has not yet been determined.

Based on the sequence of its 16S rRNA gene, the *Piscirickettsia salmonis* type-strain, LF-89 (ATCC VR 1361),

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was placed among the Gammaproteobacteria (Fryer et al. 1992). Subsequently, Mauel et al. (1999) used comparisons of 16S rRNA gene sequences and internal transcribed spaces (ITS) sequences to determine the relatedness of other *P. salmonis* isolates from Chile, Canada and Norway. They established that these strains formed a monophyletic group within the Gammaproteobacteria, although 1 Chilean isolate, EM-90, had diverged sufficiently to allow differentiation from the other isolates based on restriction fragment length polymorphism (RFLP) (Mauel et al. 1996). This group found only 1 ITS sequence in the isolates examined but, following polyacrylamide gel analysis of amplified ITS regions, Casanova et al. (2001) have suggested that *P. salmonis* may contain at least 2 rRNA (*rrn*) operons, as is commonly the case for other Gram-negative bacteria (Gürtler & Stanisich 1996, Crosby & Criddle 2003). More recently, Reid & Birkbeck (2003) have extended the information on regional variation of *P. salmonis* isolates through their comparison of 16S rDNA and ITS sequences from Scottish and Irish isolates.

In this study, routine diagnostic histopathological examination was conducted on European sea bass presenting with clinical signs of nervous disease. In paraffin sections an RLO was seen to be associated with the encephalitic lesions. Serological analyses, indirect fluorescent (IFAT) and immunohistochemistry (IHC) were then used to confirm the tentative diagnosis and to confirm whether or not the organism was antigenically related to the *Piscirickettsia salmonis* type-strain, LF-89. The DNA sequences of the 16S rDNA and ITS region were then compared with those of published *P. salmonis* strains to establish whether or not the sea bass piscirickettsia-like organism (SBPLO) might be another strain of *P. salmonis* and how closely genetically related it was to the salmonid pathogens. Furthermore, it was sought to establish if the sea bass isolate possessed at least 2 ITS regions, 1 of which contained tRNA genes in the 16S-23S spacer region.

MATERIALS AND METHODS

Fish. Juvenile sea bass *Dicentrarchus labrax* from a farm in Greece which were exhibiting abnormal swimming and whirling behaviour, and experiencing low levels of mortality, were euthanized by terminal anaesthesia in 2-phenoxyethanol and sampled for histological analysis and screening by polymerase chain reaction (PCR) for suspected *Piscirickettsia salmonis* infection. Samples of whole fish were fixed in 10% neutral buffered formalin for histology, and samples of brain and mid-gut were preserved in 100 and 70% ethanol for subsequent extraction of genomic DNA for DNA analysis.

Examination of tissue sections. ***Histology:*** Formalin fixed tissues for histological examination were embedded in paraffin, sectioned at 5.0 µm and stained with haematoxylin and eosin.

Serological analyses: Slides were prepared from 5.0 µm paraffin-wax embedded tissue sections taken from the brain and midgut of infected sea bass. Sections prepared from the liver and kidney of rainbow trout *Oncorhynchus mykiss* experimentally infected with *Piscirickettsia salmonis* LF-89 (type-strain ATCC VR 1361) were used as a positive control, while sections from non-infected sea bass were used as negative controls. Sections were dewaxed in 2 successive xylene baths (5 min each) and rehydrated in a 100% ethanol bath for 5 min followed by a 70% ethanol bath for 3 min. After rinsing with distilled water, tissue sections were encircled with wax using a PAP pen (VWR International).

Indirect fluorescent antibody test (IFAT): Sections were subjected to additional fixing in 95 % methanol for 5 min, washed with 0.01 M phosphate buffered saline (PBS), pH 7.4 and non-specific binding sites were blocked by incubation in goat serum (Sigma-Aldrich) (diluted 1/10 in PBS) for 10 min at room temperature (RT; approx. 21°C). After washing in PBS, tissue sections were incubated with rabbit anti-*Piscirickettsia salmonis* serum (kindly provided by Professor J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, OR, USA), diluted 1/100 in PBS. Tissue sections incubated with PBS only were also used as additional negative controls. Slides were washed again in PBS, then incubated with fluorescein isothiocyanate (FITC)-labelled, goat, anti-rabbit serum (Diagnostics Scotland) diluted 1/100 in PBS, for 30 min at RT. After a final washing step, slides were mounted in 50% (v/v) glycerol and examined under oil immersion with an Olympus BX50 fluorescent microscope.

Immunohistochemistry (IHC): The IHC procedure was carried out essentially as described by Alday-Sanz et al. (1994). The sections were incubated in 10% (v/v) hydrogen peroxide in methanol for 10 min at RT to block endogenous peroxidase activity. After washing with Tris-buffered saline (0.2 M TBS, pH 7.2), non-specific binding sites were blocked by incubation in goat serum, diluted 1/10 in TBS, for 10 min at RT. Slides were washed in TBS, tapped dry and incubated with anti-*Piscirickettsia salmonis* polyclonal antibody (PAb), diluted 1/100 in TBS, for 60 min in a humid chamber. Excess primary antibody was removed by washing in TBS and slides were incubated in goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, diluted 1/50 in TBS, for 30 min in a humid chamber. Sections were then incubated with 1% (v/v) hydrogen peroxide in 1.5 mg 3,3-diaminobenzidine tetrahydrochloride (DAB) and 10 ml TBS for 10 min. The reaction was

stopped by immersing the slides in tap water and slides were then counterstained with haematoxylin for 3 min. Excess stain was removed by incubating the slides in tap water for 10 min, followed by dehydration in 70% ethanol (3 min), 100% ethanol (5 min) and 2 successive xylene baths (2 × 5 min). Slides were mounted in Pertex and examined under a light microscope.

Concurrently, infected sea bass tissues were screened with an anti-*Photobacterium damselae* subsp. *piscicida* monoclonal antibody (mAb) (Aquatic Diagnostics) in IFAT and IHC. Tissues from sea bass, positive and negative for *Ph. damselae* subsp. *piscicida*, were used as controls. The monoclonal antibody was used according to the manufacturer's instructions, and either goat anti-mouse IgG-HRP (Diagnostics Scotland) or goat anti-mouse IgG-FITC (Diagnostics Scotland) diluted 1/100 in PBS was used as secondary antibody. The incubations with these antibodies were as described above.

DNA analysis. ***Isolation of genomic DNA:*** Brain tissue (0.5 g), preserved in 70 or 100% ethanol, was excised using sterile scalpels and genomic DNA was extracted using a Nucleon ST Kit (Tepnel Life Sciences PLC). Brain tissue from 2 fish was combined for each sample and 2 samples were extracted on 2 different days. As a control for possible contamination during the extraction process, concurrent extraction procedures were carried out in the absence of any tissues. Genomic DNA, extracted from cell monolayers infected with *Piscirickettsia salmonis* LF-89 or with *P. salmonis* strain SLGO-95 (kindly provided by Dr. P. A. Smith, Faculty of Veterinary Sciences, University of Chile, Santiago) was used as a positive control. Extracted DNA was stored at −20°C until required for PCR.

PCR amplification of 16S rDNA: Initially, a nested PCR amplification was carried out using the bacterial 16S primers, EubA and EubB, in the first-round amplification and *Piscirickettsia*-specific primers, PS2S and PS2AS, in the second-round amplification (Table 1) (Mauel et al. 1996). PCR amplifications were performed using 0.2 ml Ready-to-Go PCR beads (Amersham Biosciences UK), following the manufacturer's instructions, with a final concentration of 1 µM of the appropriate primer pair and 1 µl template DNA. For the second-round amplification, 1 µl of the first-round reaction was used as template. Cycling conditions were modified from Corbeil et al. (2003). Briefly, for primary PCR the mixture was denatured at 95°C for 5 min and amplified with 35 cycles of 94°C for 30 s, 50°C for 40 s, 72°C for 40 s with a final extension step of 72°C for 5 min. For nested PCR, the mixture was denatured at 95°C for 5 min followed by amplification with 35 cycles of 94°C for 30 s, 61°C for 40 s, 72°C for 40 s and a final extension step of 72°C for 5 min. Subsequently, the nested PCR was carried out using primer pairs (Table 1) designed to amplify the region between nucleotides 225 and 1475, namely PS2S and 860R (nucleotides 226–860), PS2S and 1283R (226–1283) and 860F and 1470R (860–1476). The numbering corresponds to the published sequence for the *P. salmonis* type-strain LF-89 (GenBank accession number U36941) (Mauel et al. 1999). For these amplifications, cycling parameters were as described above, except that each primer was used at 1 µM final concentration, and an annealing temperature of 56°C was used in the PCR cycle.

All amplifications were performed using a Biometra T Gradient thermocycler (Anachem) and 5 µl of each amplification reaction was examined for specificity by electrophoresis on a 1% agarose gel containing 0.5 mg ml^{-1} ethidium bromide. Molecular weight markers (DNA Molecular Weight Marker VI or DNA Molecular Weight Marker XIV, Roche Diagnostics) were added to the gel as a reference.

PCR amplification of the ITS region: A nested PCR amplification was carried out using conditions and cycling parameters as described above for the 16S rDNA PCR. However, primers PS16SA and PS23SB were used in the first round reaction, and primers PS16SH and PS23SC were used in the second round as

Table 1. Sequences and specificity of primers used for 16S rRNA gene and internal transcribed spacer (ITS) region PCR and sequencing. F: forward; R: reverse

Primer/location	Sequence (5'-3')	Specificity
Eub B (27F)[a]	AGAGTTTGATCMTGGCTCAG	Eubacterial
Eub A (1518R)[a]	AAGGAGGTGATCCANCCRCA	Eubacterial
PS2S (223F)[a]	CTAGGAGATGAGCCCGCGTTG	*P. salmonis* 16S
PS2AS (690R)[a]	GCTACACCTGCCAAACCACTT	"
850F (851F)[b]	GGATTCCCTTGAGGAGTTTAGTGG	"
850R (828R)[b]	CCACTAAACTCCTCAAGGGACTCC	"
1280R (1283R)[b]	CTTTCTCAGGTTCGCTCCAC	"
1490R (1487R)[b]	CTTCACCCCAGTCATGACCC	"
PS16SA (1387F)[a]	GCCTTGTACACAACCGCCC	*P. salmonis* ITS
PS23SB (507R)[c]	CCTTTCCCTCACGGTCAT	"
PS16SH (1519F)[b]	CCTGCGGCTGGATTACCT	"
PS23SC (203R)[c]	TAGATGTTTCAGTTCCCC	"
ITSUF (1430F)[b]	AGTGAATTGCACCAGAAGGG	"
ITSUR (303R)[c]	ATCACCCTCTATCGCCACAC	"

[a]Numbering corresponds to *Escherichia coli* 16S rRNA gene
[b]Numbering corresponds to *Piscirickettsia salmonis* LF-89 16S rRNA gene
[c]Number of bases from the 5' end of the *Piscirickettsia salmonis* 23S rRNA gene

previously described by Mauel et al. (1999) (Table 1). Subsequently, using primers ITSUF and ITSUR, complementary to *Piscirickettsia salmonis* LF-89 16S and 23S rDNA sequences (Table 1), direct PCR amplification was carried out on DNA isolated from infected sea bass. These primers were also used for nested PCR amplification of first round products obtained with primers PS16SA and PS23SB. Amplification conditions were as described above for nested 16S rDNA PCR except that each primer was used at 0.5 µM with an annealing temperature of 58°C. A faint secondary product produced by this amplification was excised from the gel and re-amplified using 30 cycles and either primer pair PS16SH/PS23SC or primer pair ITSUF/ITSUR.

DNA sequencing: In order to obtain template DNA for sequencing, the GFX™ PCR DNA and Gel Band Purification Kit was used to clean amplification reactions or to clean fragments excised from gels. Sequencing reactions were carried out using a DYEnamic™ ET terminator kit Cycle Sequencing Kit (Amersham Biosciences) and following the manufacturer's instructions. Primers used for the nested PCR reactions were diluted to 5 pmol and used as sequencing primers. Each segment of DNA was sequenced in the forward and reverse direction, following at least 3 separate PCR amplifications on different days, with the exception of the secondary ITS fragment which was amplified in a single run using 2 different primer pairs, as described above. Sequencing reactions were run on the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems), were viewed using BioEdit software (Hall 1999) and aligned using Clustal X (Thompson et al. 1994) with manual editing. Sequences obtained for the SBPLO 16S rDNA were compared with published *Piscirickettsia salmonis* sequences, sequences from fish pathogens or from other members of the Gammaproteobacteria (Table 2), while sequences obtained for ITS DNA were compared amongst *P. salmonis* strains only. Phylogenetic trees were constructed using PHYLIP version 3.6 (Felsenstein 1989). Distance matrices generated by DNADIST were determined using the assumptions of Kimura (1980). These matrices were used to generate dendrograms using the neighbour-joining method (Saitou & Nei 1987). Dendrograms were also constructed using the parsimony program DNAPARS and the maximum-likelihood program DNAML, in the PHYLIP software package. The bootstrap values were obtained from 1000 trees generated with SEQBOOT and CONSENSE within PHYLIP.

Table 2. Bacterial species and strains used in this study and the GenBank accession numbers for their 16S rDNA and ITS DNA sequences

Species/strain	GenBank accession number	DNA sequence
Piscirickettsia salmonis SBPLO	AY542956	16S rDNA
Piscirickettsia salmonis LF-89	U36941	"
Piscirickettsia salmonis SLGO-94	U55015	"
Piscirickettsia salmonis NOR-92	U36942	"
Piscirickettsia salmonis EM-90	U36940	"
Piscirickettsia salmonis ATL-4-91	U36915	"
Piscirickettsia salmonis IRE-91A	AY498633	"
Piscirickettsia salmonis IRE-98A	AY498634	"
Piscirickettsia salmonis IRE-99D	AY498637	"
Piscirickettsia salmonis SCO-95A	AY498636	"
Piscirickettsia salmonis SCO-02A	AY498635	"
Piscirickettsia salmonis SBPLO (ITS_0)	AY607584	ITS and 23S rDNA
Piscirickettsia salmonis SBPLO (ITStRNA)	AY607585	"
Piscirickettsia salmonis LF-89	U36943	"
Piscirickettsia salmonis EM-90	U36944	"
Piscirickettsia salmonis ATL-4-91	U36945	"
Piscirickettsia salmonis NOR-92	U36946	"
Piscirickettsia salmonis SLGO-94	U62104	"
Piscirickettsia salmonis C1-95	U62103	"
Piscirickettsia salmonis IRE-91A	AY498625	"
Piscirickettsia salmonis IRE-98A	AY498624	"
Piscirickettsia salmonis IRE-99C	AY498632	"
Piscirickettsia salmonis IRE-99D	AY498631	"
Piscirickettsia salmonis SCO-95A	AY498621	"
Piscirickettsia salmonis SCO-98B	AY498630	"
Piscirickettsia salmonis SCO-98C	AY498629	"
Piscirickettsia salmonis SCO-02A	AY498628	"
Piscirickettsia salmonis SCO-02D	AY498622	"
Piscirickettsia salmonis SCO-02E	AY498623	"
Piscirickettsia salmonis SCO-02F	AY498626	"
Piscirickettsia salmonis SCO-02G	AY498627	"
Vibrio anguillarum	X16895	16S rDNA
Photobacterium damselae	AB026844	"
Aeromonas salmonicida	X60405	"
Methylophaga marina	X95459	"
Beggiatoa sp.	AF035956	"
Piscirickettsia grp. Clone LA7-B48N	AF513949	"
Tilapia parasite	AF206675	"

RESULTS

Histology

In general, lesions found in infected tissue sections were necrotizing and granulomatous. Liver, kidney, gastro-intestinal tract, pancreas, muscle and subder-

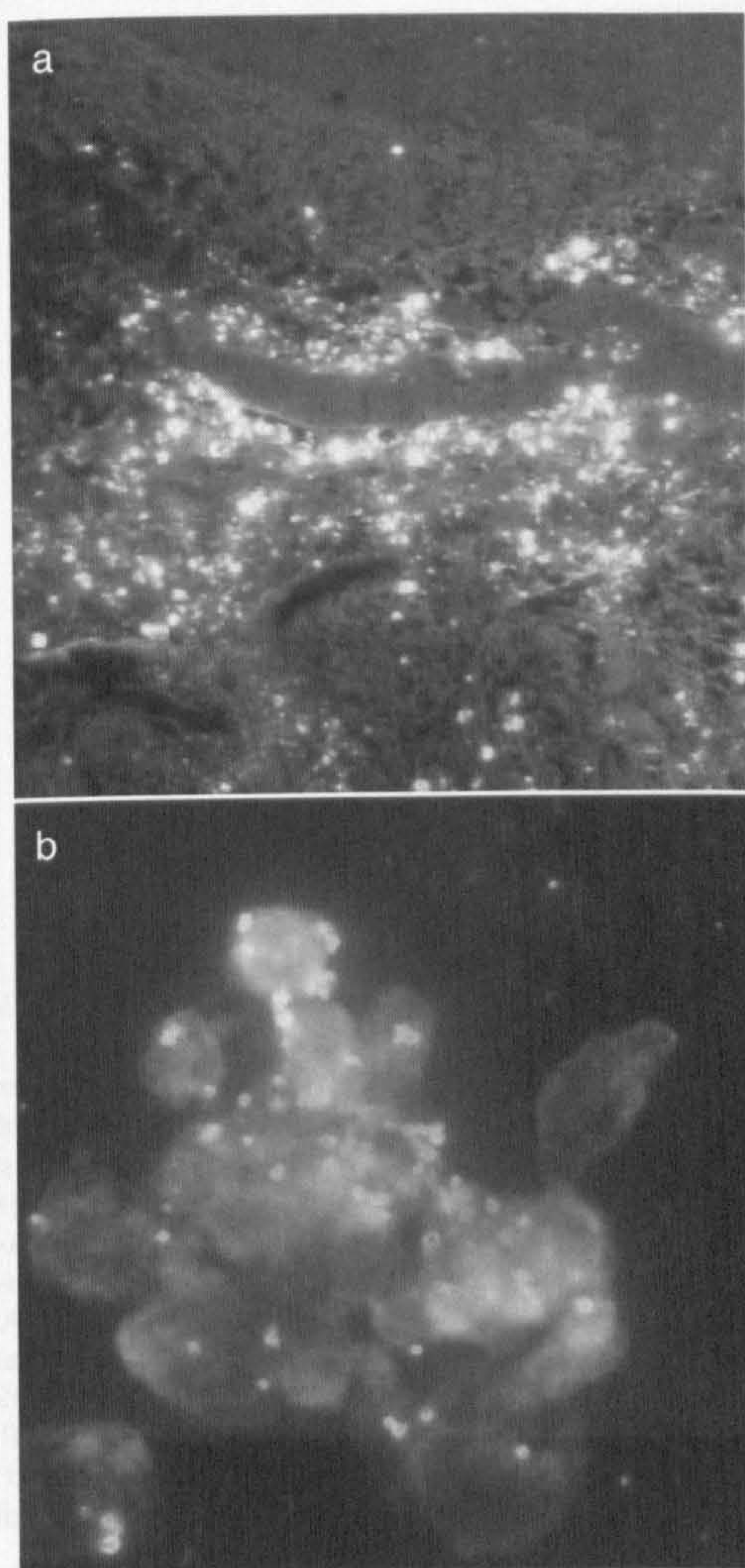

Fig. 1. *Piscirickettsia salmonis.* Indirect fluorescent antibody test, using anti-*P. salmonis* polyclonal serum to detect (a) sea bass *Piscirickettsia*-like organisms (SBPLO) in the brain of European sea bass; magnification = ×100. (b) *P. salmonis* strain SLGO-95 in CHSE-214 cell culture smear. Magnification = ×1000

mis were involved to variable degrees from one fish to another, but the organ most consistently affected was the brain. Here, lesions were also necrotizing and granulomatous, involving mostly brain stem, but in some fish they extended also to involve the tegmentum and olfactory lobes and tracts. In most fish, lesions were present in the third ventricle, involving the ciliated ependymal cells lining this, or those cells immediately beneath. The ventricular lumen sometimes was filled with debris and large numbers of eosinophilic foamy macrophages, some of which contained dense, basophilic, spherical, 1 µm-sized RLOs. These large foamy gemistocyte-like cells dominated the inflammatory response in the brain.

Serological analyses

In sections of sea bass brain tissue, ring-shaped organisms were detected by IFAT in the tegmentum using anti-*Piscirickettsia salmonis* PAb as primary antibody (Fig. 1a). The appearance of these organisms was similar to the *P. salmonis* type-strain LF-89 seen in IFAT of cell culture smears (Fig. 1b). No organisms were detected in the non-infected tissue sections or in sections incubated with anti-*Photobacterium damselae* subsp. *piscicida* MAb (not shown). In IHC, DAB deposition corresponded with areas of necrosis in the medulla oblongata (Fig. 2b) and the sensory epithelium of the olfactory organ (Fig. 2c,d). No organisms were detected in the non-infected tissue or in sections incubated with anti-*Ph. damselae* subsp. *piscicida* MAb (Fig. 2a).

Nested PCR and sequence analysis

16S rDNA

Nested PCR of DNA extracted from sea bass samples, using *Piscirickettsia salmonis*-specific primers PS2S and PS2AS, amplified a product of approximately 470 bp. A fragment of similar size was amplified from DNA extracted from cell-cultures of LF-89 and SLGO-95. Sequence analysis confirmed the sea bass product to be 16S rDNA, corresponding to nucleotides 226–673 of the published LF-89 sequence (GenBank accession number U36941) and differing from this sequence at positions 448, 450–454, 464–468, 470 and 641. Subsequent nested PCR and sequencing of the region between nucleotides 226 and 1475 confirmed the initial findings and revealed that the sea bass RLO 16S rDNA also differed from LF-89 rDNA at a further 10 positions (859, 876, 918, 928, 960, 1005, 1289, 1402, 1084 and 1103). A sequence identity matrix, constructed from the 16S rDNA sequence of the SBPLO, and *P. salmonis* strains LF-89, SLGO-95, NOR-92, ATL-4-91, EM-90, SCO-95A, SCO-02A, IRE-91A, IRE-98A and IRE-99D is shown (Table 3). This matrix was calculated from 1297 positions (including gaps) of the *P. salmonis* strains and the SBPLO. Sequence similarity between the SBPLO 16S rDNA gene and the type-strain LF-89 is 98.2%, which compares well with the sequence similarities of the Canadian strain, ATL-4 91 (98.7%) and the Norwegian strain, NOR-92 (98.9%). The similarity of the divergent Chilean strain, EM-90, is 97.1%. Both Scottish isolates (SCO-95A and SCO-02A) show a 16S rDNA sequence similarity of 99.3% with the type-strain, while the Irish strains (IRE-99D, IRE-98A and IRE-91A) reveal similarities of 98.0 to 97.1%. Dendrogram analysis of the SBPLO, *P. salmonis*

Fig. 2. *Dicentrarchus labrax*. Immunohistochemical staining of sea bass brain sections using (a) anti-*Photobacterium damselae* subsp. *piscicida* monoclonal antibody (MAb); and (b,c) rabbit anti-*Piscirickettsia salmonis* serum. Scale bar = 500 µm. Arrows indicate areas of diaminobenzidine tetrahydrochloride (DAB) deposition. Box in (c) indicates area enlarged in (d). Scale bar = 100 µm

Table 3. *Piscirickettsia salmonis*. Sequence similarities of partial ITS DNA sequences between 18 isolates of *P. salmonis* and the sea bass piscirickettsia organism (SBPLO) isolate. The comparison was made using 269 nucleotide positions, including gaps

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. LF-89	1.000	0.962	0.965	0.958	0.958	0.958	0.877	0.950	0.950	0.950	0.946	0.950	0.943	0.954	0.954	0.768	0.774	0.942	0.931
2. SLGO-94	–	1.000	0.988	0.973	0.973	0.939	0.867	0.966	0.965	0.965	0.962	0.965	0.958	0.969	0.969	0.755	0.761	0.920	0.909
3. C1-95	–	–	1.000	0.969	0.969	0.935	0.856	0.962	0.962	0.962	0.958	0.962	0.954	0.965	0.965	0.751	0.757	0.916	0.905
4. ATL-4-91	–	–	–	1.000	0.992	0.931	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.755	0.761	0.924	0.912
5. NOR-92	–	–	–	–	1.000	0.939	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.763	0.768	0.931	0.920
6. EM-90	–	–	–	–	–	1.000	0.877	0.924	0.924	0.924	0.920	0.924	0.916	0.928	0.928	0.761	0.759	0.938	0.927
7. SBPLO	–	–	–	–	–	–	1.000	0.867	0.867	0.867	0.863	0.871	0.859	0.871	0.871	0.833	0.830	0.858	0.847
8. SCO-95A	–	–	–	–	–	–	–	1.000	0.992	0.992	0.988	0.992	0.984	0.996	0.996	0.760	0.765	0.931	0.920
9. SCO-98B	–	–	–	–	–	–	–	–	1.000	0.992	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
10. SCO-98C	–	–	–	–	–	–	–	–	–	1.000	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
11. SCO-02A	–	–	–	–	–	–	–	–	–	–	1.000	0.988	0.996	0.992	0.992	0.755	0.761	0.927	0.916
12. SCO-02D	–	–	–	–	–	–	–	–	–	–	–	1.000	0.984	0.996	0.996	0.763	0.768	0.931	0.920
13. SCO-02E	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.988	0.988	0.751	0.757	0.923	0.912
14. SCO-02F	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	0.763	0.768	0.935	0.923
15. SCO-02G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.763	0.768	0.935	0.923
16. IRE-91A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.966	0.765	0.753
17. IRE-98A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.770	0.759
18. IRE-99C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.988
19. IRE-99D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000

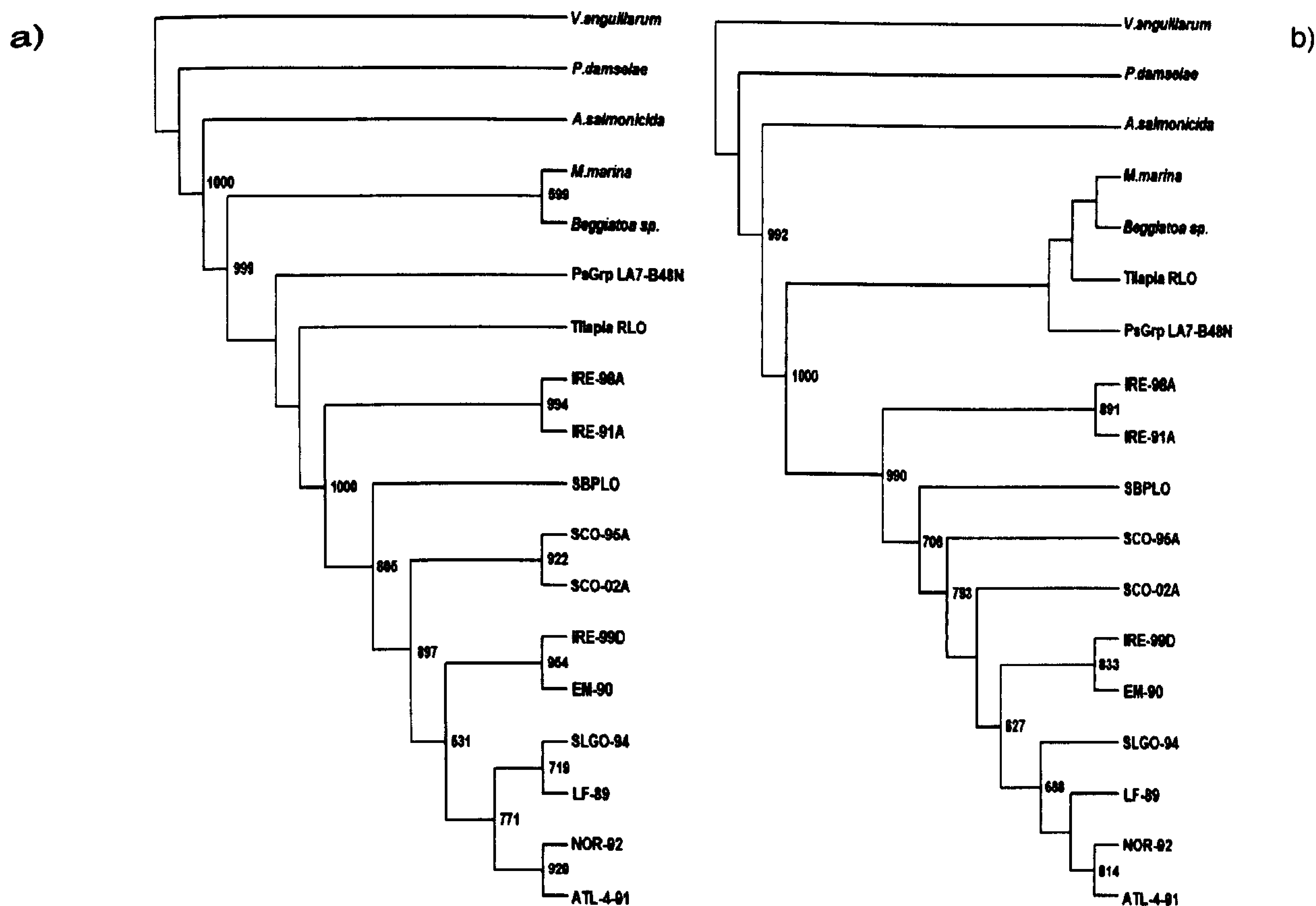

Fig. 3. Phylogenetic relationships of partial 16S rDNA sequences between the SBPLO, *Piscirickettsia salmonis* strains and members of the Gammaproteobacteria (Table 2) inferred from the (a) neighbour-joining and (b) maximum-likelihood methods using *Vibrio anguillarum* (GenBank accession no. X16895) as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 1132 positions, between nucleotide positions 226 and 1350 of *P. salmonis* LF-89. See Table 2 for full species names

and representatives of the Gammaproteobacteria 16S rDNA sequences by neighbour-joining, parsimony (not shown) and maximum likelihood methods produced similar trees (Fig. 3). The analysis places the SBPLO strain within the *P. salmonis* group (bootstrap support of 80.5 and 70.8% by the neighbour-joining and maximum-likelihood methods, respectively) and less distantly associated with the *P. salmonis* type-strain than the divergent Irish strains, IRE-91A and IRE-98A.

ITS region

Nested PCR of the sea bass RLO ITS region using primer pair PS16SA/PS23SB in the first round and primer pair PS16SH/PS23SC in the second round amplifications was successful on only 1 occasion and produced readable sequence from an amplified product of approximately 520 bp. Subsequent direct or nested PCR amplifications using primers ITSUF and ITSUR produced 2 fragments, a major product of approximately 750 bp and a faint, secondary product of approximately 1050 bp (Fig. 4). When sequenced, the major SBPLO product (ITS_0) confirmed our initial sequence obtained using primers PS16SA/PS23SB and PS16SH/PS23SC. This sequence also corresponded overall with the LF-89 ITS sequence (GenBank accession number U36943) found by Mauel et al. (1999) and with the LF-89 ITS B sequence reported by Casanova et al. (2001). The SBPLO ITS_0 differed at 32 positions from LF-89, mainly as the result of a 17-base gap in the SBPLO sequence between positions 223 and 239 (numbering calculated from the 5' end of the LF-89 ITS sequence) (Fig. 5). Comparison of the complete ITS_0 DNA sequence (between positions 1 and 292 and including gaps) reveals a similarity of 87.7 % with the corresponding LF-89 sequence (Table 4). If the 17-base gap region is excluded from the analysis for all strains, the SBPLO/LF-89 sequence similarity is increased to 93.8% but the position of the SBPLO within the range of similarities is not significantly altered (data not shown). The sequence identity matrix shows that the

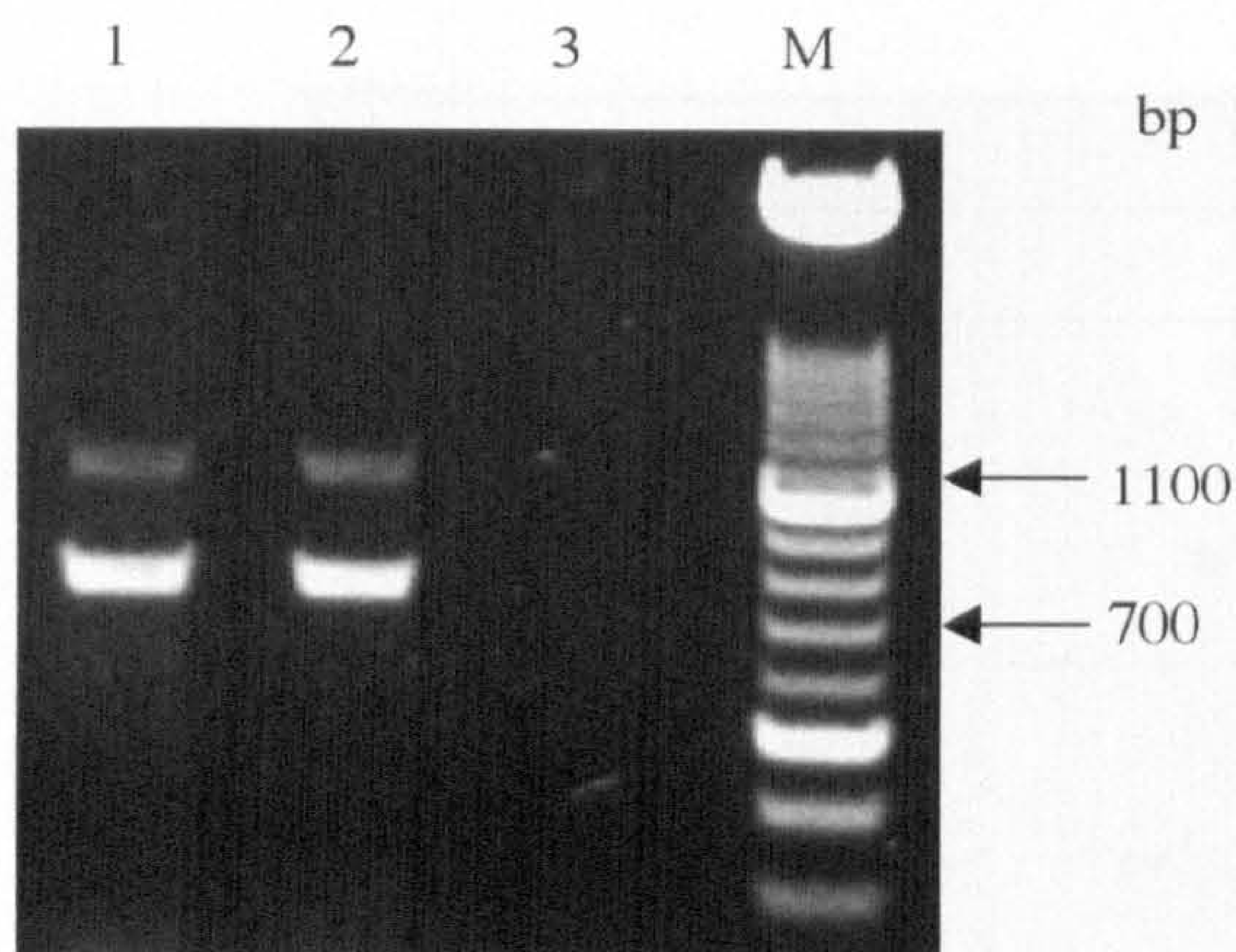

Fig. 4. *Piscirickettsia salmonis*. Products of direct PCR, using primer pair ITSUF/ITSUR, for amplification of ITS DNA. Lanes 1 and 2: DNA isolated from SBPLO-infected sea bass; Lane 3: negative control; M: molecular weight marker

SBPLO ITS_0 sequence exhibits a higher sequence identity with other members of the *Piscirickettsia salmonis* group than the Irish isolates IRE-91A and IRE-98A (76.8 and 77.4%, respectively). The Scottish isolates and the remaining Irish isolates, IRE-99C and IRE-99D, show a sequence divergence from the type-strain of less than 10%. Dendrograms inferred from the SBPLO ITS_0 and *P. salmonis* ITS sequences (nucleotide positions 49 to 310), obtained with either distance or parsimony tree-building methods, produced similar trees (Fig. 6). This analysis again placed the SBPLO strain within the *P. salmonis* group and, while this strain is divergent from most of the group, it is more closely related to the type-strain than the Irish isolates, IRE-91A and IRE-98A. Bootstrap support for this position was 100 and 99.1% for neighbour-joining and parsimony methods, respectively.

```
                                                                                    70
SBPLO ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGT-C ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
LF-89 ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
SBPLO ITSo       ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA
LF-89 ITS        ATTTAY-GAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA

                                                                                   140
SBPLO ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
LF-89 ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
SBPLO ITSo       TA-------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        TA-------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                   210
SBPLO ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
LF-89 ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                   280
SBPLO ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
LF-89 ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                   350
SBPLO ITStRNA    AATGCCGTGA AATGATTATT AG--ATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
LF-89 ITStRNA    AATGCCGTGA AATGATTATT TATAATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- -------TTT
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- -------TTT

                                                                                   420
SBPLO ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG
SBPLO ITSo       GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITS        GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG

                                                                                   490
SBPLO ITStRNA    ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITStRNA    GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
SBPLO ITSo       ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITS        GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT

                                                                                   560
SBPLO ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG
SBPLO ITSo       GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITS        GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG

                                        580
SBPLO ITStRNA    ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITStRNA    ATATAAGACT TCTTGGGGTT ATATGA
SBPLO ITSo       ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITS        ATATAAGACT TTNTGGGGTT ATATGA
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Fig. 5. *Piscirickettsia salmonis*. Sequences of the complete SBPLO ITS_0 and ITS_{tRNA} aligned with the corresponding sequences from the *P. salmonis* type-strain, LF-89. Sequences corresponding to the $tRNA^{ile}$ and $tRNA^{ala}$ genes are underlined. Dashes indicate gaps

Table 4. *Piscirickettsia salmonis*. Sequence similarities of partial 16S rDNA sequences between 10 isolates of *P. salmonis* and the SBPLO isolate. Comparison was made using 1297 nucleotide positions, including gaps

Isolates	1	2	3	4	5	6	7	8	9	10	11
1. LF-89	1.000	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
2. SLGO-94	–	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
3. ATL-4-91	–	–	1.000	0.989	0.971	0.976	0.988	0.988	0.966	0.973	0.976
4. NOR-92	–	–	–	1.000	0.972	0.977	0.989	0.989	0.966	0.974	0.976
5. EM-90	–	–	–	–	1.000	0.960	0.972	0.972	0.959	0.959	0.975
6. SBPLO	–	–	–	–	–	1.000	0.988	0.988	0.976	0.983	0.974
7. SCO-95A	–	–	–	–	–	–	1.000	1.000	0.977	0.985	0.986
8. SCO-02A	–	–	–	–	–	–	–	1.000	0.977	0.985	0.986
9. IRE-91A	–	–	–	–	–	–	–	–	1.000	0.991	0.972
10. IRE-98A	–	–	–	–	–	–	–	–	–	1.000	0.972
11. IRE-99D	–	–	–	–	–	–	–	–	–	–	1.000

Following excision from the gel, re-amplification and sequencing, the secondary SBPLO product (ITS_{tRNA}) was found to contain genes coding for $tRNA^{ile}$ and $tRNA^{ala}$, and to correspond essentially with the LF-89 ITS A sequence described by Casanova et al. (2001). Both SBPLO ITS sequences (ITS_0 GenBank accession number AY607584; ITS_{tRNA} GenBank accession number AY607585) are shown in Fig. 5.

DISCUSSION

Histological analysis of tissues from European sea bass revealed the presence of RLOs in macrophages in the brain, which was the organ most consistently affected in diseased fish. Necrotic lesions in the anterior medulla oblongata and the presence of 1 µm basophilic organisms in cytoplasmic vacuoles have previously been described for RLO infection of sea bass (Comps et al. 1996). The morphological similarity between organisms detected in the sea bass medulla oblongata and *Piscirickettsia salmonis* LF-89 cell culture smears by IFAT suggested that the sea bass pathogen was related to *P. salmonis*. Furthermore, the association of DAB deposition with areas of necrosis

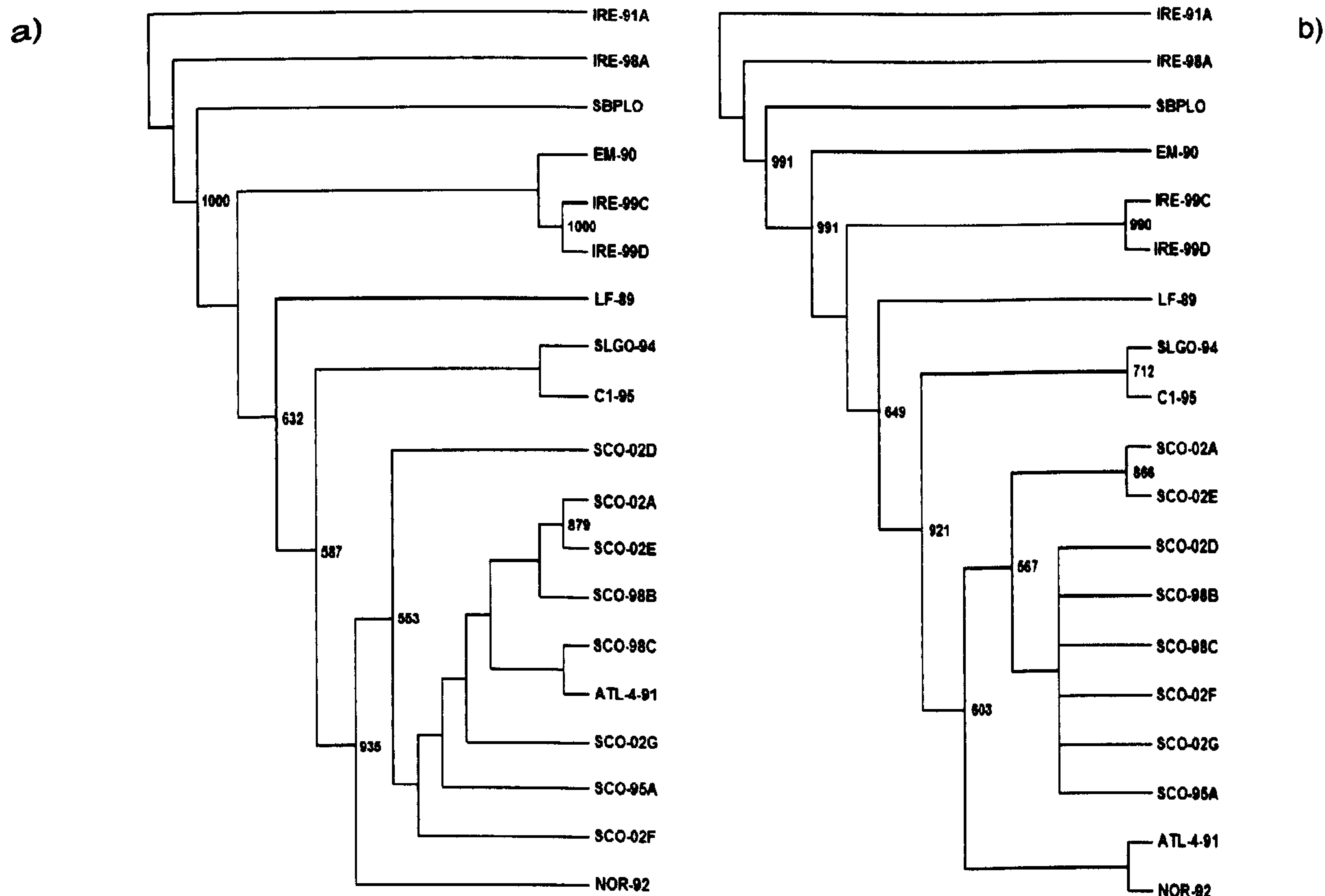

Fig. 6. *Piscirickettsia salmonis*. Phylogenetic relationships of partial ITS sequences between the SBPLO and *P. salmonis* strains inferred from the (a) neighbour-joining and (b) parsimony methods, using strain IRE-91A as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 260 positions, between nucleotides 50 and 310 from the 5' end of the *P. salmonis* LF-89 ITS sequence

seen in IHC strengthened the argument that a *P. salmonis*-related organism was responsible for the pathology found in the sea bass. While initial, on-farm diagnosis suggested that *Photobacterium damselae* subsp. *piscicida* might have played a role in the disease, evidence for involvement of *P. damselae* in the sea bass pathology was not found, as IHC using anti-*P. damselae* MAb was negative.

In salmonids, *Piscirickettsia salmonis* produces a systemic disease and, while pathological changes are found in heart, brain, intestine, ovary and gill, the most characteristic pathology is severe inflammation and necrotic lesions of the kidney, liver and spleen (Fryer et al. 1990, Rodger & Drinan 1993, Palmer et al. 1996, Olsen et al. 1997). Likewise, in diseases of white seabass (M. F. Chen et al. 2000) and grouper (S. C. Chen et al. 2000), both associated with PLOs, and in diseases of tilapia (Chen et al. 1994, Chern & Chao 1994) which were associated with RLOs, the kidney, spleen and liver also exhibited the most marked pathology, although lesions were commonly found in all tissues, including the brain. In the European sea bass, however, the most significant pathology has been associated with the brain (Comps et al. 1996 and this study) and, while antigenic similarities between the sea bass RLO and *Piscirickettsia salmonis* have been intimated by cross-reaction with anti-*P. salmonis* antiserum in IHC (Steiropoulos et al. 2002), the different pathology suggested that the organisms might not be related. Of possible interest in this regard is a RLO which was associated with brain lesions and encephalitis in Scottish farmed Atlantic salmon, but which did not react in a latex agglutination test for *P. salmonis* (Grant et al. 1996).

Since the development of molecular genetic techniques, several methods have been used to differentiate between genera, species and strains of bacteria (Grayson et al. 1999, Romalde et al. 1999, Gürtler & Mayall 2001, Houpikian & Raoult 2001). For phylogenetic studies, where differentiation between strains is used to determine their inter-relatedness and how they may have evolved from a common ancestor, methods such as DNA-DNA hybridisation or pulse-field gel electrophoresis (PFGE) allow very fine discrimination between isolates (Leclerc et al. 2000, Houpikian & Raoult 2001, Le Roux et al. 2004). However, as these methods are not practical for routine use and may prove too sensitive, their application might be most useful in differentiating between strains where a close relationship has already been demonstrated (Leclerc et al. 2000, Gürtler & Mayall 2001, Le Roux et al. 2004). Another method widely used for phylogeny is the comparison of aligned nucleotide sequences of conserved genes, which have been termed 'molecular chronometers' (Fournier et al. 1998). The rRNA genes are highly conserved across the bacterial kingdom and the 16S rRNA gene within the *rrn* operon contains highly conserved regions as well as regions that vary according to species, genera and family. Therefore, comparison of 16S rDNA sequence has become widely used for the classification of organisms (Ruimy et al. 1994, Spröer et al. 1999, Gürtler & Mayall 2001). However, in some cases, the level of conservation between strains is such that differentiation between them on the basis of 16S rDNA sequence analysis is not possible. This is the case, for example, with mammalian rickettsiae (Stothard et al. 1994) and, in these situations, workers have looked to sequencing other, less-highly conserved, genes (Roux et al. 1996, Roux et al. 1997, Fournier et al. 1998). Another alternative is the ITS region DNA found between the 16S and 23S rRNA genes in the *rrn* operon (García-Martínez et al. 1999, Leclerc et al. 2000, Houpikian & Raoult 2001, Hamid et al. 2002). Bacteria frequently possess several copies of the *rrn* operon in their genome (Gürtler & Stanisich 1996, Crosby & Criddle 2003) although there are exceptions such as the *Mycobacteria* and *Mycoplasma*, where, as a rule, only 1 or 2 copies are present (Gürtler & Stanisich 1996). It has been found that there can be considerable variation in the length and sequence of the copies of the 16S-23S ITS region even within a single genome and, therefore, the scope for comparison between strains using ITS sequences is increased (Gürtler & Stanisich 1996, Gürtler & Mayall 2001, Hamid et al. 2002). The main source of variation is the number and type of tRNA genes found in the region between the 16S and 23S rRNA genes (Gürtler & Stanisich 1996). The majority of Gram-positive bacteria do not possess tRNA genes within the ITS region but either $tRNA^{ala}$ or $tRNA^{ile}$ or both genes may be present. In Gram-negative bacteria, however, it is common to find genes for both $tRNA^{ile}$ and $tRNA^{ala}$, or only $tRNA^{glu}$ (Gürtler & Stanisich 1996, Christensen et al. 2000). A further major source of variation between the ITS regions of different strains is in the form of insertion/deletions believed to have arisen from recombination events (Pérez-Luz et al. 1998, Andersson et al. 1999, Garcia-Martinez et al. 1999, Gürtler & Mayall 2001). Because considerable variation in ITS sequence can occur not only between different strains of a genus but also between the operons of a single organism (intercistronic variation), the strength of ITS analysis may lie in fine discrimination between strains of bacteria where 16S rDNA analysis has already suggested a close relationship exists (Ruimy et al. 1994, Pérez-Luz 1998, García-Martínez et al. 1999). Furthermore, the limited number of published ITS sequences compared with the number of 16S rDNA sequences means that initial phylogenetic placement of an organism will, most likely, be based on analysis of its 16S rRNA.

In the study of fish pathogens, differences in 16S rDNA sequences have been used for classifying isolates (Romalde et al. 1999, Reid et al. 2003). Using 16S rDNA sequences, Mauel et al. (1999) demonstrated a monophyletic relationship between strains of *Piscirickettsia salmonis* from Chile, Canada and Norway, with similarities ranging from 99.7 to 98.5%. These workers also examined the DNA sequence of the ITS and of the 23S rRNA and found that, while these also showed low levels of divergence between isolates, the greater variability of these regions was reflected in the wider range of similarities; 95.2 to 99.7% and 97.9 to 99.8% respectively. Nevertheless, the phylogenetic trees derived from all 3 data sources, 16S rDNA, ITS and 23S rDNA, were in close agreement. Recently, Reid & Birkbeck (2003) compared 16S rDNA and ITS sequences from Scottish and Irish isolates and found that the Scottish isolates formed a homogenous group which was closely related to the Chilean isolates, LF-89 and SLGO-94. Irish isolates from 1999 clustered with EM-90, hitherto the most distantly related strain, while Irish isolates from 1991, 1995 and 1998 formed a new divergent group. However, while these groups found only a single *rrn* operon, Casanova et al. (2001), using different PCR primers, identified 2 ITS sequences in the type-strain, LF-89 and in EM-90, by polyacrylamide gel electrophoresis of PCR amplified ITS region DNA. One ITS sequence was the same as that described by Mauel et al. (1999), while the second ITS contained genes for $tRNA^{ile}$ and $tRNA^{ala}$. Based on this observation, it was proposed that more than one *rrn* operon might exist in these Chilean isolates.

In this study, and in work by Steiropoulos et al. (2002), serological analysis had established the likelihood of a relationship between the organism found in European sea bass and those organisms identified as *Piscirickettsia salmonis* from Chilean, Canadian and Norwegian salmonids. However, detailed analysis of genetic similarities has not previously been undertaken. It has been confirmed, through phylogenetic analysis of 16S rDNA and ITS sequences, that the organism found in European sea bass is a member of the *P. salmonis* genus which clusters with other members of this genus for which sequence information is available. Sequence similarity between the sea bass 16S rRNA gene and the *P. salmonis* type-strain is 98.2%, which compares well with sequence similarities of the Canadian and Norwegian strains. The greater variability of the 16S-23S spacer region is reflected in the lower sequence similarity of the ITS_0 sequence, but this does not affect the overall placement of the SBPLO isolate within the range of ITS sequence similarities exhibited among the currently available *P. salmonis* sequences. Although it is more divergent than the Scottish isolates and Irish isolates from 1999, both sequence similarity and dendrograms for 16S rDNA and ITS sequences suggest that the SBPLO isolate is more closely related to the majority of *P. salmonis* strains than the Irish isolates from 1991 and 1998.

The present study was conducted on a single isolate from one site in Greece. A more robust phylogenetic analysis will require the availability of further isolates from sea bass in the Mediterranean to determine whether or not these form a separate cluster within the *Piscirickettsia salmonis* group. Detailed comparisons of the tRNA-containing ITS sequence between *P. salmonis* strains may yield useful information about the geographical spread of the organism, while sequence comparisons of other genes coding for shared antigens, evident from serological analysis, may help to further elucidate relationships among the group. Ultimately, DNA-DNA hybridisation studies may suggest the requirement for creation of separate species.

The finding of RLOs in widely differing fish species, from numerous locations world-wide, suggests that these are ubiquitous parasites adapted to utilise whatever hosts are available locally. As with other pathogens, it will be important to determine whether it is environmental changes or the availability of naïve hosts which result in reduced host immunity or increased pathogenicity (host susceptibility), leading to the establishment of conditions which encourage disease outbreaks. Given the rapidly expanding aquaculture industry, with the frequent introduction of new species for farming, it seems likely that PLOs may, in the future, pose disease problems. As antibiotic treatment of disease caused by this intracellular pathogen is both costly and relatively inefficient (Fryer & Hedrick 2003), successful combatting of this disease will rely largely upon the availability of vaccines. Therefore, it will be crucial to understand the mechanisms the organism uses to evade the host immune system and, using this knowledge, develop more effective vaccines.

Acknowledgements. The authors thank D. Faichney and M. Menzies, Institute of Aquaculture, University of Stirling, for preparation of samples for histology. We thank Dr. H. Whittaker, S. Powell and J. Costa for their kindness in providing sequencing gels and data extraction. We also thank Drs. W. Starkey, M. Cairney and J. Taggart for their advice on PCR and sequencing parameters and A. Holzer for her generous help and advice on phylogenetic analyses. This work was supported by a CASE award from the Faculty of Natural Science, University of Stirling, with Schering-Plough Aquaculture as industrial partners.

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Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: May 17, 2004; Accepted: November 2, 2004
Proofs received from author(s): February 11, 2005