BACTERIAL KIDNEY DISEASE AND ITS EFFECT ON THE SALMONID IMMUNE RESPONSE

By

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Renibacterium salmoninarum, the etiological agent of bacterial kidney disease (BKD) of salmonid fish, is a pathogen of great concern among fisheries and the aquaculture industry worldwide. Previous investigations have indicated the pathogenesis of BKD is complex. It is a chronic, multisystemic, granulomatous disease with a number of potential immunomodulatory effects on the host. Given the current limitations for treatment and control of BKD, it is imperative that the pursuit of development of methods of prevention, namely management strategies and vaccination, be continued. To do so, the immunology of BKD must be elucidated in order to better understand and manipulate the associated immune responses to our advantage.

This dissertation is composed of four chapters which relate to BKD and the associated immune responses of three species of susceptible salmonid fish as follows:

Exogenous stress factors, through stress-induced immunosuppression, have been shown to influence BKD development in cultured salmonids. Chapter 1 examines the effects of two environmental stressors common to fish culture, overcrowding and overfeeding, as they affect BKD development and *R. salmoninarum* antigen prevalence among juvenile chinook salmon (*Oncorhynchus tshawytscha*).

Immunomodulatory interaction between pathogen and host in BKD is widely reported and merits further investigation. Particularly, the immunological parameters affected and the role of the extracellular protein (ECP) of *R. salmoninarum* are of interest. Chapter 2 examines the *in vivo* immune response of rainbow trout (*Oncorhynchus mykiss*) following exposure to the ECP in terms of both humoral and cell-mediated immunological parameters, including the immune response against another bacterial pathogen. Chapter 3 addresses the *in vitro* effects of the ECP upon specific splenic immunocyte functions, phagocytosis and respiratory burst activity, in brook trout (*Salvelinus fontinalis*).

The immune-complex mediated hypersensitivity reported to occur with BKD has considerable ramifications for control measures involving immunostimulation via antigen exposure. Further investigation is warranted to discern the significance and consistency of immunological hypersensivity in BKD pathogenesis. Chapter 4 examines the renal lesions, including immunopathologic changes and indications of immune-mediated disease, of brook trout exposed to *R. salmoninarum*.

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List of Abbreviations

ANOVA	Analysis of Variance
ATCC	American Type Cell Culture
BKD	Bacterial kidney disease (of salmonid fish)
DCF	2',7'-dichlorofluoroscein
DCF-DA	dichlorofluoroescin diacetate
DCFH	2',7'-dichlorofluoroscene
DFAT	direct fluorescent antibody test
ECAM	enteric coated antigen microspheres
ECP	extracellular protein
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimal Essential Media
ERM	Enteric redmouth disease
FALS	forward angle light scatter
FAT	fluorescent antibody test
FDA	U.S. Food and Drug Administration
FITC	fluoroscein isothiocyanate
Ig +	immunoglobulin positive
Ig -	immunoglobulin negative
INAD	Investigational New Animal Drug
IP	intraperitoneal
KDM2	Kidney disease media-2 (*R. salmoninarum* culture)
KDM-C	Kidney Disease Media-Charcoal
MFAT	membrane filtration fluorescent antibody test
MSA	major soluble antigen
MS-222	tricaine methanesulfate
OD	optical density
p57	57 kilodalton antigen of *R. salmoninarum*
PAS	Periodic acid-Schiff (histological stain)
PBS	phosphate-buffered saline
PBS-T20	phosphate-buffered saline with 0.05% Tween 20
PCR	polymerase chain reaction
PMA	phorbol-12-myristate 13-acetate
PMN	polymorphonuclear cell
SALS	side angle light scatter
TUF	target unmasking fluid

Foreword:

The key to discerning the pathogenesis of any infectious disease does not lie solely with an understanding of either the host or the pathogen, but rather within the realm of their interaction. It is for this reason that the study of immunology is so pivotal in medical science. It is the purpose of this dissertation to utilize this immunological approach to describe bacterial kidney disease (BKD) of salmonid fish. The chapters/manuscripts incorporated into this dissertation all attempt to describe some aspect of the interactions between the pathogen *Renibacterium salmoninarum* and its salmonid host. More specifically, the chapters all address, directly and indirectly, some aspect of the salmonid immune response to *R. salmoninarum* and discuss how this response contributes to the pathogenesis of BKD. The goals of doing so are largely twofold. First and foremost, by defining the immunologically mediated aspects of the host-pathogen interaction, we will be better equipped to manipulate this interaction advantageously. Therefore, the development of practical means of disease control involving immunomodulation would be enhanced. Secondly, this dissertation will hopefully contribute to our general knowledge base of comparative vertebrate immunopathology. By further describing aspects of the salmonid immune response to *R. salmoninarum*, we may enhance our understanding of salmonid immunity as it relates to the evolution of the vertebrate immune system.

Literature Review

A. Historical Perspective

Bacterial kidney disease (BKD) is a multisystemic, granulomatous disease of cultured and feral salmonids worldwide. The causative agent is *Renibacterium salmoninarum*, a Gram-positive diplobacillus. Bacterial kidney disease was first reported over sixty years ago with one of the earliest known outbreaks occurring in feral salmon during the spring/summers of 1930 through 1932 in the Aberdeenshire Dee and the River Spey of Scotland (Smith, 1964). In 1934, an outbreak attributed to BKD also occurred in cultured trout at a hatchery in Massachusetts (Belding and Merrill, 1935). Subsequently, other outbreaks among Pacific Northwest salmon stocks and eastern trout stocks in the U.S., as well as among salmonids in Europe, had been associated with the same as yet unclassified pathogen. Due to its morphology and physiology, the pathogen was initially classified as a *Corynebacterium* (Ordal and Earp, 1956).

By the latter half of the twentieth century, BKD (also known as Dee disease, salmonid kidney disease, corynebacterial kidney disease, white boil disease) was recognized as a significant disease among a wide variety of salmonid species across North America including the U.S.A. and Canada; Europe including the United Kingdom, Germany, Scandinavian nations, and Iceland; and Asia including Japan. Research focused on BKD had intensified along with the disease's global dissemination. Various aspects of BKD including pathogenesis, diagnosis, and control strategies were being widely investigated. Additionally, the taxonomy of the causative agent was also becoming more precisely defined. In 1980, the pathogen was reclassified to its own genus and named *Renibacterium salmoninarum* (Sanders and Fryer, 1980).

B. Characterization of *Renibacterium salmoninarum*

1. Taxonomy, Morphology, Physiology, and Artificial Culture

Taxonomic reclassification of the pathogen as *Renibacterium salmoninarum* was based on the organism's biochemical composition. Specifically, the peptidoglycan layer, cell wall polysaccharides, and the guanine + cytosine content all differed significantly enough from *Corynebacterium* and other established genera to warrant creation of the new genus *Renibacterium* (Sanders and Fryer, 1980). Stackebrandt et al. (1988) further described *Renibacterium* taxonomically as belonging to the Actinomycetes subdivision of the Coryneform group of bacteria; this study also revealed close similarities between the genus *Renibacterium* and the genera *Arthrobacter* and *Micrococcus*. *Renibacterium salmoninarum* is the only species thus far described in this genus.

Morphologically, *R. salmoninarum* is characterized as small, measuring approximately 0.4 x 0.8 µm (Bullock and Herman, 1988). The organism is strongly Gram-positive, non-motile, non-encapsulated, and non-spore forming. The shape is slightly pleomorphic. In artificial culture the bacteria tends to consistently appear as a diplobacillus (Fryer and Sanders, 1981), while cells obtained from host tissues are more prone to irregularities in shape, including clubbing (Young and Chapman, 1978). Distinctive features of *R. salmoninarum*'s biochemical composition, include a 53% guanine + cytosine content, peptidoglycan amino acids including lysine, alanine, glycine, and glutamic acid and a unique cell wall polysaccharide composition including galactose, rhamnose, N-acetyl glucosamine, and N-acetyl fucosamine (Fryer and Sanders, 1981; Fiedler and Draxl, 1986).

Physiologically, *R. salmoninarum* is described as fastidious and slow-growing, proteolytic, catalase-positive, and non-liquefactive for gelatin. *Renibacterium salmoninarum* is hydrophobic; this property represents a potential virulence factor in that it may increase cellular adhesion and uptake by host target cells (Daly and Stevenson, 1987). The organism has specific temperature requirements, growing best at 15°C, more slowly at 22°C, and not at all at 37°C. Nutritional requirements for growth are also limiting for artificial culture of the bacterium.

Culture of the organism was first achieved through the use of nutrient enhanced agar medium containing various animal tissue components (Earp et al., 1953). This type of medium containing serum or meat infusion generally yields appreciable growth of *R. salmoninarum* in approximately two weeks. Modification of the medium with 0.05 to 1% cysteine decreases this time span to 7-10 days (Fryer and Sanders, 1981). A further modification of a cysteine blood agar medium by Evelyn (1977) using the addition of peptone and the elimination of sodium chloride, tryptose, and beef extract is named Kidney Disease Medium-2 (KDM2) and further enhances the growth of *R. salmoninarum*. An additional modification resulted from a satellitism growth technique that evolved into medium supplementation with a metabolite product from spent *R. salmoninarum* cultures (Evelyn et al., 1990; Teska, 1994). Currently, the various modifications of KDM2 medium appear to be the most widely used medium for artificial growth of *R. salmoninarum*. Among these modifications are a selective KDM2 medium (Austin et al., 1983) and a charcoal based medium, KDM-C (Daly and Stevenson, 1985).

2. Antigenicity

The antigenicity of *R. salmoninarum* is described in terms of its relevance to both diagnostic parameters and disease pathogenesis. Various cell surface antigens and antigens

associated extracellularly with *R. salmoninarum* (i.e. free protein produced by *R. salmoninarum* found in the spent supernatant of broth cultures) have been identified. The most common antigen found on the cell surface and as an extracellular component is a 57 kilodalton protein (p57), also known as the major soluble antigen (MSA) or Antigen F (Getchell et al., 1985). Debreuil et al. (1990) identified this p57 antigen to be a cell surface fimbriae less than 2 nm in diameter. Various other antigens of higher and lower molecular weights are identified (Fiedler and Draxl, 1986; Getchell et al., 1985), and many are thought to be potential breakdown products of the p57 antigen (Bandin et al., 1992). The p57 antigen is generally regarded as unstable, with degradation occurring in association with elevated temperature and time. Griffiths and Lynch (1991) attributed this instability to autolysis. Still, other findings indicated that the p57 antigen is a heat stable protein (Getchell et al., 1985).

Renibacterium salmoninarum antigens, and particularly the p57 antigen, have been instrumental to the evolution of diagnostic methods for detection of BKD. Monoclonal antibodies developed against *R. salmoninarum* for serological tests recognize various epitopes of the p57 antigen (Weins and Kaattari, 1989) and have proven extremely useful in identifying the bacterium (Part D - Diagnostics) and characterizing its effects (Weins and Kaattari, 1991).

A number of physiological features of *R. salmoninarum*, including various virulence factors have been associated with its cell surface and/or extracellular protein (ECP). Autoaggregation of *R. salmoninarum* organisms and the agglutination of heterologous cell types, including rabbit erythrocytes and salmonid spermatozoa, have been attributed to the p57 antigen on the cell surface (Daly and Stevenson, 1989). Senson and Stevenson (1994) also related the autoagglutination ability of *R. salmoninarum* to the presence of cell surface p57. A variety of immunomodulatory effects associated with *R. salmoninarum* in BKD pathogenesis have also been related to the bacterial proteins. In keeping with the association of p57 with these various virulence factors, there is evidence that the overall virulence of *R. salmoninarum* is related to the presence of this antigen. Bruno (1990) identified the presence of p57 in extracts of virulent *R. salmoninarum* cells and its absence in similar extracts of avirulent cells.

Other recent research studied the structure of the p57 antigen through amino acid or codon sequencing techniques . Chien et al. (1992) described the complete sequence coding for this antigen, including indications of amino acid residues present in the precursor and mature proteins (557 amino acids, MW=57,190 D). Radacovici and Debreuil (1991) utilized enzymatic digestion

techniques and amino acid sequencing to evaluate the structure and antigenicity of fragments of the p57 protein.

C. BKD Pathogenesis

1. Susceptibility

A number of factors have significant bearing upon a salmonid host fish's susceptibility to BKD. This disease appears to be exclusively a disease of salmonids, as only salmonid fish, including Atlantic salmon (*Salmo salar*), a variety of species of Pacific salmon (*Oncorhynchus* spp.), trout (*Salmo* spp., *Salvelinus* spp.) and grayling (*Thymallus*) have been reported to be infected with *R. salmoninarum* and develop BKD (Kettler et al., 1986; Bullock and Herman, 1988). Attempts to challenge non-salmonid fish with *R. salmoninarum* are not well represented in the literature, although one such attempted challenge in the Pacific lamprey (*Lampetra tridentata*) did not show infection (Bell and Traxler, 1986). Experimental BKD infection by means of intraperitoneal injection was achieved with sablefish (*Anoplopoma fimbria*), although no naturally occurring infections are reported in this species (Bell et al., 1990).

Among the salmonid species, susceptibility to BKD differs significantly, with the Pacific salmon species being the most susceptible and rainbow trout (*Oncorhynchus mykiss*) the least (Evelyn et al., 1988 ,Bruno, 1988; Sakai et al., 1991). Genetic variability within a species may also yield intraspecific differences in susceptibility. For instance, Winter et al. (1980) reported a differential resistance to BKD among stocks of coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*) based upon genotype. Heritability estimates of BKD susceptibility among chinook salmon (*Oncorhynchus tshawytscha*) and Atlantic salmon (*Salmo salar*) indicated that the heritability component is greater for BKD than for some other bacterial diseases of these salmonid species (Beacham and Evelyn, 1992; Gjedrem and Gjoen, 1995). Genetic manipulation of stocks for BKD resistance, while promising, has not been frequently reported in the literature. There is no evidence of enhancement of BKD resistance through genetic manipulations of ploidy; Bruno and Johnstone (1990) found no differences in susceptibility between triploid Atlantic salmon and their diploid counterparts.

Age of fish appears to have some affect on BKD susceptibility, although juvenile and adult salmonids of all stages are vulnerable. Stress susceptibility and adequacy of host immunological capability are influenced by age and have a direct bearing upon subsequent BKD development. For instance, smoltification imposes a physiological stress which increases BKD susceptibility (Paterson et al., 1981b).

Bacterial kidney disease is largely regarded as a problem of cultured (hatchery) fish, although feral populations appear to be quite susceptible as well. Salmonid culture facilities have experienced losses of up to 80% of Pacific salmon stocks in British Columbia and up to 40% of Atlantic salmon stocks in Scotland; recurring BKD-related losses of approximately 10% of rainbow trout stocks per year were common for Scottish fish farms in the 1980's (Bruno, 1988). *Renibacterium salmoninarum* has been detected in feral salmonid populations worldwide, and BKD epizootics are likewise reported in feral fish (Mitchum et al., 1979).

A variety of extrinsic, i.e. environmental, factors also significantly influence a salmonid's susceptibility to BKD. Temperature is an environmental parameter which impacts upon BKD development, as it affects both the virulence of the pathogen and the immunological response of the host. Wolf and Dunbar (1959) described an increased virulence of *R. salmoninarum* in brook trout (*Salvelinus fontinalis*) and rainbow trout at 7^{o}C in contrast to 12.5^{o}C. Sanders et al. (1978) found that BKD-related mortality decreased among coho salmon and steelhead trout as water temperatures increased above 12.2^{o}C. More recently, Ortega et al.(1994) reported a correlation between decreasing water temperature and decreased mortality from BKD among rainbow trout. Similarly, BKD development and occurrence of epizootics vary seasonally, presumably related to fluctuations in water temperature (Belding and Merrill, 1935; Fryer and Sanders, 1981). Other water quality parameters including salinity are reported to affect BKD development. Significant levels of BKD mortality are noted to occur as anadromous salmonid smolts migrate from fresh to salt water (Banner et al.,1983; Banner et al.,1986; Paterson et al., 1981).

Nutritional parameters represent another extrinsic variable influencing BKD susceptibility among salmonids. Dietary supplementation with various trace minerals and vitamins is reported to substantially reduce prevalence of BKD in salmonid fish. Increased levels of iodine and fluorine have been associated with decreased BKD among rainbow trout and Atlantic salmon (Lall et al., 1985; Bowser et al., 1988). Possible correlations between other trace dietary constituents including iron, cobalt, copper, and manganese have been observed (Paterson et al.1981), and survival of sockeye salmon (*Oncorhynchus nerka*) with BKD was found to be inversely correlated with Vitamin C levels in the diet (Bell et al., 1984). In contrast, no relationship is evident between dietary levels of either Vitamin E or selenium and *R. salmoninarum* prevalence in chinook salmon (Thorarinsson et al., 1994).

2. Transmission

Renibacterium salmoninarum is transmitted directly, fish to fish, both horizontally and vertically. Horizontal transmission occurs through direct contact between infected, both diseased and

carrier, and uninfected individuals as well as through the consumption of infected tissue (Mitchum and Sherman, 1981; Wood and Wallis, 1955). Fecal-oral transmission also occurs as *R. salmoninarum* is excreted in the feces of infected host fish and may remain a viable inhabitant of organic waste in the water for up to 21 days (Austin and Rayment, 1985; Balfry, 1990). Reservoirs of *R. salmoninarum* for horizontal transmission may be clinically diseased individuals or subclinically infected carriers. Horizontal transmission via water-borne organisms in the absence of fish to fish contact has not been demonstrated and is thought to be unlikely (Kaattari et al., 1989). However, vertical transmission of *R. salmoninarum,* involving intra-ovum infection of the egg and subsequently the developing progeny, seems to be the more significant mode of naturally occurring infection (Evelyn et al., 1986a; Evelyn et al., 1984).

No specific vectors or non-salmonid reservoirs have as yet been identified in association with *R. salmoninarum*. The blue mussel, *Mytilus edulis*, is reported to clear seawater of *R. salmoninarum*, yet this mollusk does not appear to maintain viable organisms as a reservoir source(Paclibare et al., 1994). However, poor husbandry or sampling techniques used with salmonids may provide a source of mechanical vectors enhancing horizontal transmission of *R. salmoninarum*. For instance, Elliott et al. (1994) showed that coded-wire tagging of salmon may transmit the pathogen via contaminated needles.

3. Incubation and Dissemination

Time from initial exposure to fulminate clinical disease is variable (up to several months) depending on numerous conditions. Incubation varies with the virulence of the pathogen and the immunocompetence of the salmonid host, both of which may be influenced by environmental variables (water temperature, salinity etc.) as previously described. Although BKD is generally regarded as a chronic disease, acute epizootics are not uncommon, and a precise incubation period has not been defined.

Initial entry of the *R. salmoninarum* pathogen into the host is known to occur through the skin or mucous membranes such as the eye or gastrointestinal tract. The means of subsequent dissemination of the pathogen has not been determined, although it is likely that the pathogen travels through the host either intracellularly in phagocytes or extracelluarly within the circulatory system and other body fluids (Evendon et al., 1993). Whichever the case, rapid systemic dissemination following exposure is likely. Bruno (1986a) showed *R. salmoninarum* intracellularly in the kidney and spleen of host fish less than 45 minutes after exposure via intraperitoneal injection.

4. Pathology

Pathological changes in host tissue resulting from BKD infection are potentially quite severe and multisystemic. While inflammatory processes are undoubtedly responsible for these various lesions, several theories exist regarding the pathogenesis of lesions. Various explanations include: bacterial cytotoxins and/or proteolytic factors which are released from the pathogen damage host tissues; mechanical damage to host cells is caused by the bacteria, triggering the extracellular release of cell contents including enzymes and inflammatory mediators; and immunological hypersensitivity reactions, in which the host immune system attacks autologous tissue, are initiated by *R. salmoninarum* antigen (Evendon et al., 1993; Young and Chapman, 1978; Sami et al., 1992). It is quite possible that some combination of these scenarios occurs. The relative contribution from each mechanism may therefore partially explain the variability encountered in the clinical and pathological manifestations of BKD.

Gross external lesions in *R. salmoninarum*-infected fish are often variable, but may include some combination of the following: petecchial hemorrhages, particularly around the base of fins or external orifices; unilateral or bilateral exophthalmia; skin blebs or blisters (filled with bacteria and serous, serosanguinous, or purulent fluid) which may eventually rupture and/or coalesce to form large ulcerative lesions; skin abscesses; altered skin pigmentation; and abdominal distension due to free fluid accumulation in the body cavity.

Gross internal lesions may likewise be varied, but often consist of: diffuse swelling of internal organs, particularly the liver, spleen, and kidney; focal abscesses and/or diffuse purulent exudate from internal organs, particularly the liver, spleen, and kidney; petecchial hemorrhage on internal organs and the muscle of the body wall; discoloration of internal organs, usually manifesting as a pale, mottled liver, reddened spleen, and grey to white kidney; pseudomembranous covering of various internal organs; and abundant serous/serosanguinous fluid accumulation in the body cavity (Evendon et al., 1993; Bullock and Herman, 1988; Fryer and Sanders, 1981).

Histologically, BKD pathology also shows considerable variability, but is generally associated with signs of chronic inflammation. The hallmark of BKD histopathology is multisystemic, diffuse granulomatous inflammatory lesions (Fryer and Sanders, 1981). Granuloma formation may occur in various tissues and is accompanied or preceded by cellular tissue infiltration by macrophages, neutrophils, and thrombocytes. Also evident are tissue edema and degeneration of tissue structures such as renal tubules or glomeruli, culminating in multifocal necrotic centers.

The organs most commonly and severely affected include the liver, spleen, excretory kidney, and hematopoietic kidney. Additionally, the heart, gonad, gill, swimbladder, gastrointestinal tract including the pancreas, and central nervous system including sensory organs are frequently reported to show lesions induced by BKD. Extensive necrotizing myocarditis, mild to severe meningoencephalitis/ventriculitis, and similar inflammatory changes are described in these other organ systems as well (Bruno, 1986a; Speare et al., 1993). In all affected tissue, numerous *R. salmoninarum* organisms are frequently encountered both intracellularly and extracelluarly. An immunological mechanism for induction of renal lesions has been described in the literature (Section F-Immunology).

Ultrastructural lesions are consistent with the cellular damage commonly associated with severe acute to chronic inflammation. Cellular changes in BKD-induced lesions include swelling and rupture of mitochondria, dilation of endoplasmic reticulum, fatty infiltration of cytoplasm, lysosomal and nuclear morphological alterations, vacuolization, collagen deposition along basal borders, hyaline granule accumulation (renal tubule cells), and eventual disruption of cellular membrane integrity (Young and Chapman, 1978).

The morphological evidence of disease in BKD is associated with a variety of physiological alterations. BKD is associated with changes in hematology parameters including decreased hematocrit, decreased hemoglobin, decreased red cell diameter, and decreased ratio of mature to immature erythrocytes (Bruno and Munro, 1986; Suzumoto et al., 1977). This BKD-induced anemia is presumably due to erythrocyte retention in the spleen. Additionally, a leukocytosis characterized by monocytosis and neutrophilia as well as a thrombocytosis, were noted in association with BKD (Bruno and Munro, 1986). Changes in serum chemistry parameters are also reported in salmonids with BKD. These changes include hypoproteinemia, hypocholesterolemia, hyponatremia, hyperkalemia, hypoglycemia, and hyperbilirubinemia (Bruno, 1986b; Fryer and Sanders, 1981). The decreased serum protein levels are presumed to be related to either renal or hepatic impairment (i.e. excessive protein excretion or diminished protein synthesis). The hyperbilirubinemia is likely attributable to liver dysfunction. The electrolyte imbalance may be related to either extensive multisystemic cellular damage and subsequent disruption of cellular cross-membrane electrolyte gradients or to primary renal failure. In any event, these imbalances contribute significantly to alterations in intra/extravascular hydrostatic pressures and fluid balances, resulting in further physiological dysfunction including free fluid accumulation in extravascular spaces and circulatory compromise (Bruno, 1986b). Severe fluid imbalance and circulatory

failure, as well as vital organ dysfunction (primarily renal), are postulated to be the most significant causes of death from BKD (Hayakawa et al., 1989; Evendon et al., 1993).

D. Diagnosis

The detection of *R. salmoninarum* and diagnosis of BKD have been greatly refined over the past few decades. Historically, early diagnosis of BKD was accomplished through correlating observed clinical signs with positive identification of Gram-positive diplobacillary organisms in affected tissues. Subsequently, refined culture techniques allowed for bacterial isolation in artificial media to help confirm the presence of *R. salmoninarum*. These diagnostic methods, however, were often impractical and inconclusive, as Gram-staining does not preclude other coryneform bacteria and successful isolation of *R. salmoninarum* in culture medium may take over three weeks under the best of circumstances (Fryer and Sanders, 1981). Therefore, even though bacterial isolation by culture has proven quite reliable diagnostically, the time requirement makes this an undesirable option for diagnosis when used alone.

A variety of serological techniques have greatly improved the ease and reliability of BKD diagnosis. Chen et al. (1974) developed an immunodiffusion test to detection soluble antigen of *R. salmoninarum* in infected host tissue. Similarly, Kimura (1978) described a *Staphylococcus* coagglutination technique for detecting *R. salmoninarum* antigen in tissues. Bullock and Stuckey (1975) described a fluorescent antibody technique (FAT) developed against the cell surface antigen of *R. salmoninarum*. This test has proven to be a significant advancement in BKD diagnostic capability as it improved upon speed and sensitivity (Fryer and Sanders, 1981). A number of variations upon this indirect FAT have proven quite useful in clinical and research related diagnosis. These include the direct FAT (Bullock et al., 1980), a quantitative FAT (Cvitanich, 1994), a membrane filtration FAT (Elliott and Barila, 1987), and a technique for immunofluorescent detection of *R. salmoninarum* antigen in fixed, paraffin-embedded host tissue (Evenson, et al., 1994).

Pascho and Mulcahy (1987) made another formidable contribution to BKD diagnostics with the development of the enzyme-linked immunosorbent assay (ELISA) for detection of *R. salmoninarum* antigen. Further development of this test procedure, including the use of a monoclonal anti-*R. salmoninarum* antibody (Hsu et al., 1991) and the dot-blot ELISA (Sakai et al., 1987) increased its specificity, sensitivity, and practical application. Still other

serological techniques including counterimmunoelectrophoresis (Cipriano et al., 1985), immunohistochemistry of melanin-containing cells (Jansson et al., 1991), Western blot, and the immunoelectrotransfer blot (Olivier et al., 1992) are also used to detect *R. salmoninarum* antigen in infected host tissues. Comparatively, the serological techniques which identify soluble antigen of *R. salmoninarum* appear to be more sensitive than the various FAT techniques (Griffiths et al., 1991; Cipriano et al., 1985), although both of these diagnostic options are far more reliable than diagnoses based upon clinical signs, histopathology, and/or Gram-staining, particularly in asymptomatic fish. The use of monoclonal antiserum, as opposed to polyvalent anti-*R. salmoninarum* serum, also improves serological sensitivity and specificity. However, cross-reactivity with other micro-organisms is not entirely eliminated, even with the monoclonal preparations (Bandin et al., 1993c). In light of the complementary strengths and shortcomings of the various diagnostic techniques available for BKD, perhaps the most desirable approach would involve multiple techniques incorporating serology, bacterial isolation, and histology (Teska et al., 1995; White et al., 1995).

Other promising approaches to BKD diagnostics involve the use of genetic probes and gene amplification techniques. Both DNA and RNA probes have been developed for *R. salmoninarum* and are being used successfully to identify bacteria in host tissues (Leon et al., 1994a; Magnusson et al., 1994). Gene amplification, namely the polymerase chain reaction (PCR), is widely used in conjunction with genetic probes to further enhance test sensitivity (Leon et al., 1994b; Brown et al., 1994). Genetic probe diagnosis of BKD has the advantages of increased sensitivity and specificity over serological methodologies without sacrificing the component of time, as occurs with bacterial isolation techniques (Hariharan et al., 1996; Butendieck et al., 1995).

E. Control

1. Management/Husbandry

Control measures for BKD include a number of approaches. Various factors associated with management technique and animal husbandry have proven to be effective inhibitors of *R. salmoninarum* infection and disease development. Environmental parameters which act as risk factors in BKD pathogenesis, i.e. water temperature and water chemistry, may be advantageously manipulated in fish culture situations to decrease both the impact of nonspecific environmental stress and the pathogen's virulence. Nutrition may also serve as a means of BKD control as dietary modifications, particularly with regard to the trace mineral and vitamin components previously described, may diminish susceptibility of salmonids to BKD.

Likewise, genetic manipulation of stocks may be undertaken to decrease overall host susceptibility to BKD.

More specifically, impairing transmission of *R. salmoninarum* from fish to fish is an effective means of disease control. Historically, a vast reduction of the level of horizontal transmission was accomplished in the 1960's with the advent of pasteurization of fish feeds containing *R. salmoninarum*-infected salmonid tissue (Fryer and Sanders, 1981). Horizontal transmission among live fish may likewise be reduced through the utilization of water ozonization or chlorine-based disinfectants in hatcheries. (Austin, 1983; Pascho et al., 1995), as would minimizing the level of organic debris in the water of culture facilities (Austin and Rayment, 1985).

Reduction of vertical transmission by husbandry-related modifications have also been shown to be effective means of BKD control. Brood stock segregation based on *R. salmoninarum* antigen prevalence among adult salmonids has been shown to dramatically reduce the levels of antigen prevalence and BKD-related mortality among progeny (Pascho et al., 1991; Elliott et al., 1995). Similarly, Aedo and Bustos (1991) described a technique for brood stock segregation and aseptic spawning effective for obtaining *R. salmoninarum*-free fertilized eggs. Disinfection techniques commonly employed in spawning do not, however, appear to be a suitable control of vertical transmission (Evelyn et al., 1984).

2. Chemoprophylaxis/Chemotherapeutics

The use of antibiotics as preventative or treatment for BKD has met with the most success of any control measure. In the early 1950's, sulfonamide antibiotics incorporated into the diet were used to control BKD, but long term elimination of *R. salmoninarum* from the salmonid population could not be achieved with these bacteriostatic therapeutants (Elliot et al., 1989). Wolf and Dunbar (1959) examined the efficacy of ten antibiotics in treating BKD, and found the most satisfactory therapeutic regime was erythromycin at 100 mg/kg/day for 21 days. Austin (1985) similarly evaluated over 70 antibiotics for efficacy against BKD and found a few (clindamycin, kitasamycin, penicillin G, spiramycin, and erythromycin) to be effective in the treatment of clinical BKD in the earlier stages. Additionally, cephradine, lincomycin, and rifampicin were found to be satisfactory chemoprophylactic agents. This study also reported that a 10 day treatment regime with erythromycin was satisfactory, as compared to the previously advocated 21 day regime, noting that decreasing the environmental leaching of antimicrobials wherever possible would decrease the risk of selecting for antibiotic-resistant microflora. More recently, enrofloxacin has been shown to be an effective chemotherapeutant against BKD (Hsu et al., 1994). Overall,

erythromycin is widely regarded as the antimicrobial of choice in the control of BKD.

Erythromycin chemotherapy/chemoprophylaxis poses a number of important considerations. The route of administration is a crucial variable. The efficacy of oral administration of erythromycin is questionable, because of the low palatability in diets (Moffitt, 1992). Limited study has been devoted to immersion therapy, and this route of administration has not thus far been proven satisfactory (Elliott et al., 1989). Parenteral injection was shown to be efficacious in numerous studies, and appears to be the most widely used and advocated route of administration(Elliott et al., 1989; Evelyn et al., 1986b; Armstrong et al., 1989; Lee and Evelyn, 1994; Brown et al., 1990).

The age of the salmonid during erythromycin administration is crucial to treatment efficacy. Treatment of salmonid eggs with erythromycin prior to water-hardening was shown to be ineffective in eliminating *R. salmoninarum* from progeny (Evelyn et al., 1986a; Bruno and Munro, 1986). Parenteral erythromycin injection of adult brood stock prior to spawning, however, appears to be quite effective in the prevention of vertical transmission of *R. salmoninarum* (Evelyn et al., 1986b; Armstrong et al., 1989; Brown et al., 1990; Lee and Evelyn, 1994). The pre-spawning timing of therapy is also important, with the last parenteral injection given 34 to 56 days pre-spawning being the most efficacious (Evelyn et al., 1986b).

Antibiotic, particularly erythromycin, chemotherapy in the control of BKD is not without its disadvantages. There is the potential of creating iatrogenic lesions with erythromycin use in salmonids, including renal tubular lesions and lysosomal congestive overloading. These histological changes induced by therapeutic levels of erythromycin have, thus far, been shown to be largely reversible (Hicks and Geraci, 1984). The non-approved status of most chemotherapeutants, including erythromycin, in fish culture is also restrictive, although there is the possibility for INAD approval by the FDA when applicable (Moffitt, 1991). FDA restrictions imposed on the use of erythromycin, along with other therapeutic agents, is, however, certainly warranted, as concerns over the development of antibiotic resistant microbes are quite relevant to fish culture. Bell et al. (1988) already reported the development of an erythromycin-resistant strain of *R. salmoninarum*, a serious finding given the limited availability of current alternatives for BKD control.

3. Immunization

Prophylactic control of BKD through immunization has been the focus of considerable research effort, especially over the past decade. Passive immunity against BKD with serum from BKD-challenged survivors was shown to diminish BKD-related mortality (Campbell et al., 1994), but this concept is not feasible for large scale prophylactic application. Active immunization, primarily through the stimulation of humoral immune responses, has been attempted on a larger scale, but produced much conflicting data. Preparations include bacterins (heat killed, pH lysed, formalin inactivated) with and without adjuvants incorporated into monovalent or multivalent vaccines (Elliott et al., 1989). Various routes of administration utilized include direct immersion, hyperosmotic infiltration, and parenteral (intraperitoneal) injection (McCarthy et al., 1984; Paterson et al., 1981a), but none have proven conclusively to be beneficial. In one study (Sakai et al., 1989), intraperitoneal injection of a bacterin produced an agglutinating antibody titer and increased phagocytic activity of immunocytes, but subsequent experimental challenge demonstrated a lack of protective immunity. However, oral administration of a BKD preparation via enteric coated antigen microspheres (ECAMs) was associated with decreased levels of *R. salmoninarum* protein, indicating potential efficacity (Piganelli, 1995).

F. Immunology

The immunological reaction of a host salmonid to *R. salmoninarum* is integral to BKD pathogenesis. A unique feature of BKD which is pertinent to its complex immunopathology is the potential immunomodulatory effect of *R. salmoninarum* upon the host. Immunosuppression associated with BKD is described by a number of investigators. The leukoagglutination of salmonid pronephric, splenic, and circulating leukocytes and decreased antibody responses related to exposure to the p57 antigen *in vitro* are reported by Kaattari et al. (1989). Iwama and Greer (1980) showed that susceptibility to the toxic effects of a non-infectious agent, sodium pentachlorophenate, increased in juvenile coho salmon following exposure to *R. salmoninarum*. Alternatively, *R. salmoninarum* may act as an immunostimulant. Amend and Johnson (1984) suggested that *R. salmoninarum* potentiates humoral protection against *Aeromonas salmonicida* when administered as a bivalent bacterin.

Salmonid defenses against *R. salmoninarum* involving non-specific immunity have not been widely investigated. Neutrophilia is reported in association with BKD (Bruno and Munro, 1986), but neutrophil-mediated effects are not described in any detail.

Literature regarding non-specific acellular defenses such as complement and acute-phase proteins is also sparse. Rose and Levine (1992) showed a depletion of human and piscine complement in the presence of *R. salmoninarum*, suggesting that the alternative complement pathway and bacterial opsonization are involved in BKD pathogenesis. Additionally, the presence of complement *in vitro* is associated with increased intracellular survival of phagocytosed *R. salmoninarum* (Bandin et al., 1995). Decreased serum protein levels are also associated with clinical BKD (Bruno, 1986).

As previously alluded to (Part E-Control/Immunization),the humoral immune response in BKD is described in a number of investigations, yet it is questionable as to whether this response is truly protective. McCarthy et al. (1984) evaluated a variety of bacterins (formalin and pH inactivated at different concentrations) and routes of vaccination (hyperosmotic, immersion, and intraperitoneal injection) to determine efficacy against challenge infection; they concluded that the resulting humoral immunity was dependent upon the type of bacterin and route of administration; protective immunity was demonstrated with intraperitoneal injection of formalin inactivated *R. salmoninarum* bacterins, but not with the other routes. Paterson et al. (1981a) also found intraperitoneal injection to be far more efficacious a vaccine route than hyperosmotic immersion. This study also concluded that while the two year secondary immune response to *R. salmoninarum* in Atlantic salmon vaccinated as pre-yearling parr was poor, post-yearling parr vaccinated with *R. salmoninarum* cells and Freund's complete adjuvant (FCA) had decreased BKD-related lesions compared to controls. Alternatively. Sakai et al. (1993) identified a humoral response to *R. salmoninarum* in rainbow trout following vaccination with various bacterin preparations, but this response was not found to be significantly protective under any conditions. Additionally, Bruno (1987) found a lack of correlation between the level of agglutination titer and level of *R. salmoninarum* infection in Atlantic salmon. Other investigators (Lovely et al., 1994; CaboBravo, 1993) found that asymptomatically infected fish may have low to absent antibody titers. Collectively, investigations describing humoral immunity in BKD suggest that the humoral response to *R. salmoninarum* infections in salmonids does not consistently, if at all, provide protective immunity, and, in low level infective states, may not produce detectable antibody titers. Wood and Kaattari (1996) described an enhanced humoral response in chinook salmon, directed against the carbohydrate moieties of *R. salmoninarum* cells, associated with removal of the p57 antigen. This increased immunogenicity of *R. salmoninarum* without p57 lends further support to the proposed immunomodulatory nature of this antigen.

In light of these developments regarding the role of humoral immunity, the cell-mediated immune response is emerging as a significant factor in BKD pathogenesis and, ultimately, as a focus in BKD control. *Renibacterium salmoninarum* may be likened to other pathogenic bacteria such as *Mycobacterium*, *Listeria*, and *Legionella* based upon their ability to thrive intracellularly in host tissue. Immunologically, this intracellular existence is quite significant in BKD as it provides a relatively protected environment for the pathogen from the host's defenses. In addition, the intracellular location of the pathogen indicates a more prominent role of cell-mediated immunity in the disease pathogenesis (Kaattari et al., 1989). In particular, *R. salmoninarum* appears to have a tendency to locate intracellularly in salmonid phagocytes, namely macrophages (Young and Chapman, 1978). Not only does *R. salmoninarum* possess the ability to avoid host killing mechanisms and thereby survive intracellularly in salmonid phagocytes (Bandin et al., 1993a), but many investigators note the likelihood that the pathogen also multiplies intracellularly (Bandin et al., 1993b; Klein, 1992). Logistics of the intracellular existence of *R. salmoninarum* are not fully elucidated. It is theorized that the virulence factors associated with the p57 cell surface protein may contribute as the p57 protein may serve as a fimbral adhesion molecule (Dubreuil et al., 1990). The means by which *R. salmoninarum* gains entry into the cell are also undetermined; postulated mechanisms include endocytosis, Fc receptor binding (Evendon et al., 1993), and C3b receptor binding (Rose and Levine, 1992).

It is also postulated that *R. salmoninarum* directly interferes with the host's killing mechanisms, especially phagocytic respiratory burst activity, in order to avoid the host's immune defenses (Bandin et al., 1993b). Interestingly, this effect of decreased respiratory burst activity in the presence of *R. salmoninarum* appears to be related to a fish's susceptibility to *R. salmoninarum* infection, probably reflecting the decreased capacity of the host immune system to kill the bacteria (Sakai et al., 1996). Conversely, Hardie et al. (1996) showed that macrophage stimulation with macrophage activating factor(MAF) could reverse the depleted respiratory burst activity and inhibit *R. salmoninarum* growth following *in vitro* exposure to the pathogen.

Histologically, the affinity of *R. salmoninarum* for phagocytic cells and the importance of cell-mediated immunity in BKD is evidenced through the localization of *R. salmoninarum* organisms in hematopoietic tissues in association with melanomacrophage centers and sinusoidal-lining cells (Flano et al., 1996). Other typical pathological findings associated with BKD reflect a chronic, granulomatous and necrotizing disorder, further emphasizing the

importance of cell-mediated defenses (Evendon et al., 1993; Bruno, 1986). Investigations in vaccine development provide additional evidence supporting the importance of cell-mediated immunity in BKD pathogenesis, as the most efficacious experimental vaccine preparations have incorporated FCA, an immunostimulant which targets the cell-mediated immune response (Kaattari et al., 1989).

Immunological hypersensivity, the exaggerated response to a foreign agent resulting in autologous tissue damage, is another prominent feature of BKD pathogenesis. Young and Chapman (1978) described ultrastructural changes of the renal glomeruli including the appearance of flocculent deposits associated with the glomerular basement membrane and the mesangium, mesangial proliferation, thickening and irregularity of the basement membrane, and extensive inflammatory cell invasion. Their investigation noted the pathological similarities between BKD and mammalian glomerulonephritis and nephrotic syndrome which are characteristic of a Type III (immune complex deposition) hypersensitivity. Sami et al. (1992) described a chronic membranous glomerulonephritis in experimentally *R. salmoninarum*-infected rainbow trout with similar glomerulopathy and also identified immunofluorescence along the glomerular basement membrane, attributing it to immune complex deposition. These observations are supported by the work of Kaattari et al. (1989) which identified soluble immune complexes in the serum of coho salmon experimentally infected with *R. salmoninarum*.

The possibility of a Type IV (delayed, cell-mediated) hypersensitivity associated with BKD is also described (Evendon et al., 1993). Bacterial kidney disease lesions, involving extensive tissue infiltration by mononuclear immunocytes, granulomatous lesions, and necrotic foci, are typical of pathology associated with Type IV hypersensitivity (Flano et al., 1996).

Given the importance of vertical transmission in BKD epidemiology, BKD in juvenile salmonids and the ontogenetic changes associated with disease pathogenesis are important considerations. This subject, however, has received comparatively little focus in the literature. Immunological tolerance has been addressed as a significant feature related to the ontogeny of the salmonid immune system and BKD; Brown et al.(1996) showed exposure of salmonid eggs to p57 to be subsequently associated with diminished humoral immune responses against p57 in fish hatched from these eggs. Fish infected vertically and exposed to *R. salmoninarum* antigen prior to the maturation of the immune system would likely be predisposed to develop an immunological tolerance to the pathogen, impeding any immunological defense to be subsequently mounted.

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Afterword/Introduction to the Chapters:

With current limitations on the use of chemoprophylactics and therapeutics in aquatic species, it is imperative that we look to other means to achieve successful control of BKD. Management/ husbandry techniques and vaccination rank foremost among the alternatives. To accomplish this, we first need to expand our knowledge base concerning the immunology of BKD in order that we may better manipulate these immune responses to our advantage. There are a number of aspects of BKD immunology which warrant further investigation, and a number of these specific areas are addressed in the following chapters of this dissertation.

In general, these chapters address the issues of BKD and the salmonid immune response in terms of:

1- The elucidation of the relationship of specific environmental factors to BKD pathogenesis.

Environmental parameters may physiologically depress immunological function and thereby increase a fish's susceptibility to diseases. As previously mentioned, many exogenous factors which influence BKD development in feral and/or cultured have been identified and may be controlled in salmonid husbandry to reduce the severity of BKD. High levels of morbidity and mortality are reported to occur among cultured juvenile salmonids infected vertically with BKD, and the extent to which management technique may reduce BKD-related losses in these stocks warrants further investigation. Chapter 1 of this dissertation examines the effects of two environmental conditions common to fish culture, overcrowding and overfeeding, as they effect BKD development and *R. salmoninarum* antigen prevalence among juvenile chinook salmon.

2- The identification of immunomodulatory factors involved in BKD pathogenesis.

A more precise determination of the immunomodulatory mechanisms involved in the interaction between *R. salmoninarum* and its salmonid host is needed. In particular, the immunological parameters which are affected and the role of the *R. salmoninarum* ECP in these interactions are of interest. Chapter 2 of this dissertation examines the *in vivo* immune response of juvenile rainbow trout following exposure to the ECP in terms of both humoral and cell-mediated immunological parameters, including the ability to mount a secondary immune response against another bacterial pathogen. Chapter 3 addresses the *in vitro* effects of the ECP upon splenic immunocyte functions, phagocytosis and respiratory burst activity, in brook trout.

3- The definition of hypersensitivity reactions in BKD.

Immune-mediated hypersensitivity, particularly immune-complex-mediated hypersensivity, in BKD has considerable ramifications for control measures involving immunostimulation via antigen exposure. Further investigation is warranted in order to discern the importance and consistency of immunological hypersensivity in BKD pathogenesis. Chapter 4 of this dissertation examines renal lesions, including immunopathology and indications of immune-mediated disease, of brook trout exposed to *R. salmoninarum* through the consumption of infected tissues.

Most assuredly, there is an extensive list of BKD/immunology related topics which require further elucidation. It is my hope that these chapters will contribute to the overall research efforts which are attempting to better discern the relationship between bacterial kidney disease and the salmonid immune response; thus, we may better understand, and ultimately gain a greater measure of control over, this disease among cultured and feral salmonids.

Chapter 1

Effects of stocking density and feeding rate upon *Renibacterium salmoninarum* prevalence and bacterial kidney disease among chinook salmon (*Oncorhynchus tshawytscha*).

Abstract

Two groups of chinook salmon(*Oncorhynchus tshawytscha*) fingerlings, the progeny of brood stock infected either asymptomatically or clinically with *Renibacterium salmoninarum*, were maintained for twelve weeks in nine experimental groups representing three different levels of stocking density (24 g/l, 40 g/l, and 48 g/l) and feeding rate (2.8%, 5.6%, and 8.4% body weight/day). Prior to and during the investigation, renal samples from fish from each group were evaluated for *R. salmoninarum* antigen prevalence by enzyme-linked immunosorbent assays (ELISA) and direct fluorescent antibody tests (DFAT). Water quality parameters, including dissolved oxygen and unionized ammonia levels, were monitored in all groups, and mortality and growth rate data were collected throughout the twelve weeks. No significant differences in antigen prevalence were detected among the experimental groups for each progeny group, indicating a lack of effect of the potential stressors related to stocking density and feeding rate in this investigation. There was, however, a significant difference in antigen prevalence between the two progeny groups. For the progeny of asymptomatic fish, there was an initial antigen prevalence proportion of 0.10 which decreased over the twelve weeks. For the progeny of clinically diseased fish, the initial proportion of prevalence was 0.50, increasing to 0.75 over the twelve weeks. For the latter progeny group, survival and growth rate analyses indicated greater survival and growth for fish exposed to the higher density/feeding rate combinations. Significant correlation between density level and water quality parameters was evident in both progeny groups. Low correlation was found for ELISA and DFAT results.

I. Introduction

Renibacterium salmoninarum, the etiological agent of bacterial kidney disease (BKD) of salmonid fish, is a small (0.5 x 1.0 µm) Gram-positive diplobacillus which infects cultured and feral salmonids. Pathogenesis is most often chronic and multisystemic, involving granulomatous lesions and widespread bacterial dissemination with intracellular localization. Disease transmission occurs horizontally from infected to uninfected individuals or via the ingestion of infected tissues. Intra-ovum transmission of the bacterium appears to be the primary mode of infection in the wild (Evendon et al., 1993) and is similarly of considerable importance in cultured fish (Bruno and Munro, 1986; Evelyn et al., 1986).

Control of BKD has been attempted with varying degrees of success via chemical prophylaxis/therapeutics and through management strategies. Many aspects of salmonid culture management can influence *R. salmoninarum* prevalence and clinical development among stocks. For instance, dietary components such as trace minerals and ascorbate have been shown to affect salmonid resistance to BKD (Elliott et al., 1989; Bowser et al., 1988; Lall et al., 1985). Suzimoto et al. (1977) also demonstrated that genetic manipulation of fish strains may directly affect their susceptibility to BKD. Research strongly supports brood stock segregation based upon *R. salmoninarum* antigen prevalence as a management technique which may significantly reduce the prevalence of *R. salmoninarum* in progeny and subsequently decrease the level of BKD-related mortality (Evelyn et al., 1986; Elliott et al, 1995). Given the limited scope of prevention and treatment options currently available for control of BKD in cultured fish, it is imperative that husbandry related factors be identified and managed to decrease the impact of BKD among salmonid stocks.

Severity of BKD is affected by environmental variables such as water temperature, salinity, and ionic composition (Wolf and Dunbar, 1959; Sanders et al., 1978; Evendon et al., 1993). These effects may be exerted through effects upon the pathogen, the host's immune response, or a combination of the two. Two other environmental variables that might influence the presence and severity of BKD are stocking density and feeding rate. Overcrowding of fish is a common problem in hatcheries and may be directly detrimental as it influences social interaction and physiological responses. High stocking densities/overcrowding have been associated with physiological alterations in salmonids including decreased thyroid hormone levels, decreased plasma protein levels, altered plasma cortisol levels, and decreased growth rates (Leatherland and Cho, 1985; Laidley and Leatherland, 1988; Pickering and Stewart, 1984). The correlation between high stocking densities and increased mortality is another indicator that overcrowding is a significant environmental influence

(Fagerlund et al., 1981; Whalen and LeBar, 1994). Overcrowding may be indirectly problematic as it contributes to poor water quality, namely through increased levels of nitrogenous waste and decreased dissolved oxygen content.

Overfeeding is another potential environmental parameter which may influence BKD development. Similar to the effects of overcrowding, overfeeding produces excessive amounts of organic debris as sediment and particulate matter in the water. Water quality parameters such as dissolved oxygen content, nitrogenous waste levels, and total suspended solids may be secondarily altered to the detriment of the fish.

In this study, the relationship between two environmental variables, stocking density and feeding rate, and BKD-related response variables including *R. salmoninarum* antigen prevalence, growth rate, and survival was examined in two populations of *R. salmoninarum*-infected chinook salmon (*Oncorhynchus tshawytscha*) fingerlings.

II. Materials and Methods

Fertilized eggs from two populations of chinook salmon from Lake Ontario and Lake Michigan were shipped to the National Fish Health Research Laboratory (Kearneysville, WV). Brood stock that produced eggs from Lake Ontario were previously diagnosed positive for *R. salmoninarum* through serological analysis, although no evidence of clinical BKD was present. These asymptomatic brood stock were tested for *R. salmoninarum* by the enzyme-linked immunosorbent assay (ELISA) with 45% of fish being positive and by the direct fluorescent antibody test (DFAT) with 1.7% of fish being positive. The brood stock that spawned the eggs from Lake Michigan were clinically affected with BKD as evidenced by lesions in kidney and spleen tissues. Eggs were hatched in Heath incubators, and fry were reared in troughs (total volume= 415 l) supplied with spring water (12.5^{o}C, total hardness= 240 ppm). Fish were maintained on a diet of commercial salmonid growth feed (Zeigler Brothers, Inc., Gardners, PA). Seven months after hatching, 100 fingerlings from the subclinically infected brood stock (average weight=3.9 g/fingerling) and fifty fingerlings from the clinically infected brood stock (average weight= 7.9 g/fingerling) were sacrificed. Kidney tissues were aseptically removed from all specimens and frozen at -70^{o}C. For the clinically affected progeny, all renal samples were additionally utilized to prepare tissue smears on microscope slides.

For each population (subclinically and clinically infected progeny groups), nine treatment groups with three replicates each were established (Table 1). Fingerlings were arbitrarily

distributed to each of 27 circular flow-through holding tanks (35 l capacity) with a water flow rate of 1-2 l/minute. Nine tanks were stocked at each of the following density levels: 24 g fish/l (1.5 lbs/cubic foot); 40 g/l (2.5 lbs/cubic foot); 48 g/l (3.0 lbs/cubic foot). Densities were based on the premise that 24 g/l represents near ideal rearing density (personal communication, Andy Greulich, manager of Salmon River State Fish Hatchery) for cultured chinook salmon fingerlings, and therefore, the two higher densities represented two levels of overcrowding. For each density, three groups were fed at one of three rates: 2.8% body weight per day; 5.6 % body weight per day; 8.4% body weight per day. The 2.8% was considered optimal (Piper et al., 1982), and the additional levels represented overfeeding. Fish were fed three times daily with a pelleted commercial salmon diet (Zeigler Brothers, Inc., Gardners, PA). Water quality parameters including temperature, pH, dissolved oxygen content, and total ammonia were monitored and recorded two to three times per week. Temperature and dissolved oxygen measurements were recorded using a dissolved oxygen meter (YSI, Yellow Springs, OH), and pH was determined with a standard pH meter (Corning Model 220, Corning, NY). A colorimetric method (Wedemeyer and Yasutake, 1977) was employed to determine total ammonia concentration in water samples. Tanks were uniformly cleaned, generally every other day, by draining and refilling approximately 2/3 of the water volume. At each feeding and water quality sampling, fish were observed for general behavior and physical lesions, mortalities were recorded, and dead fish were collected from the experimental tanks.

Experimental tanks for both the subclinically and clinically affected progeny groups were maintained for twelve weeks. At 4, 8, and 12 weeks, all fish from each experimental tank were weighed using a digital scale (Yamamoto Corp. Model DPI-320, Colorado Springs, CO) to determine growth rates. Ten to twenty-five fish were removed from each tank for tissue sampling at the same four week intervals; the number of fish removed per tank was chosen to readjust the density to the initial density level. Fish were sacrificed using a lethal dose of tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO). Renal tissue samples were aseptically removed and placed individually into labeled, pre-weighed 2.0 ml polypropylene vials (Costar Biofreeze; Cambridge, MA). Vials were reweighed and frozen at -70^{o}C. Additionally, renal tissues from the clinical progeny groups were used to prepare cytological specimens for direct fluorescent antibody testing (DFAT; Bullock et al., 1980).

Renibacterium salmoninarum antigen prevalence in all renal tissues collected from sampled fingerlings and mortalities from both progeny groups was assessed by ELISA. Tests were performed using a modification of the technique described by Pascho and

Mulcahy (1987). Frozen renal tissue samples collected throughout the experiment were thawed at 4°C and diluted 1:15 (weight:volume) with sterile phosphate buffered saline containing 0.05% Tween 20 (PBS-T20; pH=7.4). Tissue solutions were emulsified for 30 sec. with a sterile pipette, heated to 100°C for 15 min., and centrifuged at 8800 *xg* for 10 minutes. Samples were stored at 4°C.

All ELISA buffers, antibody, antigen, reagents, and substrates were obtained from Kirkegaard & Perry, Gaithersburg, MD. Flat-bottomed microtiter plates (96 wells; Nunc-Immuno Plate, Nunc, Neptune, NJ) were coated with a 1:10 solution of coating buffer with 1:2000 coating antibody (affinity purified antibody to *R. salmoninarum*). No coating antibody was used in substrate control wells. Two hundred microliters of this solution was dispensed into each well of a microtiter plate, and the plates were covered with an adhesive seal and stored for 18 h. in a humidified chambers at 4°C. Plates were then washed with a washing buffer, and 50 µl of each sample was dispensed into the microplate wells in triplicate. Positive control antigen and negative controls prepared from renal tissue of *R. salmoninarum*-free trout were processed along with all samples, and plates were re-sealed and incubated for 3 h. at 25°C. Following incubation, plates were washed and 200 µl of a peroxidase-conjugated antiserum (peroxidase-labeled affinity purified antibody to *R. salmoninarum* diluted 1:200 in milk diluent/blocking solution was added to all wells. Plates were incubated for an additional 2 h. at 25°C. Plates were washed again and 200 µl of ABTS peroxidase substrate was added to each plate. Following an additional 20 min. of incubation, the reaction was terminated with 50 µl of stop solution added to each well (ABTS peroxidase stop solution). Plates were evaluated for optical density using a Microplate Bio-kinetics Reader (BioTek Instruments Inc. EL 312e, Winooski, VT) and an IBM compatible computer. Optical density (OD) readings were recorded for each well, and the average OD reading of three wells was calculated for each sample. Negative control sample values were averaged and the standard deviation from the mean calculated. A positive-negative threshold for samples was determined based upon the average negative control OD value plus two standard deviations from the mean. Each sample was subsequently assigned as either a "positive" or "negative" ELISA result based upon these calculations.

Additionally, renal tissues from experimental samples and mortalities from the clinical progeny group were examined by the DFAT. Briefly, the cytological slide preparations from renal tissues were coated with 1-2 drops of a solution containing 1:40 FITC-labeled antiserum (fluorescein-labeled affinity purified antibody to *R. salmoninarum*; Kirkegaard & Perry) and 1:100 Rhodamine B in phosphate buffered saline (PBS). Slides were incubated at 25°C for 20 min., rinsed in PBS, and rapidly air-

dried. Dried slides were covered with a coverslip using mounting fluid and viewed under oil immersion with a fluorescent microscope. Fluorescence with the morphology of *R. salmoninarum* cells was indicative of a positive response. Following the count of 100 microscope fields, samples were categorized numerically: 0=negative; 1= 1-5 cells per field; 2= >5 cells per field.

Statistical analysis of results was performed using SAS programs for Kaplain-Meier survival analysis for survival data, multiple linear regression for growth rates and water quality covariancy data (i.e.- dissolved oxygen and unionized ammonia levels), and logistic regression for ELISA and DFAT evaluation of antigen prevalence (SAS PROCLIFE, SAS PROCGLM, SAS PROCLOGISTIC; SAS Institute, Inc., Cary, NC). Additionally, agreement between ELISA and DFAT results for the antigen prevalence among treatment levels of the clinical progeny group was assessed using the Kappa statistic of correlation (Agresti, 1990).

III. Results

Antigen prevalence difference between progeny groups

The overall proportion (i.e.- not considering treatment group specificity) of fingerlings testing positive for *R. salmoninarum* antigen via ELISA was significantly different for the progeny of the subclinically infected brood stock versus the progeny of the clinically infected brood stock (referred to as "subclinical progeny" and "clinical progeny" for brevity) for all sampling intervals. The proportion of ELISA positive fingerlings sampled was 0.1 for the subclinical progeny at time=0, decreasing to nearly 0 for the 4, 8, and 12 week sample (4 weeks=0.034; 8 weeks=0.010; 12 weeks=0.001). For the clinical progeny group, the initial (time=0) sampling proportion was 0.5, rising to a mean of 0.73 at the 4 week sample, 0.72 at the 8 week sample, and 0.75 at the 12 week sample (Figure 1).

A wide range in ELISA OD values was found among positive samples from the clinical progeny group. Typically, negative threshold values for ELISA ranged from 0.10 to 0.15 for subclinical and clinical progeny samples. The OD values for clinical progeny samples testing positive ranged from slightly above the negative threshold (approximately 0.20) to off-scale values (>3.00). Colorimetric change was generally visible to the naked eye at OD values of >1.00. Renal samples from mortalities (clinical progeny group) all produced OD values at the higher end of the range (>1.00); most of these values were off-scale. The few positive samples from the subclinical progeny group had OD values in the lower end of the range (<0.50). In addition, ELISA testing of renal tissues from the clinical progeny brood stock supported the

observation that clinical BKD was present in the population; five of the six samples tested strongly positive (i.e. intense color change apparent/OD>2.00) for *R. salmoninarum* antigen.

Mortality/survival among the progeny groups

Differences in mortality rates between the two progeny groups were appreciable. Sixteen mortalities were recorded cumulatively for all experimental groups of the subclinical progeny, and none of these fish tested positive for *R. salmoninarum* by ELISA. In contrast, 1,218 mortalities were recorded for the experimental groups of the clinical progeny, and all of these tested positive by ELISA and/or DFAT. Mortalities for the clinical progeny group are summarized weekly in Table 2. Generally, mortality rates for all experimental groups were highest during the first few weeks of the experiment, dropping off considerably during the latter weeks. Diseased and dead fish showed similar clinical signs in the affected progeny group. These signs included petecchial hemorrhage (externally and internally), exophthalmia, hyperpigmentation, hyphema, distension of the body cavity with free serosanguinous fluid accumulation, and focal or diffuse purulent exudate of internal organs (particularly liver, spleen, and kidney).

Data acquired from mortality attributed to BKD via positive serological test results was used to formulate survival curves for the clinical progeny groups (Figure 2). Fingerling survival was similar for the five treatment groups with lower combinations of density and feeding levels (D1F1, D1F2, D1F3, D2F1, D2F2) such that the probability of fingerling survival after 12 weeks ranged between 0.55 and 0.57. For the remaining four treatment groups (D2F3, D3F1, D3F2, D3F3), the probability of fingerling survival was somewhat higher, ranging from 0.63 to 0.69.

Due to the absence of BKD-related mortality among the subclinical progeny, a survival curve was not formulated.

Growth rate differences among progeny groups

Recorded weights of fingerlings at the beginning (time=0) and end (time=12 weeks) of the experiment were used to calculate growth of fingerlings (g/fish) among the treatment levels for both progeny groups, and the effects of the experimental factors (i.e.-density and feeding rate) were assessed for both progeny groups. Growth rates for all experimental groups ranged from 6.6 to 10.3 g/fish (Tables 3a and 3b). For the subclinical progeny, no relationship between growth and the experimental factors was evident ($p=0.3445$; $F=1.11$; $df=2/24$); growth rates of experimental groups ranged from 7.9 to 9.7 g/fish and did not show consistent correlation with either density or feeding rate. For the clinical progeny group, an interaction was evident such that higher growth rates occurred in the treatment levels with higher density and feeding rates

(p=0.0545; F=4.10; df=1/23) (Figure 3). A wider range of growth rate was evident for clinical progeny experimental groups (6.6 - 10.3 g/fish).

Antigen prevalence by ELISA and DFAT: differences among treatment levels of progeny groups

The proportion of ELISA positive fingerlings among all treatment levels of the subclinical progeny group approached zero for the 4,8,and 12 week samples (< 0.035)(Table 4a). No further assessment of antigen prevalence in relationship to treatment level was warranted.

For the clinical progeny group, no relationship was apparent between antigen prevalence determined by ELISA and either density level or feeding rate. The proportion of sample fish testing positive by ELISA for each experimental group at each sampling time ranged from 0.60 to 0.93, with no evident correlation with density alone, feeding rate alone, or a combination of the two factors (p=0.842, X2=0.0832, df=3) (Table 4b). A wide range in ELISA OD values was found for positive samples within each experimental group (see above).

Direct fluorescent antibody testing for the clinical progeny group was also used to assess antigen prevalence among the experimental groups of the clinical progeny. Again, no correlation was evident between the antigen prevalence and the density and/or feeding rate variables (p=0.276, X2=2.575, df=2)(Table 5).

Measure of Agreement between ELISA and DFAT

Kappa values obtained for each sampling interval (time= 0, 4, 8, and 12 weeks) all indicated a low correlation between ELISA and DFAT results for the clinical progeny group. The Kappa statistics were calculated to be 0.20 at the 0 week sample, 0.30 at the 4 week sample, 0.24 at the 8 week sample, and 0.06 at the 12 week sample (with Kappa being equal to 1.0 with perfect correlation and equal to 0 when agreement equals that expected by chance). In contrast, a comparison of agreement between ELISA and DFAT results for mortalities from the clinical progeny group yielded a Kappa value of 1.0, representing perfect correlation (Figure 4).

Covariancy of water quality variables (dissolved oxygen and unionized ammonia concentrations)

Mean dissolved oxygen value ranged from approximately 4.0 ppm to 11.0 ppm among experimental groups with the higher values associated with the lower densities and, to a lesser degree, feeding rates. Mean unionized ammonia values, ranging from approximately 0.0001 ppm to 0.0007 ppm, were higher in experimental groups with the higher densities and feeding rates (Figures 5a and

6a). Unlike dissolved oxygen values, the unionized ammonia values encountered in this investigation generally would not be considered detrimental to fish (Dupree and Huner, 1984)(Figures 5b and 6b).

Correlation between these two water quality parameters and experimental groups was evaluated to determine covariancy (i.e.- the extent to which the treatment level and the water quality parameter vary together/interrelate). For the subclinical progeny group, significant correlation was evident between density level and dissolved oxygen content ($p=0.0001$, $F=222.53$, $df=1/24$) as well as between density level and unionized ammonia level ($p=0.0001$, $F=129.61$, $df=1/24$). Correlation was not found between feeding rate and either of the water quality variables.

For the clinical progeny group, a significant correlation was again evident between the density level and the dissolved oxygen content ($p=0.001$, $F=37.84$, $df=1/24$), but no correlation existed between feeding rate and dissolved oxygen. Both feeding rate and density level correlated significantly with unionized ammonia content for this group (density: $p=0.001$, $F=24.51$, $df=1/24$; feeding rate: $p= 00546$, $F=4.08$, $df=1/24$).

IV. Discussion

The ELISA results for overall antigen prevalence differences between the two progeny groups were consistent with previous reports in the literature regarding vertical transmission of *R. salmoninarum*. Brood stock segregation of chinook salmon based on serological indicators of antigen prevalence (ELISA and DFAT) has been demonstrated to significantly reduce both antigen prevalence (Pascho et al., 1991) and mortality (Elliott et al., 1995) in the offspring. In this study, the apparent increases in *R. salmoninarum* antigen prevalence as determined by ELISA and mortality level associated with the clinical progeny group over the subclinical progeny group lend further support to the importance of brood stock segregation as an effective means of BKD control among cultured salmonids. Interpretation of ELISA results based upon the nine treatment levels for each group was, however, less conclusive. No apparent correlation between stocking density and/or feeding rate and the prevalence of *R. salmoninarum* antigen was demonstrated in this study.

Correlation of the secondary variables (dissolved oxygen and unionized ammonia content of water) with treatment levels of density and feeding rate was evident in this investigation. The most consistent correlation was observed between the density level and the two water quality parameters; namely, increased stocking density coincided with elevated levels of unionized ammonia and decreased levels of dissolved oxygen for both progeny groups.

Water quality variables, particularly the dissolved oxygen contents below 6.0 ppm which occurred in the higher density treatment groups, represent potential stress factors directly associated with density and feeding rate (Dupree and Huner, 1984); however, stress was not manifested through increased antigen prevalence or decreased growth/survival in response to these variables.

The relationship, if any, between excessive levels of stocking density and feeding rate and increased *R. salmoninarum* antigen prevalence among chinook salmon fingerlings was not substantiated by this investigation. The principle of environmental stress impacting resistance to disease and antigen prevalence is well established (Snieszko, 1974). While this relationship, as it relates to BKD pathogenesis, was not conclusively supported by this study, it certainly cannot be dismissed. As no parameter reflecting stress levels (i.e. endogenous serum cortisol levels) was assessed in this investigation, stress differences among the treatment groups were not definitively established. Mazur et al. (1993) conducted a similar study examining the effects of stocking density and feeding rate upon *R. salmoninarum* antigen prevalence in chinook salmon smolts held in seacages, obtaining significantly different results; that study reported significant density effects upon antigen prevalence such that higher stocking density correlated with increased antigen prevalence. Differences in these and our findings may be explained by a number of factors related to experimental design, including life stage of the fish (smolts versus fingerlings), the habitat (freshwater enclosed tanks versus cages in saltwater), and antigen prevalence assessment (DFAT and ELISA versus the quantitative fluorescent antibody technique). Additionally, the Mazur et al. (1993) investigation utilized treatment level densities below their standard density whereas our study used treatment level densities at and above a designated standard (24 g/l). Our investigation attempted a laboratory simulation of salmonid culture conditions rather than an experiment conducted on site at a salmonid culture facility. It is quite possible that given other environmental conditions (water flow, other water parameters, alternative treatment levels for potential stressors), the outcome may have been substantially different.

Otherwise, the level of infection represented by the two progeny groups may have been inappropriate for illustration of the impacts of environmental parameters on BKD pathogenesis and antigen prevalence. For instance, the infection level of the subclinical progeny group may have been too low in the majority of fish for disease progression to be evident, even with the impact of external stressors. Likewise, individuals in the clinical progeny group may have been so highly infected with *R. salmoninarum* that any stress and related immunosuppressive facilitation of disease pathogenesis was not a factor.

Alternatively, the possibility exists that the findings correctly support the notion that environmental variables play a minimal role in BKD pathogenesis of vertically infected juvenile salmonids. As vertical transmission involves intra-ovum infection by *R. salmoninarum* prior to the development of protective immunity in the offspring, immunological tolerance may be a factor in pathogen dissemination and subsequent disease outbreak. Brown et al. (1996) showed that exposure of coho salmon (*Oncorhynchus kisutch*) as unfertilized eggs to the p57 antigen of *R. salmoninarum* diminished the subsequent ability of these fish to mount a humoral immune response against p57. With immunological tolerance for *R. salmoninarum*, external factors which would typically diminish immunological function would not influence BKD pathogenesis as fully as would be expected for immunologically competent fish. Different results would therefore be expected with fish infected horizontally. Mazur et al. (1993) also discusses the probability that horizontal (fecal-oral) transmission occurred among fish in that investigation.

Both serological tests employed in this study, ELISA and DFAT, yielded dissimilar results. While the test methodologies display 100% correlation in confirming mortality due to BKD, agreement between the tests applied to asymptomatic, clinically diseased, or healthy fish was poor. Both DFAT and ELISA are highly utilized and considered reliable diagnostic methodologies common to both clinical medicine and scientific research. Factors related to each of these tests specifically adapted for detection of *R. salmoninarum* may, at least in part, explain the inconsistency between results. The ELISA detects soluble antigen of *R. salmoninarum* that is extracted from tissue, while the DFAT detects whole cells (i.e. cell surface antigen) of *R. salmoninarum*. The overall ability to detect soluble antigen in homogenized tissue samples would presumably be greater than the ability to detect cells from a tissue smear representing a comparably far smaller tissue sample. This difference has been cited as a contributory factor to the overall greater sensitivity of ELISA as compared to DFAT (Meyers et al., 1993; Dixon, 1987). On the other hand, inherent features of this ELISA for *R. salmoninarum* antigen may also undermine its reliability. The use of a polyclonal antiserum to *R. salmoninarum* may allow for cross-reactivity with similar antigens, producing false positive results; likewise, cross-reactivity with other bacterial species has been reported for the DFAT (Brown et al., 1995; Wood et al., 1995; Austin et al., 1985). Additionally, the complexity of the ELISA (in terms of equipment, reagents, and procedure) compared to DFAT allows for greater error in technique and interpretation of results. There is considerable precedence for discrepancies of results among serological tests, ELISA and DFAT in particular, in the literature. Indeed, Meyers et al.(1993) describes considerable dissimilarities in results

obtained from ELISA and DFAT as methods of assessing prevalence of *R. salmoninarum* among salmonid stocks. Complete agreement between ELISA and DFAT applied to the mortality specimens in this current study suggests that levels of bacterial cells and soluble antigen in fish with lethal BKD are sufficiently elevated to be readily detected serologically by either method. The otherwise poor correlation between the techniques suggests that levels of *R. salmoninarum* organisms and soluble antigen in subclinically/early clinically diseased fish do not necessarily coincide.

In this study, treatment groups with a combination of higher density and feeding rates were associated with better survival and growth in the clinical progeny group. Assuming that the higher levels of density and feeding rate are valid representations of elevated environmental stress, this is an unusual finding; there is not substantial precedence for physiological benefit from external stressors in fish culture. Previous literature generally has shown an inverse correlation or lack of correlation between various environmental stressors and survival/growth of fish (Kjartansson et al., 1988; Fagerlund et al., 1981). One possible explanation is that this investigation was confounded by the presence of increased levels of organic debris, including excessive uneaten feed and fecal matter, in the treatment level tanks with the higher combinations of density and feeding rate. As an obligate pathogen, *R. salmoninarum* has limited survival capabilities outside the host related to an affinity for organic debris, including fecal matter (Austin and Rayment, 1985; Mitchum and Sherman, 1981). It is possible that horizontal transmission of *R. salmoninarum* was decreased in experimental groups with increased organic debris (i.e.- the higher treatment levels) since the debris provided an alternative foci of bacterial colonization. As all experimental tanks were cleaned generally every other day, the bacteria would have been routinely removed from the system in these treatment groups. The overall antigen prevalence data for the clinical progeny group (Figure 1) reflects an increase in the proportion of ELISA positive fish throughout the course of the experiment, possibly indicating a component of horizontal transmission. Alternatively, individual fingerlings could simply have been experiencing increased antigen loads with time, increasing the proportion of fish with antigen levels surpassing the negative threshold for ELISA. Furthermore, this hypothesis was not supported by results for antigen prevalence among the treatment levels (i.e.- antigen prevalence did not decrease in higher treatment levels) as determined by either ELISA or DFAT. Further investigation into the bacterial load in organic debris from the different treatment levels would be warranted to better elucidate the potential impact of organic debris upon survival and growth of cultured salmonids as well as upon *R. salmoninarum* antigen prevalence in tissues.

Table 1. Organizational scheme for experimental groups.

Organization of the nine experimental groups for each progeny population, representing three treatment levels each of stocking density (D1-D3) and feeding rate (F1-F3). The lowest levels of each (D1/F1; 24 g fish/l and 2.8 % body weight/day) represent "ideal" levels while the other levels represent degrees of overcrowding and overfeeding.

Stocking density (g fish/l)	**Feeding rate (% body weight/day)**		
	2.8 %	**5.6 %**	**8.4 %**
24 g/l	D1/F1	D1/F2	D1/F3
40 g/l	D2/F1	D2/F2	D2/F3
48 g/l	D3/F1	D3/F2	D3/F3

Table 2. BKD-related mortalities from the clinically infected progeny group

BKD-related mortalities, tallied on a weekly basis, for each of the nine experimental groups (D1-3 = density levels, D1 = 24 g/l, D2 = 40 g/l. D3 = 48 g/l; F1-3 = feeding rates, F1 = 2.8% body weight/day, F2 = 5.6 %, F3 = 8.4%) among the clinically diseased population of chinook salmon fingerlings. The total cumulative number of BKD-related mortalities was 1, 218 fingerlings.

Group	Time (week)												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
D1F1	26	18	11	8	12	8	4	5	6	3	2	1	104
D1F2	27	13	10	11	13	11	3	5	5	2	0	3	103
D1F3	35	22	16	3	7	5	5	7	1	4	3	4	112
D2F1	40	32	14	18	16	5	5	5	5	2	4	4	150
D2F2	43	34	19	15	11	7	6	6	10	5	5	6	167
D2F3	27	17	14	13	14	9	4	13	7	7	4	7	136
D3F1	28	18	15	17	13	9	6	11	4	7	5	4	137
D3F2	33	15	16	23	25	13	8	5	12	8	5	6	169
D3F3	22	25	16	14	12	8	6	5	9	3	8	12	140
Total:	281	194	131	122	124	75	47	62	59	41	36	47	1,218

Table 3a. Growth among the asymptomatic progeny population.

Mean growth (g/fish) for the experimental groups of the asymptomatic progeny population over twelve weeks. D1-3 represent the density levels while F1-3 represent the feeding rates.

Stocking Density (D: g fish/l)	Feeding rate (F; % body weight/day)		
	F1 (2.8%)	F2 (5.6%)	F3 (8.4%)
D1 (24 g/l)	8.0	9.7	7.9
D2 (40 g/l)	8.6	9.1	8.1
D3 (48 g/l)	9.3	9.7	8.4

Table 3b. Growth among the clinical progeny population.

Mean growth (g/fish) for the experimental groups of the clinical progeny population over 12 weeks. D1-3 represent the density levels while F1-3 represent the feeding rates.

Stocking density (D; g fish/l)	Feeding rate (F; % body weight/day)		
	F1 (2.8%)	F2 (5.6%)	F3 (8.4%)
D1 (24 g/l)	8.3	8.9	9.1
D2 (40 g/l)	7.9	8.3	10.3
D3 (48 g/l)	6.6	9.6	9.5

Table 4a. ELISA results for the asymptomatic progeny group.

ELISA data summary for the nine experimental groups at 4, 8, and 12 weeks and the time 0 control sample for the asymptomatic progeny population. Values represent the proportion of fish with positive ELISA results within each group. D1-3 represent the density levels (D1 = 24 g/l, D2 = 40 g/l, D3 = 48 g/l) and F1-3 represent the feeding rates (F1 = 2.8% body weight/day, F2 = 5.6%, F3 = 8.4%).

Time (week)	Group (D,F)	Proportion
0	---	0.09
4	D1F1	0.02
	D1F2	0
	D1F3	0.04
	D2F1	0.09
	D2F2	0.04
	D2F3	0.04
	D3F1	0
	D3F2	0.07
	D3F3	0
8	D1F1	0
	D1F2	0
	D1F3	0
	D2F1	0
	D2F2	0
	D2F3	0.07
	D3F1	0.02
	D3F2	0
	D3F3	0
12	D1F1	0.10
	D1F2	0
	D1F3	0
	D2F1	0
	D2F2	0
	D2F3	0
	D3F1	0
	D3F2	0
	D3F3	0

Table 4b. ELISA results for the clinical progeny group.

ELISA data summary for the nine experimental groups at 4, 8, and 12 weeks and the time 0 control sample for the clinical progeny population. Values represent the proportion of fish with positive ELISA results within each group. D1-3 represent the density levels (D1 = 24 g/l, D2 = 40 g/l, D3 = 48 g/l) and F1-3 represent the feeding rates (F1 = 2.8% body weight/day, F2 = 5.6 %, F3 = 8.4%).

Time (week)	Group (D,F)	Proportion
0	----	0.50
4	D1F1	0.67
	D1F2	0.60
	D1F3	0.80
	D2F1	0.63
	D2F2	0.83
	D2F3	0.80
	D3F1	0.83
	D3F2	0.63
	D3F3	0.77
8	D1F1	0.93
	D1F2	0.84
	D1F3	0.70
	D2F1	0.77
	D2F2	0.71
	D2F3	0.63
	D3F1	0.63
	D3F2	0.70
	D3F3	0.60
12	D1F1	0.76
	D1F2	0.71
	D1F3	0.73
	D2F1	0.73
	D2F2	0.79
	D2F3	0.77
	D3F1	0.81
	D3F2	0.71
	D3F3	0.77

Table 5. DFAT results for the clinical progeny group.
DFAT data summary for the experimental groups at 4, 8, and 12 weeks and the time 0 control sample from the clinical progeny population. Values represent the proportion of fish within each DFAT classification (0 = negative, 1 = 1-5 cells per field, 2 = greater than 5 cells per field) for each experimental group. D1-3 represent the density levels (D1 = 24 g/l, D2 = 40 g/l, D3 = 48 g/l) and F1-3 represent the feeding rates (F1 = 2.8% body weight/day, F2 = 5.6%, F3 = 8.4%).

Time (week)	Group (D,F)	Proportion per DFAT category		
		0	1	2
0	---	0.12	0.56	0.32
4	D1F1	0.50	0.33	0.17
	D1F2	0.56	0.27	0.17
	D1F3	0.42	0.27	0.31
	D2F1	0.40	0.37	0.23
	D2F2	0.43	0.30	0.27
	D2F3	0.50	0.42	0.08
	D3F1	0.37	0.37	0.26
	D3F2	0.59	0.33	0.08
	D3F3	0.12	0.60	0.28
8	D1F1	0	0.60	0.40
	D1F2	0.20	0.52	0.28
	D1F3	0	0.80	0.20
	D2F1	0.08	0.56	0.36
	D2F2	0.28	0.58	0.14
	D2F3	0.18	0.62	0.20
	D3F1	0.20	0.60	0.20
	D3F2	0.10	0.60	0.30
	D3F3	0.12	0.38	0.60
12	D1F1	0.65	0.30	0.05
	D1F2	0.44	0.38	0.18
	D1F3	0.58	0.32	0.10
	D2F1	0.60	0.28	0.12
	D2F2	0.60	0.30	0.10
	D2F3	0.70	0.27	0.03
	D3F1	0.54	0.42	0.04
	D3F2	0.70	0.28	0.02
	D3F3	0.50	0.40	0.10

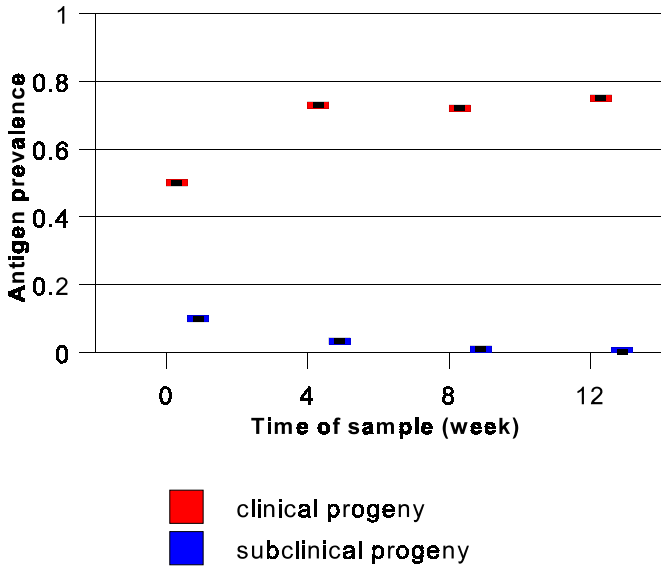


Figure 1: A comparison of *R. salmoninarum* antigen prevalence as determined by ELISA for two progeny groups.

The y-axis displays the proportion of fish sampled within each population which tested positive for the antigen.

Figure 2: Survival analyses for the nine treatment groups of the clinical progeny population.
(ch1fig2.pic; 828K)

The density variable is represented by the columns (left=24 g/l, middle= 40 g/l, right=48 g/l) and the feeding rate variable is represented by the rows (top=8.4%, middle=5.6%, bottom=2.8%). Note the highest survival rates at the end of the 12 week interval were experienced by the groups with the higher levels of density and feeding (survival curves furthest top and right).

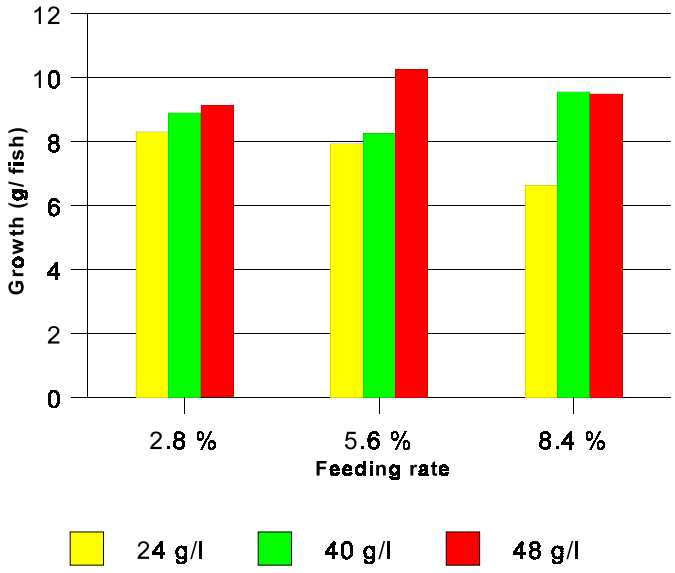


Figure 3: Growth rates for the treatment groups from the clinical progeny population.

Each bar color represents a different density level at each feeding rate. Note the increase in growth rate corresponding to increased density for each feeding rate, especially apparent for the 5.6% and 8.4% feeding rates.

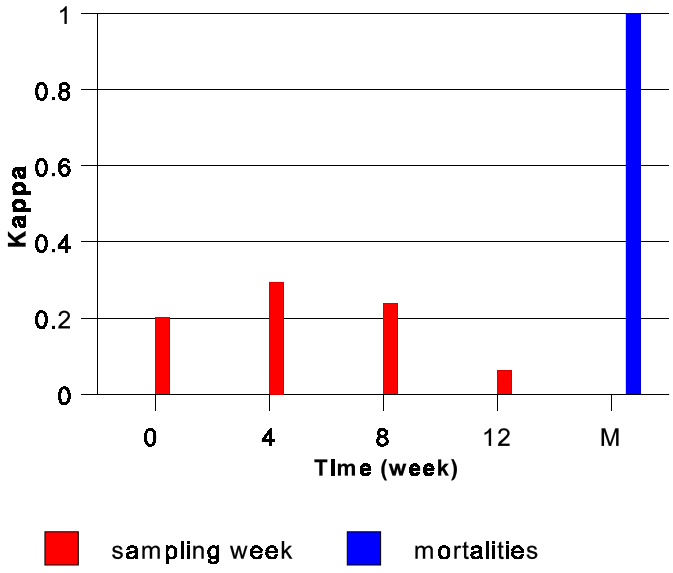


Figure 4:

Correlation between the ELISA and DFAT results for the clinical progeny population. (Perfect correlation is represented by a Kappa value of 1.00 and absence of correlation is represented by a Kappa value of zero.)

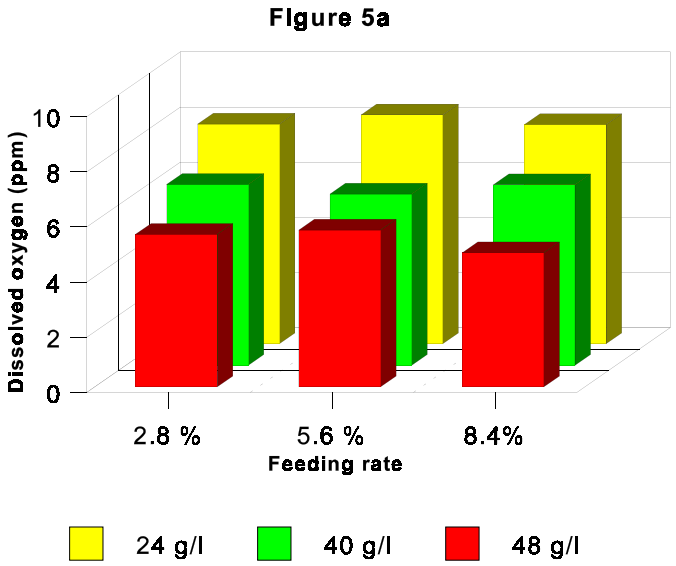


Figure 5: Covariancy of water quality parameters, dissolved oxygen (5a) and unionized ammonia (5b), with the treatment groups for the subclinical progeny population.

Each bar color represents a different density level at each feeding rate.

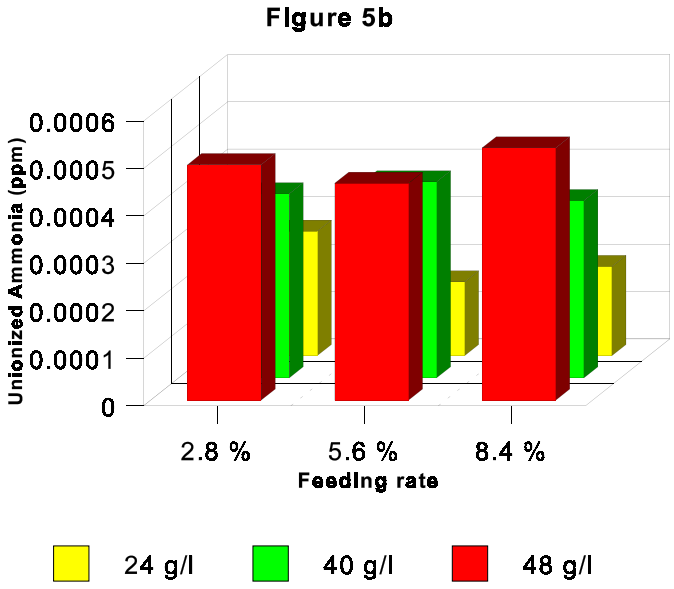


Figure 6a

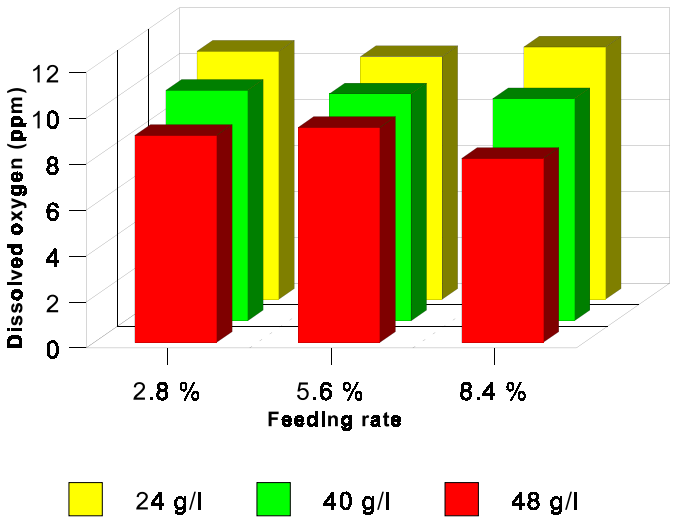
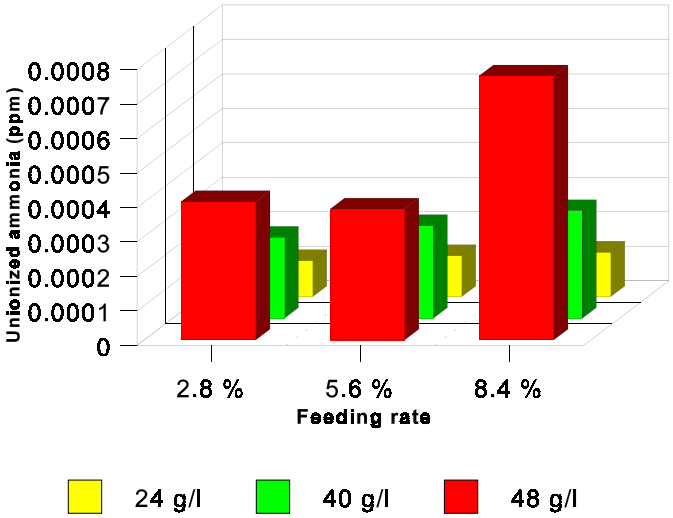


Figure 6: Covariancy of water quality parameters, dissolved oxygen (6a) and unionized amonia (6b), with the treatment groups for the clinical progeny population. Each bar color represents a different density level at each feeding rate.

Figure 6b



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Chapter 2

In vivo effects of the extracellular protein of *Renibacterium salmoninarum* upon immunological parameters in juvenile rainbow trout (*Oncorhynchus mykiss*).

Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo* to the extracellular protein (ECP) of *Renibacterium salmoninarum* were evaluated in terms of various immunological parameters, including the ability to mount a secondary immune response against another pathogen of salmonids. Seven groups of one hundred trout were each treated as follows: intraperitoneal (IP) injection of ECP (150 µg ECP/fish) and IP vaccination against *Yersinia ruckeri* with a formalin-killed bacterin (4 groups); vaccination alone; ECP injection alone; IP injection of phosphate buffered saline as a control. For the four groups receiving both the ECP and vaccination, the timing was offset so that the ECP was given: 1 day prior to vaccination; simultaneously with vaccination; 8 days post-vaccination; or 22 days post-vaccination. Twenty-two days post-vaccination, all groups were challenged with *Y. ruckeri*. At 12 sampling times, pre and post-challenge, the following immunological parameters were evaluated in seven fish from each of the seven groups: hematocrit, total serum protein, total leukocyte count, differential leukocyte count, microagglutination titer, and splenic phagocytosis. Post-challenge mortalities were recorded and cultured for *Y. ruckeri*. Results of the investigation indicated immunomodulatory effects of the ECP upon some of the select parameters. The ECP-exposed fish showed post-challenge elevations in leukocyte counts and small lymphocyte counts, representing a potentially immunostimulatory effect. The ECP exposure precluded a post-challenge increase in monocyte numbers, representing a potentially immunosuppressive effect of the ECP. Mortality related to *Y. ruckeri* challenge occurred only in unvaccinated fish, with fewer mortalities in the group receiving the ECP alone than in the control group, possibly indicating ECP-related nonspecific immunostimulation. No effects related to ECP exposure were evident for the other parameters examined.

I. Introduction

Bacterial kidney disease (BKD) of salmonid fish is a chronic, granulomatous disease often associated with high levels of morbidity and mortality. The causative agent, *Renibacterium salmoninarum*, is a Gram-positive diplobacillus. This organism has a number of unique features involved in disease pathogenesis and immunomodulation. The extracellular protein (ECP) produced by *R. salmoninarum* contains a number of soluble antigens. The ECP may be isolated from both bacteria in artificial media and from BKD-infected salmonid tissues at concentrations approaching 1 mg/ml (Turaga et al., 1987a; Kaattari et al., 1989), and it has been extensively investigated as a factor in BKD pathogenesis. Antigenically, the most prominent component of the ECP is a 57 kD protein (p57) deemed the major soluble antigen or Antigen F (Getchell et al., 1985). Other antigenic ECP constituents, with molecular weights both higher and lower than the 57 kD antigen, have been identified by a number of investigators (Getchell et al., 1985; Fiedler and Draxl, 1986; Wiens and Kaattari, 1989; Griffiths and Lynch, 1991; Bandin et al., 1992). A number of *R. salmoninarum* virulence factors have been attributed to the ECP, particularly to the p57 antigen. Included in these are the hydrophobicity and autoaggregation of *R. salmoninarum* cells, agglutination of rabbit erythrocytes, and agglutination of salmonid spermatozoa (Daly and Stevenson, 1987; Daly and Stevenson, 1989). The p57 protein has also been associated with cellular adhesion by Dubreuil et al. (1990) who identified this antigen to be a cell surface fimbriae.

Various immunomodulatory effects have also been ascribed to the ECP, particularly to the p57 antigen. *In vitro* leukoagglutination of salmonid leukocytes from the pronephros, spleen, and peripheral circulation, and induction of macrophages *in vitro* are among the effects associated with the p57 antigen of *R. salmoninarum* (Kaattari et al., 1989). Similarly, Turaga et al. (1987b) described immunomodulation associated with soluble antigens of *R. salmoninarum*, characterized as an *in vitro* decrease in antibody responses to an unrelated antigen. Other immunomodulatory mechanisms have been associated with BKD without regard to any association with soluble antigens; these include the intracellular survival and reproduction of *R. salmoninarum* (Bandin et al., 1993) and the involvement of immunological hypersensitivity in BKD pathogenesis (Sami et al., 1992; Evendon et al., 1993).

The pervasion of any *in vivo* immunomodulation by *R. salmoninarum* and the importance of these immunomodulatory features in epizootics of BKD or other diseases is largely undetermined. Moreover, the general *in vivo* effects of the soluble antigens of *R. salmoninarum* have not been fully elucidated. Shieh (1988) identified a lethal extracellular toxin produced *in vivo* by *R. salmoninarum*. In contrast, Bandin et al. (1991) concluded that no

in vivo toxic effects associated with the ECP of *R. salmoninarum* were evident.

Immunomodulatory mechanisms enacted by *R. salmoninarum* upon the host may be reflected by the host's ability to mount an effective immune response to another pathogen following exposure to the immunomodulatory agent. The objective of this investigation was to examine the *in vivo* immunomodulatory effects of the *R. salmoninarum* ECP in salmonid fish as they influence the ability of the salmonid host to mount an immune response against another lethal pathogen.

II. Materials and Methods

Preparation of the Extracellular Protein

Extracellular protein was recovered by a modification of the methods of Hsu et al. (1991) and Rockey et al. (1991). An isolate of the ATCC #33209 strain of *R. salmoninarum* was subcultured and incubated on KDM2 agar at 15 ^{o}C for 21 days. Samples from the cultures were Gram-stained and serologically evaluated via the direct fluorescent antibody test (DFAT) for *R. salmoninarum* (Bullock et al., 1980) in order to confirm pure cultures of the organism. The plates were rinsed with sterile water, and the bacterial colonies agitated to remove them from the agar. The bacterial suspension was centrifuged for 20 min. at 600 *xg* and 10^{o}C. The supernatant was recovered, and protein concentration was determined by the method of Bradford (1976). Sterile phosphate buffered saline (PBS; pH=7.2) was used to dilute the solution to the final concentration of 750 µg/ml. The extracellular protein solution was maintained at 15^{o}C for 24 h. preceding injection into sample fish.

Preparation of *Yersinia ruckeri* bacterin

A formalin-killed particulate *Yersinia ruckeri* (causative agent of enteric redmouth disease, ERM, of salmonids) antigen preparation was developed by a modification of the method of Anderson and Dixon (1989). Briefly, the 11.40 isolate of Serotype 1 *Y. ruckeri* (Schill et al., 1984) was subcultured in tryptic soy broth medium. Bacterial cells were harvested from log phase growth via centrifugation (150 *xg* for 15 min.), and the pelleted cells washed three times in PBS. Cells were resuspended in PBS to a 1% solution, and formalin (0.4% final volume) was added. The suspension was continuously stirred for 24 h. and stored at 4^{o}C. Prior to vaccination, the suspension was readjusted with PBS to a concentration yielding 30% transmission at 525 nm on a spectrophotometer.

Preparation of *Y. ruckeri* bath suspension

An isolate of *Y. ruckeri* (11.40) was cultured on tryptic soy agar, suspended in sterile PBS, and injected into ten juvenile rainbow trout to confirm viability and pathogenicity. Bacterial cultures from renal tissues were taken from the resulting mortalities, and *Y. ruckeri* was re-isolated and biochemically identified. Eight liters of tryptic soy broth were inoculated with the pathogenic isolate. After 48 h. incubation at 22^{o}C, bacterial cells were precipitated via centrifugation (6000 *xg* for 15 min.) and resuspended in PBS to a total volume of 1400 ml. Cell concentration was quantified via dilution plate counts (Cipriano et al., 1991), and the suspension was divided into seven 200 ml aliquots. One aliquot was added to each experimental tank at the time of challenge.

Experimental groups and sampling chronology

One hundred juvenile rainbow trout (*Oncorhynchus mykiss*; average weight = 65 g) were arbitrarily distributed into each of seven circular 285 l tanks supplied with spring water (12.5^{o}C). Fish were maintained on a commercial diet of pelleted salmonid feed (Zeigler Brothers, Inc., Gardners, PA). Each group was treated with an experimental regime as follows: vaccination with the *Y. ruckeri* bacterin (0.2 ml IP); injection with the *R. salmoninarum* ECP solution (0.2 ml IP; 150 µg ECP/fish); injection with the bacterin and the ECP (4 groups; 0.2 ml IP each solution); and injection with sterile PBS (0.2 ml IP). For the four groups injected with both bacterin and ECP, the chronology of the injections was varied as follows: simultaneous injection with bacterin and ECP; injection of ECP one day prior to vaccination; injection with ECP 8 days following vaccination; injection with ECP at time of challenge (22 days following vaccination). Seven fish were removed from the appropriate groups for sampling (as described below) at the following times post-ECP injection/vaccination: 12 hours, 1 day, 2 days, 4 days, 8 days, 16 days, and 22 days. For the two groups in which the vaccination and ECP injection were offset by 8 and 22 days, sampling was done at the aforementioned time intervals post-ECP injection. Fish were sacrificed with a lethal dose of tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO).

Twenty-two days following vaccination (after the 22 day sample was collected), all groups of fish were challenged with *Y. ruckeri* in an 80 l bath suspension of 6.5×10^{10} organisms/l for 20 minutes. Post-challenge sampling was resumed for all experimental groups as before with additional samplings at: 23 days (1 day post-challenge), 26 days (4 days post-challenge), 30 days (8 days post-challenge), 38 days (16 days post-challenge), and 52 days (30 days post-challenge). All mortalities were recorded and cultured for *Y. ruckeri*.

Immunological sampling methodologies

Hematocrit/serum protein determination:

Whole blood was obtained via veinupuncture of the caudal vessels using heparinized tuberculin syringes with 22 gauge needles. The blood was transferred to capillary tubes and centrifuged in a microhematocrit centrifuge for 3 minutes. Hematocrit values were assessed using a Critocap reader (Sherwood Medical, St. Louis, MO), and serum protein was measured with a protein refractometer (Aloe-Hitachi, Japan).

Total leukocyte count:

Ten microliters of whole blood from the veinupuncture was dispensed into a solution of 790 µl PBS with 200 µl Wright's stain (1:100 dilution), and the cells were gently agitated to provide even distribution. Leukocytes were counted using a hemacytometer, and the counts were extrapolated to obtain the white blood cell concentration in peripheral blood (Klontz, 1994).

Leukocyte differential count:

Ten microliters of whole blood from each fish was spread evenly across a microscope slide and allowed to air dry. Slides were methanol-fixed and stained with Leukostat staining solutions (Fisher Diagnostics, Pittsburgh, PA). One hundred leukocytes from each sample were categorized cytologically according to type: small lymphocyte, large lymphocyte, polymorphonuclear cell (PMN), monocyte (Yasutake and Wales, 1983; Campbell, 1988). The percentage of each cell type present was multiplied by the total leukocyte count for the given sample to determine the total differential cell counts.

Microagglutination antibody titer:

The remaining blood from each fish was placed into a Vacutainer (red top) tube (Becton-Dickinson Vacutainer Systems, Rutherford, NJ) and held for six to eight hours at 4^{o}C. The tubes were centrifuged at 300 *xg* for 10 min., and the serum was removed and stored at -70^{o}C. Microagglutination titers against *Y. ruckeri* were determined in all samples simultaneously at the completion of all sampling. Log_2 dilutions of the serum samples were carried out to twelve dilutions in V-bottom microtiter plates (50 µl/well). Fifty microliters of the *Y. ruckeri* antigen preparation (30% transmission at 525 nm) previously described were added to each well, and microtiter plates were sealed and incubated at 22^{o}C for 18 hours. Following incubation, the titers were determined, calculated as the reciprocal of the lowest reactive dilution (i.e. the well with the last agglutination reaction). Negative control wells with PBS were run with the experimental serum samples.

Phagocytosis assay:

Phagocytosis capabilities of splenic phagocytes for *Y. ruckeri* were assessed via a modification of the technique described by Anderson (1992a). Briefly, spleens were aseptically removed from all fish and placed in cold (4°C) sterile Eagle's Minimal Essential Media (EMEM; Earle's salts containing 2% newborn calf serum, 100 mg/ml gentomycin, and 0.016 M Tris; Wolf and Quimby, 1973). Spleens were finely macerated, and the cellular debris resuspended in cold, sterile EMEM. Cell suspensions were washed twice in EMEM via centrifugation for 5 min. at 90 *xg* and 4°C. Following the second wash, cells were resuspended in 0.51 ml of EMEM. Ten microliters of the suspensions were removed for determination of approximate leukocyte concentration with a hemacytometer as previously described, and the cell suspensions were adjusted to 10 million leukocytes/ml. Approximately 5 million leukocytes (0.5 ml suspension) per sample were placed on sterile microscope slides and incubated at 4°C for 2 hours. Following incubation, the slides were gently washed in sterile PBS, and 0.5 ml of the *Y. ruckeri* antigen preparation (30% transmission at 525nm) diluted 1:10 in PBS was added to each slide. The slides were again incubated for 2 hours at 4°C, gently rinsed in PBS, and allowed to air dry. Once dry, the slides were methanol-fixed and stained with the Leukostat staining solutions. Stained slides were examined and phagocytic cells were identified based on cellular morphology. One hundred phagocytes were counted per slide, and phagocytosis was assessed based on the percentage of phagocytic cells which had adhered or engulfed rod-shaped bacteria. Five slides per experimental group per sampling time were examined. In addition, two slides per group from all post-challenge samples were assessed without the addition of the antigen preparation; these slides were prepared in order to detect any background presence of *Y. ruckeri* in the infected fish.

Survival:

Mortalities were removed daily from all experimental groups throughout the experiment. Renal tissues from all post-challenge mortalities were cultured on eosin/methylene blue agar, and any bacterial colonies were analyzed biochemically to identify the microorganism(s).

Statistical analysis

Statistical analyses were performed with the analysis of variance (ANOVA) for the data obtained by the following techniques: hematocrit, serum protein, leukocyte count, differential leukocyte count, antibody titer, and phagocytosis. Additionally, for the antibody titer and differential leukocyte count data, the natural logarithms of the experimental values were substituted into the statistical calculations to normalize the sample data. For the survival data, the Fisher's exact test was used to evaluate

survival in all groups; further comparison of groups with mortalities was completed using the log-rank statistic. All statistical analyses were done with SAS programs (PROCGLM, PROCLIFETEST; SAS Institute, Inc., Cary, NC). Data for the following four groups: PBS-injected, bacterin-injected, ECP-injected, and simultaneous bacterin and ECP-injected, were analyzed as a unit. Data for the four groups injected with ECP and bacterin at different time intervals were also analyzed separately to evaluate any differences based on chronology of the ECP exposure.

III. Results

Data is summarized in Tables 1-9 in the appendices of this chapter.

Exposure to the ECP appeared to have no significant effect ($p<0.05$) on either hematocrit or serum protein values. For both parameters, however, there was an interaction between vaccination status and challenge with *Y. ruckeri*, such that both hematocrit and serum protein values decreased significantly post-challenge in the experimental groups that were not vaccinated. Such decreases were not noted in the groups which were vaccinated (Figure 1).

Total leukocyte count analysis revealed a significant post-challenge leukocytosis only in the experimental groups exposed to the ECP ($p<0.0062$, $F=7.59$, $df=1$). Chronological evaluation of the ECP effect (i.e.-statistical analysis of the 4 groups vaccinated and injected with ECP at various times) showed that the leukocyte count decreased as the ECP/bacterin injection interval increased; there was a significant difference between the earliest (time = 1 day prior to vaccination) and latest (time = 22 days/at challenge) treatments, but not between the intermediate treatments ($p<0.0025$, $F=4.99$, $df=3$; Figure 2).

Differential leukocyte counts also revealed significant treatment-based differences in the total numbers of the four major types of white blood cells identified. In keeping with the total leukocyte count results, the small lymphocytes count increased significantly post-challenge in the experimental groups exposed to the ECP, but not in the unexposed groups ($p<0.0139$, $F=6.11$, $df=1$). Chronological evaluation also showed the small lymphocyte count to decrease with an increasing ECP injection/ vaccination interval, with a significant difference only between the earliest and latest groups ($p<0.0012$, $F=5.57$, $df=3$). A post-challenge increase in small lymphocyte count was also evident for the vaccinated fish ($p<0.0.04$, $F=4.25$, $df=1$; Figure 3). For the large lymphocytes, no significant changes were evident based upon the exposure to ECP or the chronology of this exposure. An interactive effect between

vaccination and challenge was however evident, such that the large lymphocyte count increased significantly post-challenge in unvaccinated fish but not in the vaccinated fish ($p<0.0001$, $F=22.76$, $df=1$; Figure 4). Similarly, there was no apparent effect of ECP exposure upon PMN count, but an interactive effect based upon vaccination and challenge was again evident. Unvaccinated fish showed a significantly increased PMN count post-challenge, while the vaccinated counterparts showed a decreased post-challenge PMN count ($p<0.0001$, $F=34.72$, $df=1$; Figure 5). Finally, the monocyte count showed a significant increase post-challenge only in fish not exposed to ECP ($p<0.0246$, $F=5.10$, $df=1$); no ECP effect based upon chronology was evident. Additionally, a significant post-challenge increase in monocyte count was evident in unvaccinated fish but not in the vaccinated fish ($p<0.0001$, $F=16.94$, $df=1$; Figure 6).

The microagglutination antibody titer data revealed no significant differences in titers based upon exposure/chronology of exposure to the ECP. There was, however, a significant elevation in the post-challenge antibody titers among vaccinated fish ($p<0.0001$, $F=32.46$, $df=1$; Figure 7).

Phagocytosis data showed no significant changes based upon either ECP exposure or vaccination. There was an overall significant decrease in the percentage of phagocytes which had adhered or engulfed bacteria post-challenge compared to pre-challenge ($p<0.0014$, $F=10.40$, $df=1$; Figure 8).

The survival data showed that mortalities in the 30 day post-challenge period occurred only in the groups receiving PBS alone (7 mortalities) and the group receiving the ECP without the bacterin (2 mortalities). Post-mortem renal cultures and biochemical analyses confirmed the presence of *Y. ruckeri* in all mortalities. Statistical analysis revealed significantly different survival rates among the seven groups ($p<0.0001$). Further analysis of the groups with mortalities via survival estimates showed the difference in survival between the two groups not to be statistically significant ($p<0.05$), although survival was considerably lower for the PBS only group than the ECP-exposed group ($p=0.0799$; Figure 9).

IV. Discussion

As a potential immunomodulatory agent, the *R. salmoninarum* ECP has the possibility of effecting not only BKD development but also other disease processes in salmonid culture. Given the typical chronic, slowly progressive nature of BKD, long term presence of *R. salmoninarum* soluble antigen within the tissues of a host is quite likely. Thus, this antigen may substantially effect the immune system of a salmonid host, altering the immunological response to

other pathogens. In this respect, subclinical BKD may therefore be regarded as a factor in epizootics of other salmonid diseases. The question remains as to whether any ECP-related immunomodulation is immunostimulatory, immunosuppressive, or both.

Results of this investigation support the concept of immunomodulation brought about by the *R. salmoninarum* ECP. The effect, however, is not consistent among the various immunological parameters examined. There appeared to be no ECP-related effects upon hematocrit, serum protein, antibody microagglutination titer, or phagocytosis capabilities at the 150 µg/fish ECP concentration used in this investigation. The absence of significant changes in the hematocrit values indicates no ECP-related changes in erythrocyte production or utilization to a degree which substantially affected the hematocrit. Similarly, no effect was noted on serum protein, indicating that total levels of the various immunological proteinaceous components of salmonid serum, including immunoglobulin, acute phase proteins, lectins, and antibacterial proteinases (Alexander and Ingram, 1992), were unaltered. Lack of response in the antibody titer suggests that ECP does not play a prominent immunomodulatory role in specific humoral immunity *in vivo*. Moreover, the absence of significant alterations in the phagocytic capabilities of the splenic phagocytes suggests that this functional aspect of cell-mediated immunity is also not affected by the ECP in this investigational framework. The lack of effects cannot, however, conclusively demonstrate that these aforementioned immunological parameters are completely unaffected in a naturally occurring infection due to experimental limitations imposed by the use of a single concentration of ECP and uncertainty regarding *in vivo* longevity of this ECP following a single dosage.

The total leukocyte count and differential analysis demonstrated significant changes relating to exposure to the ECP. The increased leukocyte count post-challenge was most likely related directly to the post-challenge increase in small lymphocyte numbers in ECP-exposed fish, as small lymphocytes represented a consistently large percentage of the total leukocyte count. Interestingly, this relationship appeared strongest (i.e. highest lymphocyte counts) when the vaccination and ECP injection were performed close together, with the count diminishing as the ECP was given later (8 and 22 days post-vaccination). These results are difficult to define in terms of precise immunomodulatory effects as small lymphocytes were not further differentiated according to function. For instance, morphological assessment alone did not distinguish the Ig+ from Ig- lymphocytes, and this distinction has considerable ramifications for immunological function (Kaattari, 1992). It is also uncertain whether the increase in small lymphocyte numbers in peripheral circulation in this investigation represents an increased total cell production or altered leukocyte

trafficking. It is likely that the elevation in small lymphocyte numbers represents an immunostimulatory response as decreased lymphocyte counts have been associated with stressors such as exogenous glucocorticoid administration and immunosuppression (Campbell, 1988).

The monocyte count also changed significantly, increasing post-challenge only in the absence of the ECP. As in mammalian species, salmonid monocytes are precursors to macrophages, playing an important role in phagocytosis, cytokine production, and antigen presentation (Secombes and Fletcher, 1992). The results of this investigation suggest that the ECP may act as an immunosuppressive agent upon the monocyte population, preventing a post-challenge monocytosis. Indicators of monocyte/macrophage function (i.e. phagocytosis assay), however, failed to corroborate additional immunosuppressive effects for this cell population.

The survival analysis data provides further suggestion of immunomodulation by the ECP. The only two groups experiencing *Y. ruckeri* related mortalities post-exposure were the unvaccinated groups injected with PBS or ECP; however, the experimental group given only ECP yielded just two mortalities while the PBS-injected group had seven mortalities in the 30 days post-challenge. The upper confidence limit for survival of the ECP-injected group is inclusive of 100% (proportion=1.00) survival, experienced by the vaccinated fish; however, it must be noted that a statistically significant difference in survival ($p < 0.05$) was not observed between the ECP-injected and PBS-injected groups. These results may possibly indicate an immunostimulatory effect of the ECP, contributing to an overall enhanced immunological capability and resulting in comparatively better survival in the ECP-injected fish than in the control group. This notion is not without precedent; Amend and Johnson (1984) similarly found that a pH-lysed *R. salmoninarum* bacterin was able to potentiate protection against *Aeromonas salmonicida* when given as a bivalent vaccine along with the *A. salmonicida* bacterin. It is interesting to note that other bacterial preparations, including the lipopolysaccharide of Gram-negative organisms and the killed *Mycobacterium* incorporated in complete Freund's adjuvant have been shown to be immunostimulatory in piscine species (Anderson, 1992b). The immunostimulatory effects of *Mycobacterium* antigens are particularly intriguing in comparison to the potential for analogous effects with *R. salmoninarum* antigens, given the similar pathogenesis of the infections induced by the two agents (i.e. chronic granulomatous diseases involving facultatively intracellular pathogens and prominent cell-mediated immune responses).

Regarding the intracellular localization of *R. salmoninarum*, it is important to note that this investigation presented ECP to the host tissues presumably in the extracellular space. It is quite possible that intracellular exposure of salmonid immunocytes to the ECP would produce an entirely different result. Therefore, based on these and previous findings, further investigation into the possibility of immunological potentiation by the *R. salmoninarum* ECP is warranted.

Additional findings in this investigation pertaining to the effects of vaccination upon the immunological parameters also proved interesting. Decreased post-challenge hematocrit and serum protein levels in unvaccinated fish (the groups experiencing mortalities) may be the effects of clinical ERM, a hemorrhagic disease. Differential leukocyte alterations including the increases in numbers of large lymphocytes, PMN's, and monocytes in unvaccinated fish may again be a response to ERM and the resulting bacteremia; increased numbers of small lymphocytes post-challenge in vaccinated fish may represent an increased level of "memory" cells in the secondary immune response. Again, without the differentiation of functional lymphocyte populations, however, this theory remains speculatory. The post-challenge increase in antibody microagglutination titer in vaccinated fish was expected as the secondary humoral response against *Y. ruckeri* antigen in salmonids is well documented (Austin and Austin, 1993). The lack of a vaccination-related stimulation of phagocytosis was also not unexpected as cell-mediated immunity generally is poorly induced by bacterins (Tizard, 1987).

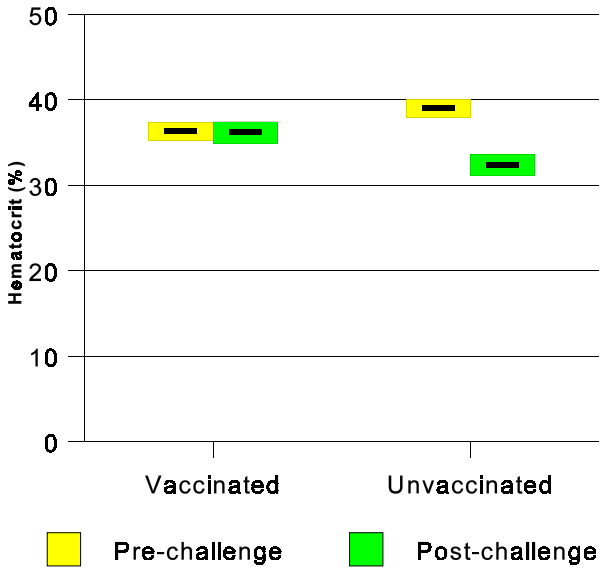

Figure 1a: Hematocrit.

Hematocrit values for vaccinated and unvaccinated fish, pre and post-challenge. A significant decrease in hematocrit was evident post-challenge in unvaccinated fish only. (Bar = mean value/ Box = 95% confidence interval)

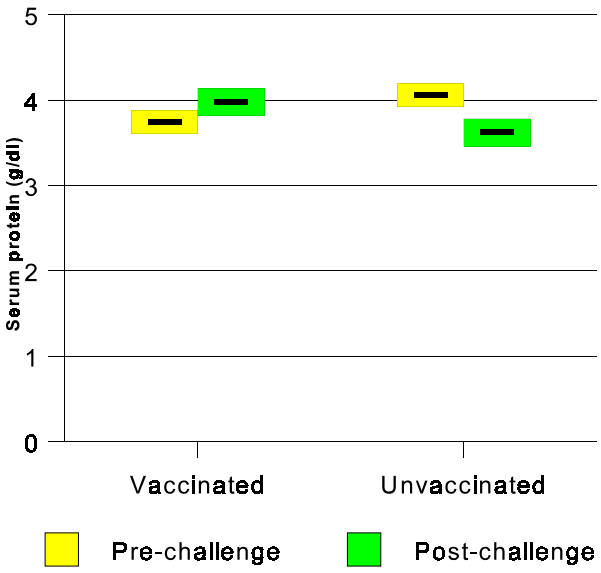

Figure 1b: Serum protein

Serum protein values for vaccinated and unvaccinated fish, pre and post-challenge. A significant decrease in serum protein was evident after challenge in unvaccinated fish only. (Bar = mean value/ Box = 95% confidence interval)

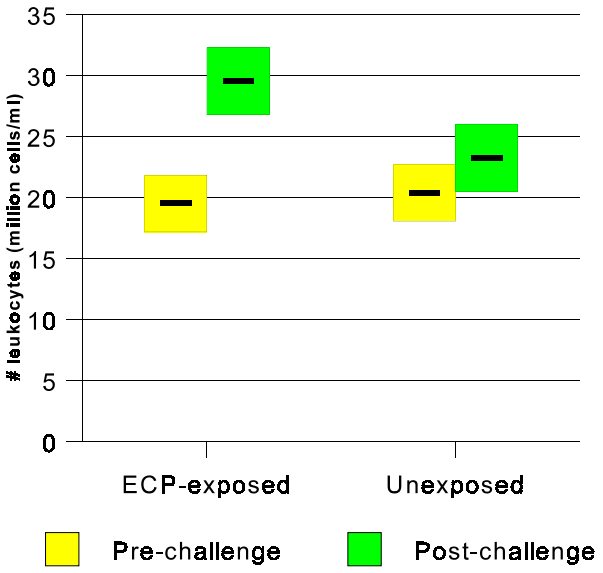

Figure 2a: Leukocyte count variation with ECP exposure, pre and post-challenge.

A significant increase in the total leukocyte count was evident post-challenge for fish exposed to the ECP, but not for unexposed fish.
(Bar = mean value/ Box = 95% confidence interval)

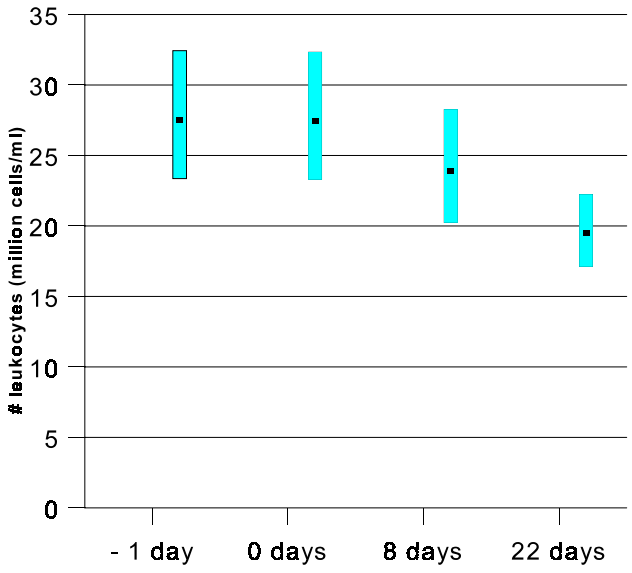

Figure 2b: Leukocyte count variation with the chronology of the ECP-exposure.

A significantly lower total leukocyte count was evident for fish exposed to the ECP 22 days post-vaccination compared tofish exposed 1 day prior to or simultaneously with vaccination.
(Bar = mean value/ Box = 95% confidence interval)

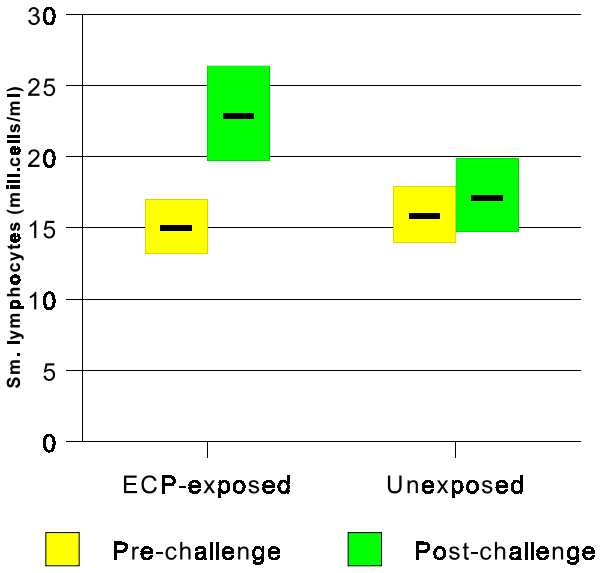

Figure 3a: Small lymphocyte counts and ECP exposure.

A significant increase in the small lymphocyte count was evident post-challenge for fish exposed to the ECP. (Bar = mean value/ Box = 95% confidence interval)

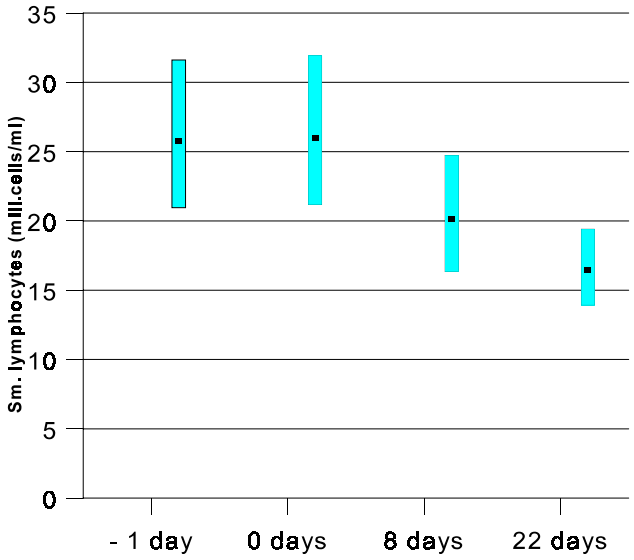

Figure 3b: Small lymphocyte count based upon the chronology of the ECP exposure.

A significant decrease in small lymphocyte counts was evident in the fish exposed to the ECP 22 days after vaccination compared to fish exposed 1 day prior to or simultaneously with vaccination. (Bar = mean value/ Box = 95% confidence interval)

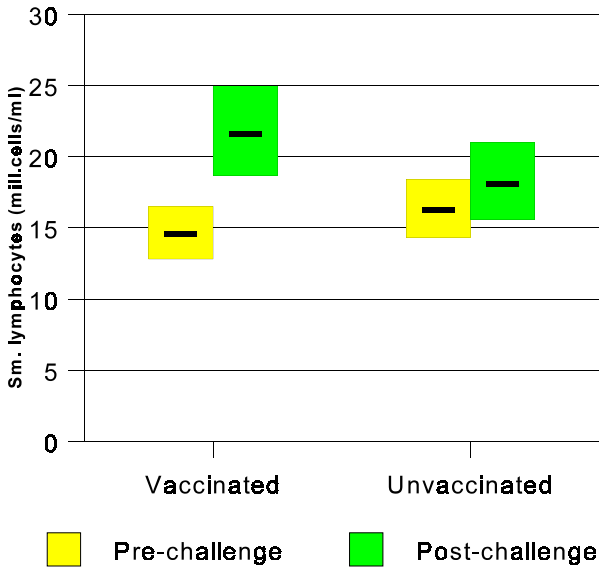

Figure 3c: Small lymphocyte count and vaccination status.

A significant increase in small lymphocyte count was evident post-challenge for fish vaccinated against *Y. ruckeri*, but not for unvaccinated fish.
(Bar = mean value/ Box = 95% confidence interval)

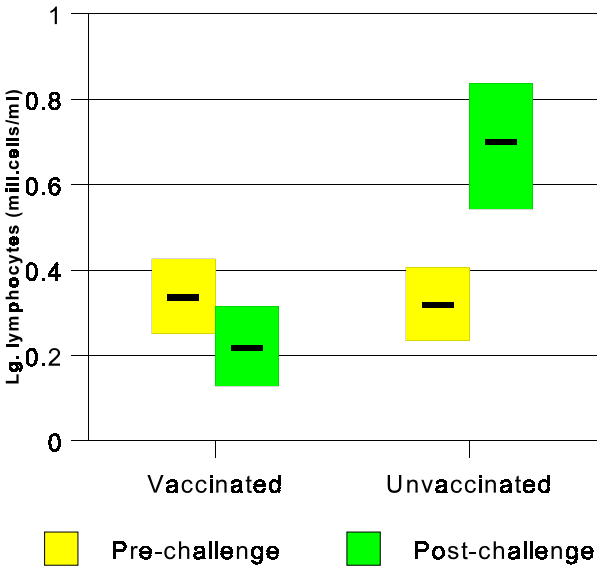

Figure 4: Large lymphocyte count.

A significant increase in the large lymphocyte differential cell count was evident post-challenge for unvaccinated fish, but not for vaccinated fish
(Bar = mean value/ Box = 95% confidence interval)

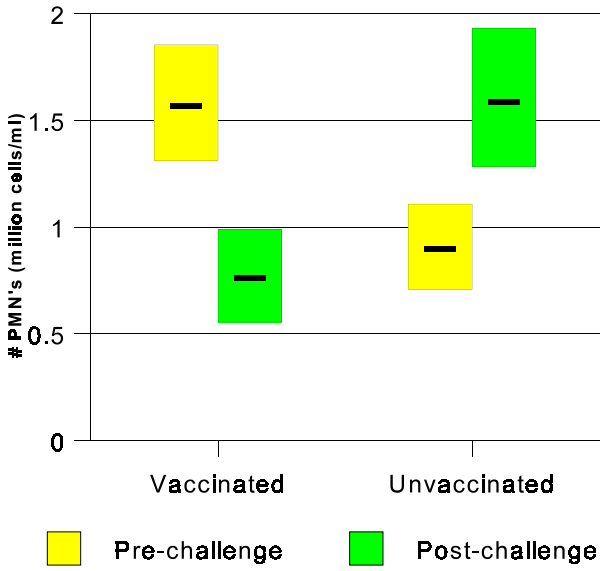

Figure 5: PMN cell count.

A significant decrease in the PMN differential cell count was evident post-challenge for vaccinated fish, while a significant post-challenge increase in this value was evident for the unvaccinated fish.
(Bar = mean value/ Box = 95% confidence interval)

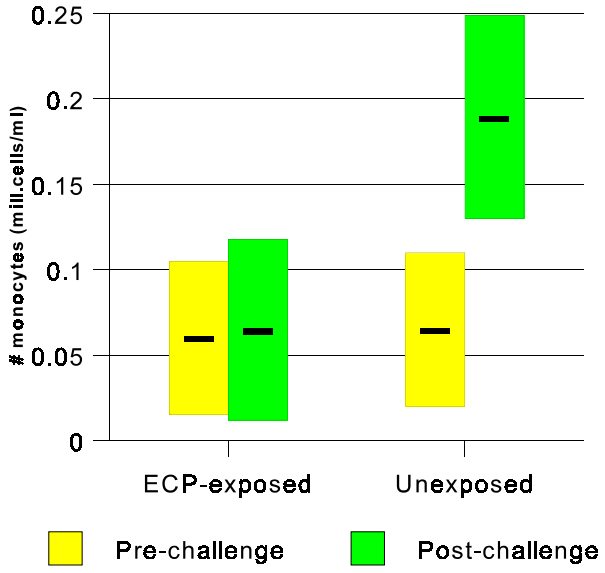

Figure 6a: Monocyte counts based upon ECP exposure.

A significant increase in the differential monocyte count was evident post-challenge in fish not exposed to the ECP, but not for the ECP-exposed fish.
(Bar = mean value/ Box = 95% confidence interval)

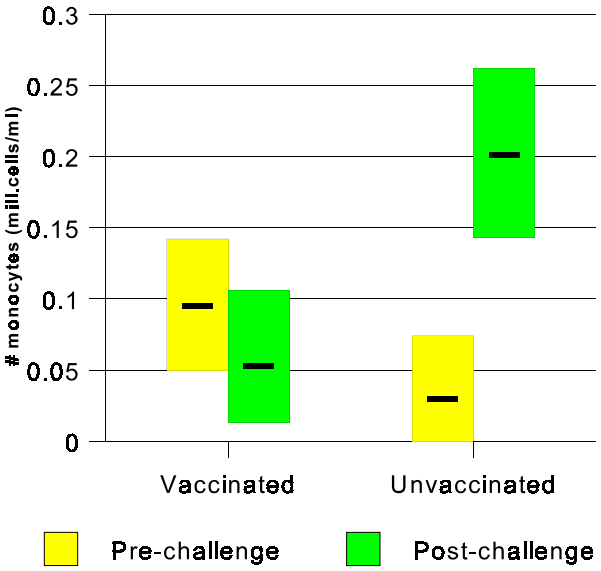

Figure 6b: Monocyte counts based upon vaccination status.

A significant increase in the monocyte differential count was evident post-challenge only for unvaccinated fish.
(Bar = mean value/ Box = 95% confidence interval)

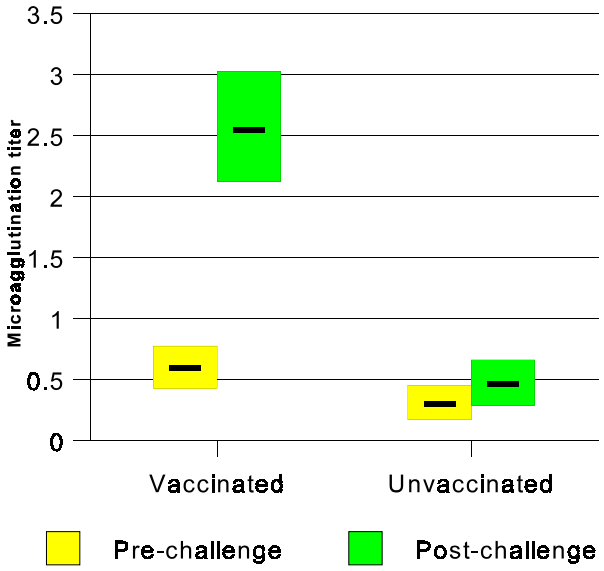

Figure 7: Microagglutination antibody titer.

Statistical evaluation of the microagglutination titer showed a significant post-challenge increase in titer for vaccinated fish. (Bar = mean value/ Box = 95% confidence interval)

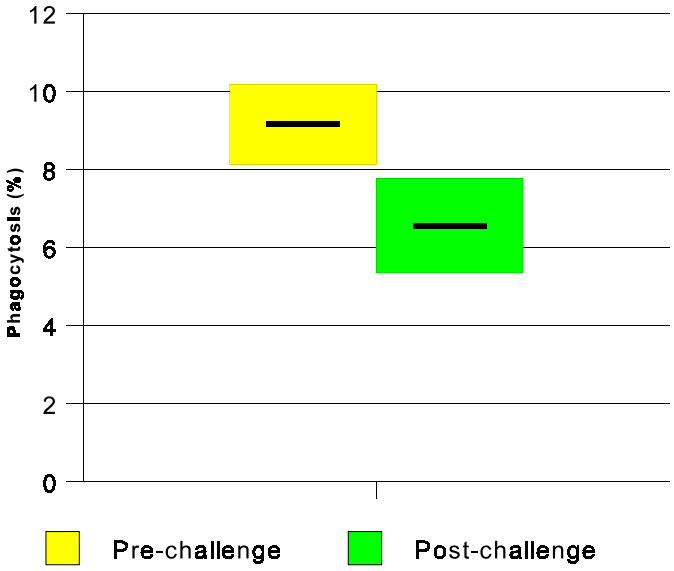

Figure 8: Phagocytosis.

Phagocytosis capabilites of splenic phagocytes of fish from all experimental groups. A statistically significant decrease in phagocytosis was evident post-challenge.
(Bar = mean value/ Box = 95% confidence interval)

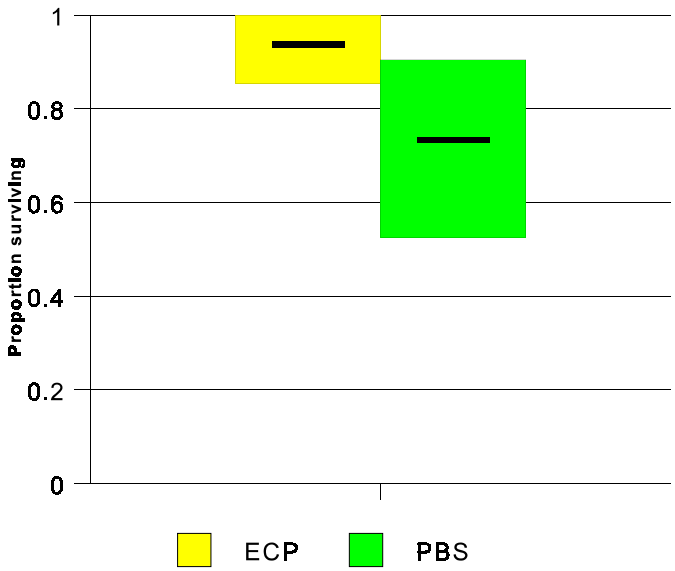

Figure 9: Survival.

A survival estimate (proportion) for the two experimental groups with unvaccinated fish. ECP = ECP-injected, PBS = PBS-injected (control). Thiry days post-challenge, These two groups were the only to suffer ERM-related mortalities. While survival was higher for the ECP-injected group, the difference in survival between these two groups was not significant at p = 0.05.
(Bar = mean value/ Box = 95% confidence interval)

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Appendices: Data Summary Tables

Data summaries for the immunological parameters evaluated are contained in the following tables of this appendix. Numbers in cells of the tables represent the mean values for all fish sampled at each sampling interval in each treatment group, followed by the standard deviations (in parentheses). For the two groups in which the ECP was given 8 and 22 days post-vaccination, all pre-challenge sample times are based upon ECP injection alone, and therefore these pre-challenge samples are not chronologically equivalent to the other groups.

(* Microagglutination titer numerical data is based on reciprocals of $\log_2$ dilutions. Mean values with standard deviations are not represented in Appendix C; instead, all titer values for each experimental group and sampling interval are listed.)

Appendix A: Hematocrit (%)

time	vaccinated		unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	3.8 (3.1)	34.6 (3.8)	39.4 (4.8)	40.7 (3.7)	34.2 (5.8)	33.8 (3.1)	37.3 (8.8)	30.9 (4.3)
1 d	35.3 (3.7)	32.4 (2.9)	39.6 (3.7)	38.8 (5.7)	35.8 (3.9)	35.3 (3.7)	37.8 (4.4)	33.3 (5.7)
2 d	39.3 (7.1)	38.4 (4.4)	40.3 (4.5)	42.4 (3.9)	34.9 (4.9)	39.3 (7.1)	43.2 (4.2)	35.6 (5.5)
4 d	36.1 (3.5)	38.4 (5.3)	36.7 (4.2)	41.3 (6.4)	36.1 (4.5)	36.1 3.5)	39.6 (6.1)	34.8 (5.2)
8 d	39.0 (4.1)	38.8 (7.6)	37.4 (4.2)	36.1 (4.1)	39.3 (5.6)	39.0 (4.1)	35.4 (6.4)	33.6 (6.8)
16 d	34.8 (3.2)	36.7 (6.8)	36.6 (2.1)	40.1 (5.2)	36.3 (7.1)	34.8 (3.2)	35.6 (2.9)	34.4 (4.8)
22 d	34.7 (4.3)	35.8 (3.5)	37.6 (7.0)	39.3 (5.7)	38.3 (9.3)	34.7 (4.3)	---	---
* Time of Challenge								
23 d	35.7 (4.3)	35.1 (4.0)	33.3 (3.6)	35.3 (5.5)	33.6 (2.0)	35.7 (4.3)	30.8 (6.0)	33.3 (5.7)
26 d	37.0 (4.9)	39.3 (4.3)	36.7 (4.8)	37.1 (6.9)	34.0 (3.3)	37.0 (4.9)	36.8 (6.4)	34.8 (5.2)
30 d	40.3 (7.3)	40.6 (2.1)	30.6 (4.4)	33.1 (3.2)	38.1 (5.5)	40.3 (7.3)	39.4 (5.5)	33.6 (6.8)
38 d	31.1 (2.1)	35.1 (4.0)	30.0 (5.6)	25.3 (6.2)	39.7 (6.9)	31.1 (2.1)	36.0 (2.7)	34.4 (4.8)
52 d	34.8 (3.6)	32.7 (4.1)	28.4 (8.4)	34.0 (7.8)	36.6 (6.8)	34.8 (3.6)	39.4 (3.1)	37.1 (5.3)

Appendix B: Total serum protein (g/dl)

time	vaccinated		unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	3.7 (0.8)	3.8 (0.8)	4.3 (0.7)	4.6 (0.2)	3.4 (0.9)	3.7 (0.8)	3.1 (1.1)	4.2 (0.7)
1 d	4.1 (0.7)	3.3 (0.6)	4.7 (0.1)	4.3 (0.3)	3.8 (0.8)	4.1 (0.7)	3.6 (0.6)	3.6 (0.7)
2 d	4.1 (1.1)	3.5 (0.6)	4.3 (0.4)	4.2 (0.5)	3.3 (0.5)	4.1 (1.1)	3.4 (0.5)	3.9 (0.8)
4 d	3.4 (0.4)	4.0 (0.6)	3.7 (0.3)	3.9 (0.4)	3.6 (0.4)	3.4 (0.4)	3.4 (0.8)	3.5 (0.9)
8 d	3.8 (0.6)	4.1 (1.2)	3.7 (0.6)	3.8 (0.9)	3.4 (0.6)	3.8 (0.6)	3.8 (0.4)	4.1 (0.4)
16 d	3.3 (0.7)	3.5 (1.0)	3.6 (0.5)	3.7 (0.8)	3.5 (0.7)	3.3 (0.7)	3.6 (0.8)	4.0 (0.8)
22 d	3.8 (0.4)	3.7 (0.5)	3.7 (0.6)	4.0 (0.6)	3.9 (0.9)	3.8 (0.4)	---	---
*** Time of challenge**								
23 d	4.0 (0.5)	4.1 (0.6)	3.8 (0.6)	3.3 (0.7)	3.8 (0.4)	4.0 (0.5)	3.3 (0.5)	3.6 (0.7)
26 d	3.8 (0.4)	3.5 (0.7)	3.5 (0.3)	3.5 (0.4)	2.9 (0.9)	3.8 (0.4)	3.6 (0.5)	3.5 (0.9)
30 d	4.4 (0.5)	3.8 (0.2)	3.3 (0.4)	3.4 (0.6)	3.4 (0.7)	4.4 (0.5)	4.1 (0.4)	4.1 (0.4)
38 d	4.3 (0.2)	4.7 (0.6)	4.0 (0.8)	4.3 (0.5)	4.4 (0.6)	4.3 (0.2)	4.2 (0.3)	4.0 (0.8)
52 d	3.6 (0.3)	3.4 (0.5)	3.1 (0.7)	3.9 (0.4)	3.4 (0.9)	3.6 (0.3)	3.9 (0.3)	4.2 (0.3)

Appendix C: Microagglutination antibody titers

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	2,1,1,1, 1,1,1	1,1,3,0, 3,1,2	2,1,0,4, 1,4,1	0,0,0,0, 0,1,1	2,2,1,2, 1,0,3	2,1,1,1, 1,1,1	2,0,0,0, 0,1,0	1,0,2,7 6,2,5
1 d	1,0,1,0, 0,1,0	0,0,0,1, 0,0,1	0,0,0,1, 0,1,3	0,0,0,2, 0,0,2	0,0,0,2, 3,2,2	1,0,1,0, 0,1,0	0,0,2,0, 0,2,0	0,0,2,4, 4,0,1
2 d	0,0,0,1, 1,0,0	0,0,0,0, 2,2,1	0,1,0,0, 1,0,1	0,0,0,0, 0,1,1	0,2,0, 0,0,	0,0,0,1, 1,0,0	0,0,0,0, 0,0,0	0,5,7,5, 5,0,3
4 d	1,0,0,1, 1,0,1	1,0,0, 0,1,0	0,0,0,0, 0,2,0	0,0,0, 0,1,0	0,0,0,0, 0,0,0	1,0,0,1, 1,0,1	1,0,0, 0,1,0	0,0,0,0, 0,0,0
8 d	1,0,0,0, 1,0,0	0,0,1, 0,0,0	0,0,0,0, 0,0,0	0,1,0,0, 0,0,0	0,0,0, 0,0,0	1,0,0,0, 1,0,0	0,0,0,0, 0,0,0	5,1,0,6, 3,2,0
16 d	0,0,3,1, 3,3,2	2,0,1,4, 0,0,1	0,0,0, 0,0,1	0,0,0, 0,0,1	0,3,0, 1,3	0,0,3,1, 3,3,2	0,1,0, 4,3	5,5,3,3, 7,4,6
22 d	2,0,0,5, 0,2,5	1,4,1,0, 0,0,1	0,1,0,0, 1,0,0	0,1,0,1, 1,2,0	0,0,4,0, 3,0,4	2,0,0,5, 0,2,5	---	---
*Time of Challenge								
23 d	1,1,1,0, 4,0,1	1,6,6,0 7,0,4	0,0,0,0, 0,0,0	0,0,0,0 0,1,0	0,1,1,0, 0,2,7	1,1,1,0, 4,0,1	1,1,6,3, 3,6,2	0,0,2,4, 4,0,1
26 d	6,5,7,0, 6,4,2	1,2,1,3, 3,6,7	0,0,1,0, 0,0,2	0,0,1,2, 0,0,0	4,6,3,1, 4,3,4	6,5,7,0, 6,4,2	6,6,6,2, 3,2,5	4,1,5,4, 0,4,5
30 d	0,5,2,6, 5,4,6	1,1,5,0, 5,2,5	7,1,4,1, 1,0,1	0,1,0,0, 1,1,1	5,4,3,4, 5,6,4	0,5,2,6, 5,4,6	5,1,1,6, 1,0,4	5,1,0,6, 3,2,0
38 d	1,7,7,1, 4,5,5	6,0,4,2, 3,1,1	0,3,0,0, 0,0,2	0,0,0,0, 0,0,0	0,0,5,5, 7,5,6	1,7,7,1, 4,5,5	7,0,5,4, 3,8,5	5,5,3,3, 7,4,6
52 d	0,0,4,4, 5,6,6	3,5,3,4, 1,5,10	3,1,2,3, 2,1,2	0,0,1,0, 0,3,2	4,0,0,3, 5,2,5	0,0,4,4, 5,6,6	2,0,4,3, 4,1,7	1,1,0,0, 3,4,3

Appendix D: Total Leukocyte Count (million cells/ml)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	9.7 (2.8)	11.3 (5.3)	18.3 (6.3)	17.1 (5.2)	12.8 (3.5)	9.8 (2.8)	18.4 (5.7)	18.0 (5.9)
1 d	12.6 (7.9)	14.7 (6.9)	13.3 (6.9)	9.5 (6.2)	16.1 (5.4)	12.6 (7.9)	35.3 (8.5)	17.5 (6.1)
2 d	17.2 (5.3)	19.3 (7.5)	18.2 (6.6)	18.1 (5.8)	30.1 (7.9)	17.2 (5.3)	37.2 (14.0	17.3 (10.2)
4 d	21.5 (3.7)	26.7 (10.1)	21.3 (11.3)	23.3 (8.1)	13.1 (6.5)	21.5 (3.7)	34.4 (12.9	27.2 (4.3)
8 d	23.1 (10.5)	20.1 (8.5)	16.8 (8.0)	27.6 (10.1)	19.0 (9.9)	23.1 (10.5)	22.2 (3.1)	21.8 (5.8)
16 d	27.6 (16.1)	27.7 (14.7)	20.0 (7.7)	26.6 (10.0)	27.7 (9.1)	27.6 (16.1)	20.3 (6.8)	19.3 (5.7)
22 d	25.2 (9.9)	24.3 (11.0)	27.8 (5.3)	18.7 (6.1)	33.1 (13.0)	25.2 (9.9)	---	---
*** Time of Challenge**								
23 d	24.8 (11.7)	15.8 (4.6)	28.6 (12.6)	23.3 (9.5)	43.6 (13.9)	24.8 (11.7)	17.7 (5.6)	17.5 (6.1)
26 d	38.8 (19.1)	15.8 (4.6)	28.6 (12.6)	23.3 (9.5)	43.6 (13.9)	24.8 (11.7)	17.7 (5.6)	17.5 (6.1)
30 d	34.1 (18.6)	30.3 (6.2)	17.7 (6.1)	14.7 (5.0)	31.5 (10.6)	34.1 (18.6)	34.9 (11.3)	21.8 (5.8)
38 d	16.8 (8.4)	10.0 (9.5)	13.7 (6.7)	24.2 (9.8)	23.2 (12.0)	16.8 (8.4)	12.8 (2.1)	19.3 (5.7)
52 d	44.7 (12.8)	35.9 (16.9)	33.9 (11.2)	28.3 (15.3)	18.8 (7.3)	44.7 (12.8)	32.5 (10.5)	29.1 (7.7)

Appendix E: Small Lymphocyte Count (million cells/ml)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	8.3 (2.7)	9.5 (4.8)	15.1 (5.2)	16.2 (5.3)	8.7 (4.1)	8.3 (2.7)	15.4 (5.8)	14.0 (5.2)
1 d	9.1 (7.0)	9.6 (5.2)	10.8 (5.4)	9.0 (5.9)	11.7 (6.0)	9.1 (6.9)	29.0 (8.4)	14.9 (5.4)
2 d	12.0 (4.6)	13.0 (5.7)	17.2 (6.2)	16.5 (5.9)	24.1 (5.6)	12.0 (4.6)	31.8 (12.3)	15.5 (9.3)
4 d	18.4 (3.3)	22.8 (9.0)	19.7 (11.2)	22.1 (8.0)	10.7 (4.9)	18.4 (3.3)	31.4 (11.6)	25.4 (3.6)
8 d	24.4 (7.2)	18.9 (8.4)	15.6 (7.5)	26.3 (9.7)	16.7 (9.2)	24.4 (9.2)	21.4 (3.5)	21.0 (6.2)
16 d	26.9 (16.2)	26.2 (14.0)	18.8 (8.0)	25.5 (9.7)	26.4 (8.6)	26.9 (16.2)	17.8 (5.7)	18.3 (5.3)
22 d	21.4 (7.2)	22.6 (10.3)	25.8 (5.8)	17.1 (5.8)	30.7 (13.1)	21.4 (7.2)	---	---
* Time of Challenge								
23 d	23.1 (10.7)	14.5 (4.1)	25.2 (10.0)	19.5 (10.2)	40.6 (13.7)	23.1 (10.7)	15.5 (5.3)	14.9 (5.4)
26 d	37.7 (18.6)	22.4 (10.7)	40.0 (10.8)	25.2 (5.4)	31.6 (7.5)	37.8 (18.6)	35.3 (14.1)	25.4 (3.6)
30 d	33.0 (19.1)	28.6 (6.4)	15.5 (5.8)	11.7 (5.0)	30.2 (10.4)	33.0 (19.1)	33.0 (6.0)	21.0 (6.2)
38 d	15.3 (7.8)	9.3 (8.6)	10.7 (7.0)	18.1 (10.6)	21.8 (11.0)	15.3 (7.8)	11.9 (2.4)	18.3 (5.3)
52 d	42.9 (12.1)	34.8 (16.5)	28.1 (7.9)	28.1 (13.6)	18.1 (6.9)	42.9 (12.1)	31.7 (10.2)	27.7 (7.9)

Appendix F: Large Lymphocyte Count (million cells/ml)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	16 d	22 d
12 h	0.12 (0.08)	0.18 (0.28)	0.42 (0.22)	0.29 (0.18)	0.88 (0.93)	0.12 (0.08)	0.55 (0.73)	1.22 (0.59)
1 d	0.71 (0.61)	0.65 (0.59)	0.59 (0.58)	0.18 (0.14)	0.54 (0.39)	0.71 (0.61)	0.91 (1.04)	0.86 (0.70)
2 d	0.34 (0.23)	0.66 (0.84)	0.12 (0.12)	0.35 (0.21)	0.70 (0.34)	0.34 (0.23)	0.54 (0.68)	0.39 (0.25)
4 d	0.37 (0.29)	0.69 (0.88)	0.14 (0.22)	0.40 (0.18)	0.56 (0.56)	0.37 (0.29)	0.38 (0.75)	0.47 (0.45)
8 d	0.23 (0.26)	0.44 (0.40)	0.42 (0.31)	0.10 (0.14)	0.62 (0.54)	0.23 (0.26)	0.31 (0.43)	0.35 (0.22)
16 d	0.12 (0.13)	0.24 (0.25)	0.29 (0.19)	0.51 (0.59)	0.60 (0.63)	0.12 (0.13)	0.41 (0.60)	0.25 (0.23)
22 d	0.18 (0.21)	0.55 (0.54)	0.72 (0.58)	0.44 (0.59)	0.39 (0.47)	0.18 (0.21)	---	---
* Time of Challenge								
23 d	0.20 (0.26)	0.46 (0.54)	0.46 (0.40)	0.69 (0.22)	1.29 (0.63)	0.20 (0.26)	0.46 (0.32)	0.86 (0.70)
26 d	0.07 (0.14)	0.14 (0.25)	0.15 (0.25)	0.55 (0.50)	0.86 (0.46)	0.07 (0.14)	0.70 (0.77)	0.47 (0.45)
30 d	0.30 (0.34)	0.46 (0.38)	1.05 (1.52)	1.16 (0.92)	0.25 (0.29)	0.30 (0.34)	0.50 (0.62)	0.35 (0.22)
38 d	0.26 (0.39)	0.19 (0.33)	1.19 (1.80)	2.09 (1.86)	0.11 (0.19)	0.26 (0.39)	0.18 (0.15)	0.25 (0.23)
52 d	0.36 (0.40)	0.12 (0.23)	1.62 (3.07)	0.80 (0.77)	0.27 (0.23)	0.36 (0.40)	0.11 (0.19)	0.21 (0.27)

Appendix G: Polymorphonuclear Cell Count (million cells/ml)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	1.27 (0.46)	1.61 (1.48)	2.82 (2.92)	0.70 (0.41)	2.50 (2.64)	1.27 (0.46)	2.26 (1.91)	2.68 (1.33)
1 d	2.63 (2.00)	4.18 (3.65)	1.84 (1.64)	0.37 (0.29)	3.72 (1.24)	2.63 (2.00)	4.95 (3.22)	1.43 (0.55)
2 d	4.69 (3.33)	5.24 (4.07)	0.79 (0.77)	1.23 (0.93)	5.07 (3.23)	4.69 (3.33)	4.68 (2.49)	1.43 (1.68)
4 d	2.49 (1.77)	3.05 (1.93)	1.45 (1.73)	0.71 (0.39)	1.77 (1.62)	2.49 (1.77)	2.68 (3.76)	1.28 (1.14)
8 d	0.85 (0.79)	0.65 (0.50)	0.74 (0.36)	1.17 (1.10)	1.34 (1.68)	0.85 (0.79)	0.52 (0.40)	0.86 (0.76)
16 d	0.56 (0.86)	1.15 (0.77)	0.89 (0.96)	0.58 (0.41)	0.68 (0.56)	0.56 (0.86)	1.07 (1.57)	0.73 (0.53)
22 d	0.80 (0.83)	1.13 (0.72)	1.26 (0.64)	1.17 (1.26)	1.90 (0.90)	0.80 (0.83)	---	---
* Time of Challenge								
23 d	1.52 (1.82)	0.72 (0.35)	2.73 (3.11)	2.24 (1.28)	1.33 (0.90)	1.52 (1.82)	1.52 (1.09)	1.43 (0.55)
26 d	1.01 (1.52)	0.31 (0.25)	1.18 (0.60)	0.99 (0.23)	2.24 (2.47)	1.01 (1.52)	1.13 (1.25)	1.28 (1.14)
30 d	0.81 (1.18)	1.25 (1.17)	1.22 (0.93)	1.74 (1.28)	0.99 (0.99)	0.81 (1.18)	1.40 (0.78)	0.86 (0.76)
38 d	1.26 (0.90)	0.49 (0.62)	1.64 (1.12)	3.41 (2.56)	1.19 (1.04)	1.26 (0.90)	0.69 (0.69)	0.73 (0.53)
52 d	1.35 (0.98)	0.85 (0.52)	3.99 (3.22)	1.26 (1.50)	0.45 (0.54)	1.35 (0.98)	0.71 (0.69)	1.09 (0.69)

Appendix H: Monocyte Count (million cells/ml)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	0.02 (0.05)	0 (0)	0.03 (0.07)	0.03 (0.07)	0.12 (0.27)	0.02 (0.05)	0.16 (0.27)	0.02 (0.05)
1 d	0.21 (0.30)	0.29 (0.40)	0.09 (0.18)	0 (0)	0.09 (0.15)	0.21 (0.30)	0.48 (0.59)	0.26 (0.19)
2 d	0.18 (0.31)	0.34 (0.41)	0.04 (0.10)	0.04 (0.10)	0.20 (0.32)	0.18 (0.31)	0.15 (0.26)	0.04 (0.11)
4 d	0.26 (0.50)	0.13 (0.36)	0 (0)	0.06 (0.10)	0.14 (0.31)	0.26 (0.50)	0 (0)	0.08 (0.13)
8 d	0 (0)	0.07 (0.13)	0.05 (0.10)	0 (0)	0.03 (0.05)	0 (0)	0 (0)	0 (0)
16 d	0 (0)	0.08 (0.20)	0.05 (0.10)	0 (0)	0 (0)	0 (0)	0 (0)	0.06 (0.11)
22 d	0 (0)	0.03 (0.08)	0.08 (0.14)	0 (0)	0.08 (0.14)	0 (0)	---	---
* Time of Challenge								
23 d	0.02 (0.04)	0.09 (0.11)	0.22 (0.34)	0.52 (0.25)	0.36 (0.96)	0.02 (0.04)	0.22 (0.28)	0.26 (0.19)
26 d	0 (0)	0.28 (0.32)	0.05 (0.15)	0.14 (0.19)	0.18 (0.30)	0 (0)	0 (0)	0.08 (0.13)
30 d	0 (0)	0 (0)	0.02 (0.04)	0.13 (0.23)	0.05 (0.12)	0 (0)	0 (0)	0 (0)
38 d	0.05 (0.09)	0.02 (0.04)	0.17 (0.26)	0.56 (0.56)	0.10 (0.17)	0.05 (0.09)	0.04 (0.06)	0.06 (0.11)
52 d	0.08 (0.22)	0.07 (0.18)	0.16 (0.33)	0 (0)	0.06 (0.12)	0.08 (0.22)	0.05 (0.13)	0.10 (0.26)

Appendix I: Phagocytosis (%)

Time	Vaccinated		Unvaccinated		ECP given at:			
	ECP	no ECP	ECP	no ECP	-1 d	0 d	8 d	22 d
12 h	8.6 (5.4)	12.4 (5.4)	16.8 (5.8)	16.8 (8.8)	18.4 (5.9)	8.6 (5.4)	6.8 (2.2)	3.8 (1.6)
1 d	11.0 (5.1)	7.8 (5.8)	10.6 (6.0)	11.4 (1.3)	11.2 (5.9)	11.0 (5.1)	5.4 (3.0)	7.4 (4.3)
2 d	11.6 (1.7)	6.6 (2.9)	14.0 (4.2)	8.6 (5.3)	4.8 (4.0)	11.6 (1.7)	7.2 (3.0)	5.6 (2.3)
4 d	5.6 (3.1)	7.6 (4.9)	6.6 (3.1)	10.0 (5.6)	14.4 (3.4)	5.6 (3.1)	5.2 (3.4)	7.8 (5.4)
8 d	1.2 (1.8)	10.6 (6.3)	8.6 (4.9)	5.4 (4.0)	11.6 (9.8)	1.2 (1.8)	7.4 (4.3)	2.0 (1.2)
16 d	6.0 (2.1)	10.2 (7.6)	5.2 (4.2)	6.4 (3.4)	13.6 (6.3)	6.0 (2.1)	5.2 (6.6)	10.2 (6.2)
22 d	9.4 (5.3)	17.8 (11.3	4.4 (3.4)	5.2 (3.3)	10.0 (3.2)	9.4 (5.3)	---	---
* Time of Challenge								
23 d	9.6 (8.1)	11.0 (4.1)	8.8 (5.8)	5.2 (4.3)	4.4 (4.5)	9.6 (8.1)	11.0 (7.4)	7.4 (4.3)
26 d	11.0 (5.5)	10.4 (8.9)	3.4 (4.9)	3.6 (4.8)	9.4 (6.1)	11.0 (5.5)	12.4 (8.8)	7.8 (5.9)
30 d	3.8 (2.7)	2.2 (1.6)	9.4 (9.5)	14.8 (9.8)	4.8 (3.8)	3.8 (2.7)	3.0 (2.9)	2.0 (1.2)
38 d	3.2 (1.9)	11.2 (1.9)	8.8 (3.9)	8.0 (5.6)	4.8 (4.1)	3.2 (1.9)	5.2 (1.3)	10.2 (6.2)
52 d	3.6 (1.1)	1.2 (1.3)	0 (0)	2.0 (1.9)	0.4 (0.9)	3.6 (1.1)	1.8 (2.0)	1.2 (1.6)

Chapter 3

In vitro effects of the extracellular protein of *Renibacterium salmoninarum* on phagocyte function in brook trout (*Salvelinus fontinalis*)

Abstract

Renibacterium salmoninarum is a facultative intracellular pathogen often found in host phagocytes where it appears to successfully avoid the host fish's immunological defenses. The objective of this investigation was to determine whether the soluble extracellular protein produced by *R. salmoninarum* may contribute to the immunomodulation in bacterial kidney disease via inhibition of host phagocyte respiratory burst and/or phagocytosis mechanisms. Splenic cells from healthy adult brook trout (*Salvelinus fontinalis*) were incubated with two different concentrations of extracellular protein or viable *R. salmoninarum*. Splenic cell cultures were then evaluated for respiratory burst activity via flow cytometry with the dichlorofluorescin diacetate assay and for phagocytosis via light microscopic assessment of microsphere engulfment. Respiratory burst activity was inhibited in all experimental groups as compared to controls, while no differences were noted in phagocytic abilities in cells among the treatment groups.

I. Introduction

Renibacterium salmoninarum, a Gram-positive diplobacillus, is the causative agent of bacterial kidney disease (BKD) of salmonid fish. Affecting various species of both feral and cultured salmonids, BKD is a chronic granulomatous disease producing high levels of morbidity and mortality.

Bacterial kidney disease pathogenesis is complex, involving immunomodulatory effects of the pathogen upon the host. *Renibacterium salmoninarum*, similar to other pathogens such as *Mycobacterium* spp. and *Listeria* spp., has the ability to exist and even multiply intracellularly inside macrophages. Young and Chapman (1978) first described the intracellular existence of this bacteria, prior to its classification as *Renibacterium*. Flano et al. (1996) further characterized the chronology of BKD immunopathogenesis, including the intracellular existence of *R. salmoninarum* in hematopoietic tissue of experimentally infected coho salmon (*Oncorhynchus kisutch*). The ability of *R. salmoninarum* to resist killing and thereby survive and even replicate intracellularly inside rainbow trout macrophages over the course of several days was also observed by Klein (1992) and Bandin et al. (1993a). The significance of these findings to the pathogenesis of BKD is largely undetermined. Evendon et al. (1993) suggested that the enhanced intracellular survival of *R. salmoninarum* in phagocytic cells supports the likelihood that cellular transport plays a role in the pathogen's dissemination. However, Flano et al. (1996) proposed that the bacteria merely incubates intracellularly for a period whereas systemic dissemination occurs via freely circulating organisms.

In recent years, many investigations have examined the immunomodulatory effects of *R. salmoninarum* on salmonid phagocytes. Rose and Levine (1992) found that complement-mediated opsonization of *R.salmoninarum* enhanced bacterial adherence to and phagocytosis by rainbow trout macrophages. Similarly, Bandin et al. (1993b) found that bacteria coated with serum had enhanced survival inside rainbow trout phagocytes. Brattgjerd et al. (1996) determined that the immunostimulatory effects of M-Glucan on salmonid macrophage activities including phagocytosis did not extend to enhancement of macrophage uptake of *R. salmoninarum* in Atlantic salmon (*Salmo salar*). Investigations describing the respiratory burst activity of macrophages with regard to *R. salmoninarum* revealed that the superoxide anion response of rainbow trout macrophages was significantly decreased in the presence of *R. salmoninarum in vitro* (Bandin et al., 1993a). However, *R. salmoninarum* organisms are susceptible to killing by free radicals (H_2O_2) *in vitro* (Hardie et al., 1994). Sakai et al. (1996) found a significantly lower chemiluminescent response of salmonid phagocytes against *R. salmoninarum* when compared against a non-salmonid fish, indicating potentially diminished ability of salmonid phagocyte-mediated killing of *R. salmoninarum*.

The extracellular protein (ECP) produced by *R. salmoninarum* has also received considerable attention in the literature with regard to its role in BKD pathogenesis. The major soluble antigen of the ECP is most widely reported to be a relatively unstable 57 kD antigen found as a cell surface component or as a free protein, although discrepancies exist in the literature regarding stability and molecular weight (Griffiths and Lynch, 1991; Fielder and Draxl, 1986). *Renibacterium salmoninarum* ECP has been predictably encountered in association with both artificial microbial cultures and BKD-infected salmonid tissues. Tissue concentrations of ECP as high as 1.0 mg/ml have been reported (Kaattari et al., 1989). Both *in vivo* and *in vitro*, the ECP has been associated with a number of potential immunomodulatory effects. Among these are hemagglutination of rabbit erythrocytes (Daly and Stevenson, 1987), decreased *in vitro* antibody response of coho salmon, decreased hematocrit (Turaga et al., 1987), leukagglutination of salmonid leukocytes, and activation of macrophages (Kaattari et al., 1989). Still others have reported a complete lack of biological activity of the ECP (Bandin et al., 1991).

The purpose of this investigation was to determine whether the ECP of *Renibacterium salmoninarum* directly affects the function of salmonid phagocytes *in vitro*. In particular, respiratory burst activity and phagocytosis, two critical physiological processes of cell-mediated immunity, were examined in splenic phagocytes exposed to *R. salmoninarum* extracellular protein.

II. Materials and Methods

A. Extraction of *Renibacterium salmoninarum* extracellular protein

Extracellular protein in solution was recovered by a modification of the methods of Hsu et al., 1991 and Rockey et al., 1991. An isolate of the ATCC #33209 strain of *R. salmoninarum* obtained from the National Fish Health Research Laboratory in Kearneysville, West Virginia was subcultured and incubated on KDM2 agar plates at 15 ^{o}C for 20 days. Samples from the cultures were Gram-stained and serologically tested via the direct fluorescent antibody test (DFAT) for *R. salmoninarum* (Bullock et al., 1980) in order to confirm pure cultures of *R. salmoninarum*. Ten plates were rinsed with approximately 24 ml of sterile water, and the bacterial colonies were agitated to remove them from the agar. The bacterial suspension was centrifuged for 20 min. at 3000 *xg* and 10 ^{o}C. Twenty-two milliliters of supernatant were recovered and filter sterilized through a 0.2 µm filter (Acrodisc, Gelman Sciences; AnnArbor, MI). The bacterial cellular pellet was weighed to determine the protein:cell ratio. The supernatant fraction containing the ECP was frozen at - 20 ^{o}C for three weeks. Upon initiation of the experiment, the solution was thawed and

maintained at 4 °C. Protein concentration was determined with a modified enhanced BCA assay (Pierce; Rockford, IL), and the pH of the solution was determined with a standard pH meter.

B. Collection and incubation of splenic phagocytes

Sixteen healthy adult brook trout, *Salvelinus fontinalis*, from stock maintained at the National Fish Health Research Laboratory weighing approximately 200 g each (approximate splenic weights=0.5 g) were sacrificed via a lethal dose of tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO). The spleens were aseptically removed and placed in cold (4 °C) sterile Eagle's Minimum Essential Media (EMEM; Earle's salts containing 2% newborn calf serum, 100 mg/ml gentomycin, and 0.016 M Tris; Wolf and Quimby, 1973). Splenic cell suspensions were prepared via a modification of the method of Anderson (1992). Briefly, spleens were finely macerated and the cellular debris was resuspended in cold, sterile EMEM. Cell suspensions were washed twice in EMEM via centrifugation for 5 min. at 100 *xg* and 4 °C. Following the second wash, cells were resuspended in 1.01 ml of EMEM. Ten microliters of the suspensions were removed for determination of approximate leukocyte concentration with a hemacytometer (Klontz, 1994). Concentrations of all 16 trout cell cultures were adjusted to 10 million leukocytes/ml with additional EMEM and a minimum of 2 ml total volume per sample. Each cell culture was then divided into four aliquots of 0.5 ml, arbitrarily labeled Groups A through D. Treatments (0.5 ml each) were added to each group as follows:

Group A: sterile phosphate buffered saline

Group B: ECP solution at a final (incubation) concentration of 0.1 mg/ml

Group C: ECP solution at a final concentration of 1.0 mg/ml

Group D: *R. salmoninarum* cells in phosphate buffered saline, equivalent to the production of 0.1 mg of ECP.

Treated cell cultures were incubated with gentle agitation in polysterene round-bottomed tubes for approximately 18 h. at 4 °C.

C. Determination of phagocyte H_2O_2 production in respiratory burst activity

Respiratory burst assessment was performed via the method of Bass et al. (1983) as modified by Holladay et al. (1996). Following incubation, ten of the splenic cell cultures in each of Groups A through D were prepared for evaluation of phagocyte respiratory burst activity with the dichlorofluoroescin diacetate (DCF-DA) assay for flow cytometry. A 200 µl aliquot from each of the 40 samples was incubated in a polysterene round-bottomed tube

for 15 min. at 4 °C with 5 µl of DCF-DA (Molecular Probes, Eugene, OR; 5mM). Cells were subsequently stimulated by the addition of 10 µl phorbol-12-myristate 13-acetate (PMA; Sigma, St. Louis, MO; 100 ng/ml) and incubated similarly for 30 minutes. Immediately following incubation, the splenic cell suspensions were filtered through a 45 µm nytex filter to remove large acellular debris prior to flow cytometry. Background fluorescence, determined using unstained cells from each treatment group, was minimal and was subtracted from respective populations incubated with the fluorescent probe.

Flow cytometric analysis was performed on a Coulter EPICS V Flow Cytometer interfaced with an MDADS data analysis computer (Coulter Electronics, Hialeah, FL) and a hard copy display provided by Tektronix 4612 Video Hard Copy Unit (Tektronix, Wilsonville, OR). Standardization of the flow cytometer was performed using fluorescent calibration beads (Coulter) with 480 nm laser (Coherent, Palo Alto, CA) and excitation set at 300 mW. A population of large cells containing phagocytes was isolated on a two-parameter histogram of forward angle light scatter(FALS) and side angle light scatter(SALS), based on the high FALS (cell size) and SALS (granularity) characteristics of these cells. A 452-515 laser blocker was employed to block laser light from other fluorescent measurements. The fluorescent distribution was displayed as a 256 channel, single parameter histogram. Green fluorescence was measured through 550 nm longpass dichromic and 525 band pass filters. A gated amplifier was used to electronically exclude small particles and cellular debris from evaluation. For each of the 40 treated samples and 4 unstained controls, 5,000 events were collected and analyzed.

D. Determination of cellular phagocytosis

The remaining six samples of Groups A through D were utilized to evaluate cellular phagocytosis with a modification of the methods of Sakai et al. (1989) and Anderson (1992). Briefly, Fluoresbrite microspheres (1.16 µm; Polysciences, Inc., Warrington, PA) were adjusted to a concentration of 75,000 microspheres/µl in sterile phosphate buffered saline, and 1 ml of the microsphere suspension was sonicated for 30 sec. to disrupt aggregation. One milliliter of additional EMEM was added to each of the 24 treated splenic cell cultures, followed by 20 µl of the microsphere suspension. Cells were resuspended with agitation and incubated for an additional 24 h. at 4 °C. Following incubation, the splenic cells were centrifuged for 5 min. at 100 *xg* and 10 °C, washed in 1.5 ml EMEM, and centrifuged again as before. The supernatant was discarded, and the cell pellet was resuspended in 0.5 ml of EMEM. Each splenic cell culture was distributed evenly over two sterile microscope slides (0.25 ml/slide) and allowed to air dry at room temperature in the dark for 12 hours. Once dry, the slides were

fixed and stained using Leukostat (a Wright's stain modification; Fisher Diagnostics, Pittsburgh, PA).

Evaluation of phagocytosis was accomplished using conventional light microscopy. Microscope slides were viewed with oil immersion (magnification = 100x), and phagocytic cells (monocytes/macrophages and granulocytes) were identified based on morphology, and differentiated based on adherence/engulfment of microspheres (Figure 1). One hundred phagocytes were counted for each of the 24 groups, and the percentage of cells phagocytosing microspheres was recorded for each sample. The count was repeated in triplicate, and the mean percentage of phagocytosing cells was recorded for each of the samples.

III. Results

A. Extracellular protein

Protein concentration in the twenty-two ml of the solution containing the ECP was 2.0 mg/ml, yielding a total recovery of 44.0 mg protein. The weight of the corresponding bacterial cell pellet was 1.46 g, indicating a protein:cell ratio of 30.14 mg/g for this culture. The pH of the protein solution was determined to be 8.05.

B. DCF-DA assay for Respiratory Burst Activity

In the DCF-DA procedure, endogenous cellular esterases hydrolyze acetate groups from the DCF-DA molecule following passive diffusion of parent compounds into cells. This process converts DCF-DA into a non-fluorescent 2',7'-dichlorofluoroscene (DCFH) probe that is effectively trapped inside the cell. Subsequent production of H_2O_2 by PMA-activated cells during the oxidative respiratory burst oxidizes DCFH to the highly fluorescent 2',7'-dichlorofluoroscein (DCF) product, which can be readily detected and quantitated by flow cytometry (Bass et. al., 1983).

Flow cytometric analysis of the ten samples from each of four treatment groups produced numerical values for mean levels of cellular fluorescence as well as histograms displaying both mean cellular fluorescence and dot-plot representations of forward and side angle light scatter for the gated cell population examined for each sample. Statistical analysis of results revealed significant differences in the mean level of fluorescence (indicating significant differences in respiratory burst activity of phagocytes) in control Group A versus the three treatment groups (Dunnett's t-test, $p < 0.05$). Although there was no statistically significant difference among the three treatment groups, mean cellular fluorescence for Group C (treated with 1.0 mg/ml extracellular protein) was less than the fluorescence for Group B (0.1 mg/ml ECP) and Group D (*R. salmoninarum* cells equivalent to

the cell weight producing 0.1 mg/ml ECP in the initial protein harvest procedure). Groups B and D produced similar results numerically and graphically (Table 1; Figure 2).

C. Phagocytosis of Fluorescent Microspheres

Evaluation of cellular phagocytosis for the six samples in each of the four treatment groups revealed no significant differences in phagocytic capabilities among the four groups (Dunnett's t-test, $p<0.05$; Table 2; Figure 3).

IV. Discussion

An understanding of the immunomodulatory effects of a pathogen upon its host is critical to the development of protective measures against disease. The majority of literature regarding immunological prophylactic control of BKD have emphasized humoral factors. In recent years, however, this emphasis is shifting as the importance of cell-mediated protection in BKD pathogenesis is becoming apparent. For instance, Kaattari et al. (1989) reported that the greatest resistance to BKD development during vaccine trials occurred in salmonids vaccinated with Freund's complete adjuvant, a non-specific macrophage activator. This finding, coupled with the consistent difficulties encountered in attempts to develop a vaccine for BKD utilizing humoral protective mechanisms (Evendon et al., 1993), suggests that additional focus on the cell-mediated immune response to *R. salmoninarum* is warranted.

Our investigation provides further evidence that the immunomodulatory effects of *R. salmoninarum* may be exerted, at least in part, through cell-mediated immunity. Specifically, the extracellular protein of the bacteria may inhibit phagocytic cell functions essential to their killing ability. Respiratory burst activity, the production of oxygen free radicals in aerobic microbicidal defense, is a well established occurrence in piscine phagocytes, both mononuclear cells and granulocytes (Secombes and Fletcher, 1992). Bandin et al. (1993a) observed that the intracellular survival of microbial pathogens is a virulence mechanism common to virulent and avirulent strains of *R. salmoninarum*, and that the macrophage superoxide anion response as stimulated by PMA was reduced in the presence of *R. salmoninarum*. These previous findings coupled with results of the present investigation suggest that the inhibition of respiratory burst activity via the extracellular protein may be related to *R. salmoninarum*'s resistance to intracellular killing by phagocytes.

This study provides no evidence of *R. salmoninarum* ECP interference with phagocytosis. For a facultative intracellular pathogen which finds its way inside the host cell, interference with the phagocytosis processes of the cell may be

counterproductive to achieving intracellular existence. A number of facultative intracellular pathogenic bacteria, including *Mycobacterium tuberculosis*, *Brucella abortus*, and *Listeria monocytogenes* are readily phagocytosed by host cells and rely on chemical interference or protective cell wall components to avoid intracellular killing. Moreover, certain properties of *R. salmoninarum* have been reported as potential enhancement factors in phagocytosis, including the bacteria's hydrophobicity, leukagglutination, and complement-mediated opsonization (Rose and Levine, 1992).

An interesting feature of our current results is the suggestion of a dose-dependent relationship between the concentration of ECP and the degree of inhibition of the respiratory burst. As the concentration of ECP in salmonids with BKD can vary considerably (up to 1.0 mg/ml), this relationship could have a significant bearing upon BKD pathogenesis. There is some precedence for dose-dependency in the immunomodulatory effects of the ECP in that Kaattari et al. (1989) reported ECP induced activation of large, esterases-positive, vacuolated cells (presumably macrophages) in a dose-dependent manner. As results do not statistically confirm a dose-dependent relationship in this investigation, further investigation is warranted to confirm or dispute it. Clarification of other aspects of the ECP-phagocyte interaction in *R. salmoninarum* infected salmonids could prove quite beneficial to BKD control.

Table 1: Mean cellular fluorescence(%) of splenic cells in the 10 samples for each of the four treatment groups.

Group A = PBS control; Group B = 0.1 mg/ml ECP; Group C = 1.0 mg/ml ECP; Group D = *R. salmoninarum* cells.

Fish #	A	B	C	D
1	18.5	9.3	6.4	7.6
2	13.6	10.2	8.0	8.9
3	33.9	11.3	8.7	13.0
4	57.1	29.4	18.6	29.4
5	18.3	12.1	9.5	10.1
6	13.2	7.5	6.4	6.2
7	13.5	10.2	4.9	6.8
8	22.6	10.1	11.4	8.4
9	17.1	8.0	11.2	9.6
10	22.2	12.1	12.3	13.8

Table 2: Mean cellular phagocytosis (%) of microspheres for the six splenic cell isolates in each of four treatment groups.

Group A = PBS control; Group B = 0.1 mg/ml ECP; Group C = 1.0 mg/ml ECP; Group D = *R. salmoninarum* cells.

Fish #	A	B	C	D
1	10.7	12.7	11.0	11.7
2	16.0	15.3	12.3	9.7
3	10.3	13.0	9.0	10.0
4	13.7	12.7	13.3	4.7
5	6.7	8.0	13.0	11.0
6	8.0	5.3	9.3	12.3

Figure 1. Phagocytosis of fluorescent microspheres by splenic phagocytic cell of a brook trout.

(ch3fig1.jpg; 507 K)

Figure 2. Mean cellular fluorescence (respiratory burst activity) of splenic phagocytes in the four treatment groups.

(ch3fig2.pic; 264 K)

Group A= PBS control; Group B= 0.1 mg/ml ECP; Group C= 1.0 mg/ml ECP; Group D= *R. salmoninarum* cells. * denotes significant difference from control group (Group A).

Figure 3. Mean cellular phagocytosis of fluorescent microspheres in the four treatment groups.

(ch3fig3.pic; 288 K)

Group A= PBS control; Group B= 0.1 mg/ml ECP; Group C= 1.0 mg/ml ECP; Group D= *R. salmoninarum* cells.

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Chapter 4

BKD-induced renal lesions of brook trout (*Salvelinus fontinalis*) exposed via ingestion of infected tissues

Abstract

Brook trout (*Salvelinus fontinalis*) exposed to *Renibacterium salmoninarum* via ingestion of infected tissues were evaluated for histological evidence of renal lesions. The fish were fed *R. salmoninarum*-infected chinook salmon tissues incorporated into the feed at a dose of 1.69×10^{11} bacterial cells/fish. Renal tissues from five exposed trout were evaluated at 1, 4, 7, and 10 weeks post-exposure while unexposed trout were sampled at 0 and 4 weeks. Enzyme-linked immunosorbent assay results were positive for *R. salmoninarum* in renal tissues at the 4, 7, and 10 week sample intervals for exposed fish, and no unexposed fish tested positive. A variety of renal glomerular, tubular, and interstitial parameters were evaluated microscopically. Histological lesions were identified at 7 and 10 weeks post-exposure and included fibroepithelial crescent formation, glomerular sclerosis, dilation of Bowman's space, glomerular basement membrane thickening, mesangial matrix proliferation, tubular vacuolization and necrosis, inflammatory cell infiltration of the interstitium, fibrous connective tissue deposition, and granuloma formation. Renal tissue evaluated via immunofluorescent technique with fluorochrome-labeled anti-trout immunoglobulin showed no evidence of immunoglobulin deposition along glomerular or tubular basement membrane. These findings indicate that significant renal lesions developed in the *R. salmoninarum*-infected fish exposed by the gastrointestinal route within 7-10 weeks post-exposure. While the results bear similarities to lesions reported in association with other routes of exposure and are consistent with an immune-mediated component, immunofluorescence did not confirm any immunological hypersensitivity.

I. Introduction

Renibacterium salmoninarum is the etiological agent of bacterial kidney disease (BKD), a subacute to chronic, multisystemic, granulomatous disorder of salmonids. *Renibacterium salmoninarum* is a facultatively intracellular pathogen with an affinity for the renal tissue of susceptible salmonid hosts. Natural transmission of BKD is largely vertical (Evelyn et al., 1984), although horizontal transmission via direct contact between infected and uninfected salmonids and through consumption of infected tissues or feces also occurs (Murray et al., 1992; Balfry et al., 1996; Austin and Rayment, 1985). Some investigators (Elliott et al., 1994; Mitchum and Sherman, 1981) have postulated that vectors may play a role in the horizontal transmission of BKD. Experimental infection with *R. salmoninarum* in salmonids has been accomplished by a number of routes including immersion baths, feed preparations containing viable organisms, and cohabitation with infected fish (Wood and Wallis, 1955; Murray et al., 1992). However, the most common and reliable method of experimental challenge is parenteral injection of *R. salmoninarum*.

The kidney is a primary target organ in BKD pathogenesis. Grossly, *R. salmoninarum*-infected salmonid kidneys may appear uniformly pale and swollen or have focal abscesses (Fryer and Sanders, 1981). Histologically, lesions characteristically contain intracellular and extracellular *R. salmoninarum*, diffuse granulomas, necrosis of glomeruli and tubules, interstitial infiltration with immunocytes, and interstitial edema (Bruno, 1986).

Renal ultrastructure in BKD, described by Young and Chapman (1978), confirms significant glomerular and tubular damage. Recently, Sami et al. (1992), investigating the renal immunopathogenesis associated with BKD in fish infected intraperitoneally with *R. salmoninarum*, described a chronic membranous glomerulonephritis and identified putative immunoglobulin deposits along the glomerular basement membrane via both electron microscopy and indirect immunofluorescence. This appeared to indicate that this glomerular lesion was associated with immune complex-mediated inflammation. Previous investigations (Young and Chapman, 1978) had also noted similarities between BKD renal lesions and the pathogenesis of immune-mediated glomerulonephritis in mammals, including subendothelial dense deposits resembling immune complexes, mesangial proliferation, mesangial dense deposits, and basement membrane thickening and irregularity. Further corroboration of an immune complex-mediated component to BKD was provided by Kaattari et al. (1989) with the identification of soluble immune complexes in the serum of *R. salmoninarum*-infected salmonids.

In mammals, the pathogenesis of immune-complex glomerulopathy has been extensively studied. Immune complex-mediated glomerulonephritis, characterized as a Type III immunological hypersensitivity reaction, involves the deposition of circulating, soluble immune complexes in the glomerular filtration barrier. Complex deposition is followed by glomerular inflammation and renal tissue damage, and is mediated by both complement and cellular phagocytosis mechanisms (Tizard, 1987). The initial formation of soluble immune complexes is dependent upon a number of factors related to antigen presentation. Soluble immune complexes generally form in circumstances of significant antigen or antibody excess. Therefore, it is presumable that factors which determine the degree of antigen exposure and/or antibody production will affect the likelihood of soluble complex formation (Kumar et al., 1992). Furthermore, the route of antigen presentation may ultimately influence the formation of soluble immune complexes. Paul (1984) stated that presentation of antigen via the gastrointestinal tract may impact any subsequent immune response as hepatic passage and phagocytosis may, to some degree, filter out any available antigen.

We report here the renal lesions associated with *R. salmoninarum* infection via gastrointestinal exposure (i.e.- ingestion of infected tissues). As gastrointestinal transmission of *R. salmoninarum* has consistently produced clinical disease in cultured salmonid populations (Wood and Wallis, 1955), it follows that this means of infection represents an alternative, albeit less practical, to parenteral injection for the experimental induction of BKD.

II. Materials and Methods

Sixty yearling brook trout (*Salvelinus fontinalis*) were maintained at the National Fish Health Research Laboratory in Kearneysville, WV in each of two circular 700 l tanks supplied with running spring water (12.5°C). Both groups were maintained on a diet of commercial pelleted salmonid feed (Zeigler Brothers, Inc., Gardners, PA). Over a four day period, the experimental group was fed the viscera of chinook salmon (*Oncorhynchus tshawytscha*) fingerlings infected with *R. salmoninarum* while the control group was fed only the pelleted ration. Abdominal viscera (liver, spleen, intestine, and kidney tissues) was aseptically removed from the dead fingerlings, finely macerated, and homogenized in a stomacher for three 1-minute cycles. Forty-eight grams of the viscera (6 g/feeding over 2 feedings/day) were fed to the sixty trout in the experimental group, thoroughly mixed with their standard pelleted ration. The concentration of *R. salmoninarum* in the infected viscera was determined via the membrane filtration fluorescent antibody test (MFAT; Elliott and Barila, 1987), and an average infective dose per fish was calculated. At the sampling

intervals of 1 week, 4 weeks, 7 weeks, and 10 weeks post-exposure, five fish were removed from the experimental group and sacrificed via a lethal dose of tricaine methanesulfonate anesthetic (MS-222; Sigma Chemical Co., St. Louis, MO). Fish were necropsied aseptically, gross organ structure was noted, and renal (mesonephric) tissue was collected from the mid-kidney region. Five control fish were sacrificed and necropsied at the sampling intervals of 0 weeks and 4 weeks in a similar manner. Samples of renal tissue were placed in 10% neutral buffered formalin fixative and also frozen at -70°C. Upon completion of all samplings, the frozen renal tissues were thawed and tested by the enzyme-linked immunosorbent assay (ELISA) using a modification of the method described by Pascho and Mulcahy (1987). Formalin-fixed renal tissue samples were paraffin-embedded and prepared for histological evaluation. Sectioned specimens were stained with hematoxylin & eosin (H&E) and Periodic acid-Schiff (PAS) stains (Prophet et al., 1992). Conventional light microscopy was used to examine all histological specimens.

Renal tissue from each specimen was evaluated for the following criteria:

1. Glomeruli:
* mesangial cellularity- the mean number of mesangial cells per glomerular cross section (8 glomeruli examined)
* mesangial matrix- presence or absence of increased mesangial matrix (based upon the ratio of matrix to mesangial cells, categorized as present if greater than 1:1)
* sclerosis- presence or absence of glomerular sclerosis (adherence of the glomerular capillary bed to Bowman's capsule)
* Bowman's space dilation- presence or absence of dilation of Bowman's space ("present" if the glomerulus filled less than 3/4 of Bowman's space in cross-section)
* crescent formation- presence or absence of fibroepithelial crescents (cellular proliferation) along Bowman's capsule
* basement membrane thickening- presence or absence of any thickening or irregularities along the glomerular basement membrane

2. Tubules:
* hyaline- presence or absence of eosinophilic-staining hyaline protein in the tubular lumen
* tubular dilation- presence or absence of dilation of proximal or distal renal tubules
* vacuolization- vacuoles present or absent in renal tubular cells ("present" if vacuoles were readily apparent in tubule cells every few high power fields or more frequently)
* necrosis- necrosis of renal tubules present (evident every few high power fields or more frequently) or absent

* basement membrane thickening- presence or absence of any thickening or irregularities(i.e.-splitting, incontinuity) of the tubular basement membrane

3. Interstitium:
* tubule density- the mean number of renal tubules per high power field (over 10 fields)
* pigmentation- presence or absence of abundant pigmentation based on the ratio of pigmented to nonpigmented interstitium (greater than 1:4 constituted "present")
* fibrous connective tissue- the presence or absence of peri-tubular/peri-glomerular fibrous connective tissue
* granulomas- the presence or absence of granulomas

Immunofluorescence of renal tissues was evaluated by a modification of the method described by Tanimoto and Ohtsuki (1996). Briefly, paraffin embedded tissue sections on microscope slides were deparaffinized with xylene and absolute ethanol, placed in a hot bath (95°C) of target unmasking fluid (TUF; Signet Laboratories, Inc., Dedham, MA) for 10 minutes, and rinsed in water. Tissue sections were then stained for 45 minutes with fluorochrome-labeled affinity purified goat anti-trout immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) at 37°C. Stained slides were again rinsed in water, coverslips applied, and the tissue sections evaluated using fluorescent microscopy. Unstained tissue sections from the trout served as negative controls while splenic tissue sections were positive controls.

Statistical evaluation of results was performed with nonparametric analyses with $p=0.05$. The Kruskal-Wallis One-Way ANOVA was applied to numerical data (tubule density/glomerular cellularity) while the Fisher's exact test was used for the remaining categorical data (Norman and Streiner, 1994).

III. Results

The concentration of *R. salmoninarum* inoculum in the homogenized viscera from the fingerlings was 2.11×10^{11} cells/g tissue, which translated into an infective dose of approximately 1.69×10^{11} bacterial cells/trout. Results of the ELISA for the control and experimentally infected fish are summarized in Table 1. Briefly, the ELISA was positive for *R. salmoninarum* in the 4-weeks post-exposure group with sampled fish testing positive at both subsequent sampling intervals as well. No tissue from control fish or fish tested 1 week post-exposure were ELISA positive for *R. salmoninarum*.

Gross observations revealed little evidence of disease. No external or internal lesions involving organs other than the kidney were noted in any fish. A swollen and pale grey kidney was noted for one specimen from the 7 week post-exposure group.

Histological observations are summarized in Table 2. Statistically significant ($p<0.05$) histological changes were noted in the renal tissue samples from the 7 and the 10 week post-exposure groups. Tubular vacuolization and necrosis, granulomas, and decreased tubular density were all noted at both the 7 and 10 week intervals (Figures 1, 2, and 3). Interstitial fibrous connective tissue, glomerular sclerosis, fibroepithelial crescent formation, and dilation of Bowman's space were significantly greater at the 10 week post-exposure sample time (Figures 1, 4, and 5). Mesangial matrix proliferation and glomerular basement membrane thickening/irregularity were significantly higher in the 7 week sample group (Figure 6) compared to the control group, but not significantly higher in the 10 week sample fish. Additionally, the presence of PAS-staining debris resembling tubular basement membrane within the cytoplasmic vacuoles of interstitial cells (macrophages) in close proximity to degenerating tubules were observed in both the 7 and 10 week post-exposure groups (Figure 4). Bacteria were seen occasionally in the interstitium of exposed fish, both intracellularly and extracellularly.

Minimal, nonspecific immunofluorescence was observed in the renal samples. Occasional fluorescence of interstitial cells was noted for all sample time intervals, inclusive of control tissue samples. One tissue sample from the 10 week post-exposure interval displayed a mild, focal granular fluorescence associated with a single glomerulus. However, no aggregated deposits of fluorescing material were seen in glomeruli of either infected or non-infected fish.

IV. Discussion

This investigation has shown significant renal disease in the kidneys of *R. salmoninarum*-infected trout exposed by ingestion of infected tissues. These results are similar to findings previously reported in association with experimentally induced and naturally occurring BKD (Bruno, 1986; Young and Chapman, 1978).

Histological parameters were selected for this study based upon their reliability as indicators of renal pathology/immunopathology in mammals as well as their known association with BKD-induced renal lesions in salmonids (Bruno, 1986). Tubule density correlated inversely with interstitial cellularity and reflected the degree of inflammatory cell invasion of the interstitium. Likewise, the presence of granulomas and/or fibrous connective tissue, especially as peri-tubular or peri-

glomerular deposits, indicated a chronic state of granulomatous inflammatory disease. Pigmented melanomacrophages/melanomacrophage aggregates of the renal interstitium are reported to have a prominent role in inflammatory processes among fish (Wolke, 1992). The amount of vacuolization and necrosis of renal tubules, tubule dilation, and the presence of hyaline protein in the tubular lumen are all indicative of tubular cell viability and renal tubule function (Kumar et al., 1992). The glomerular parameters evaluated in this investigation served as more specific indicators of glomerulonephritis-induced disease potentially related to an hypersensitivity reaction. Mesangial cellularity and abundance of the mesangial matrix are factors which often increase in association with mammalian proliferative glomerulonephritis (Slauson and Lewis, 1979). Additionally, shrinkage of the glomerular tuft/dilation of Bowman's space, glomerular sclerosis, and fibroepithelial crescent formation are changes reported to occur in glomerulonephritis (Wilson et al., 1982). Basement membrane thickening and irregularity (i.e.-splitting, focal proliferation) associated with either the glomeruli or tubules may indicate a membrane-associated immunologic disease such as Type II or Type III hypersensitivity reactions (Keane and Michael, 1988; Robertson et al., 1977).

Inflammatory changes in the renal interstitium were evident both 7 and 10 weeks post-exposure, as shown through the increased abundance of interstitial inflammatory cells, the infiltration of fibrous connective tissue, and granuloma development. Degenerative tubular changes such as the vacuolization and necrosis of tubular epithelial cells also became readily apparent during this time. Glomerular changes consistent with glomerulonephritis, including mesangial matrix proliferation, sclerosis, crescent formation, and dilation of Bowman's space, were also seen at 7 and 10 weeks post-exposure. Basement membrane thickening was observed chronically in both tubules and glomeruli, from 4 weeks post-exposure onward in glomeruli and from 7 weeks post-exposure onward in tubules (although statistical significance associated with this lesion was found only for glomerular membrane thickening in the 7 week sample).

The ELISA results confirmed *R. salmoninarum* antigen present in renal tissue as early as 4 weeks post-exposure, the sampling interval prior to the appearance of significant renal lesions. While all renal samples were ELISA positive at 4 weeks post-exposure, only 60% and 40% of tissue samples tested positive at 7 and 10 weeks respectively. This finding is possibly related to the method of exposure acting as a potential confounder of the investigation; trout were merely offered the ration containing infected viscera and were not force-fed the inoculum, therefore equal dosage among sample fish was not assured. Moreover, it is

possible that alternative routes of exposure (i.e.- trans-gill or trans-epithelium) occurred to a lesser degree via liberation of *R. salmoninarum* from the feed into the water column. Alternatively, tube-feeding the infected ration would be more likely to have resulted in equal exposures among fish but would also have introduced an artificial stress factor with a potentially significant effect upon host immune response.

Moreover, for the 10 week post-exposure group, ELISA results do not correspond completely with histological indicators of disease as three fish with pathologic changes including granulomas, glomerular sclerosis, and dilation of Bowman's space did not have positive ELISA results. Absence of detectable levels of soluble antigen in fish having lesions consistent with BKD might reflect either antigen production or availability (i.e. intracellular sequestration of bacteria) at this stage in disease pathogenesis. Additionally, this inconsistency between pathology and ELISA may support an immune-mediated basis for renal pathogenesis in BKD, indicating that unbound *R. salmoninarum* soluble antigen is seemingly not a prerequisite for the development of lesions.

Overall, the histological observations in this investigation were consistent with pathologic changes induced by immune-mediated hypersensitivity. Glomerular changes resemble those encountered in membranoproliferative glomerulonephritis of mammals. Similar lesion formation has been described in association with BKD in salmonids infected by other routes of exposure (Sami et al., 1992; Young and Chapman, 1978). Thickening and irregularity of the basement membrane of both glomeruli and tubules, as well as the appearance of phagocytosed tubular basement membrane in interstitial cells suggests that a Type II hypersensitivity involving the formation of anti-basement membrane antibody may also be associated with BKD; however, our investigation did not prove this conclusively. This type of hypersensitivity reaction (i.e. anti-basement membrane antibody formation) has not been reported among any species of fish. Our immunofluorescence results were not indicative of either a Type II (i.e. linear fluorescence along the basement membrane) or Type III (i.e. granular fluorescence along the glomerular basement membrane) hypersensitivity reaction. While one focal region of granular fluorescence was noted (10 weeks post-exposure), this finding was not consistent among the infected fish. The inability of the immunofluorescence technique applied in this investigation to demonstrate immune complex formation/ deposition in renal tissues does not necessarily rule out any immunological hypersensitivity component to the renal disease.

Table 1. ELISA results for control and post-exposure groups.

Sample Group	# Positive ELISA/Total # Tested
Control	0/10
1 week	0/5
4 weeks	5/5
7 weeks	3/5
10 weeks	2/5

Table 2: Summary of histological observations for the control and post-exposure groups.
Mesangial cellularity and tubule density are represented by the mean value observed for all fish followed by the standard deviation (parentheses). All other data are categorically summarized as the number of "positive" (i.e.- lesion present) fish sampled/total number sampled for each group. "*****" indicates a statistically significant difference in the parameter for the sampling interval compared to the unexposed control group.

Glomeruli	**Control**	**1 week**	**4 weeks**	**7 weeks**	**10 weeks**
mesangial cellularity	53(12)	45(8)	48(8)	46(11)	41(2)
mesangial matrix	4/10	0/5	3/5	5/5*	2/5
sclerosis	2/10	0/5	0/5	2/5	5/5*
Bowman's space dilation	0/10	0/5	0/5	1/5	4/5*
fibroepithelial crescents	0/10	0/5	1/5	2/5	4/5*
basement membrane thickening	0/10	0/5	1/4	3/4*	1/4
Tubules					
hyaline protein	1/10	0/5	0/5	0/5	0/5
dilation	0/10	0/5	1/5	2/5	2/5
vacuolization	3/10	3/5	3/5	5/5*	5/5*
necrosis	3/10	2/5	3/5	5/5*	5/5*
basement membrane thickening	0/10	0/5	0/4	2/4	1/4
Interstitium					
tubule density	14.2(2.6)	19.2(5.0)	13.8(0.8)	8.8(3.7)*	9.6(1.5)*
pigment	4/10	2/5	1/5	0/5	2/5
granulomas	0/10	0/5	0/5	3/5*	3/5*
fibrous connective tissue	0/10	2/5	2/5	2/5	4/5*

Figure 1. Decreased tubule density/interstitial cell infiltration in a 10 week post-exposure sample.
(ch4fig1.jpg; 933 K)
Tubular vacuolization and necrosis as well as dilation of Bowman's space in the glomerulus are also apparent.

Figure 2. Severe tubular necrosis of two adjacent renal tubules 10 weeks post-exposure with cellular debris present in tubule lumens.
(ch4fig2.jpg; 899 K)

Figure 3. Granuloma formation in renal tissue 7 weeks post-exposure. (ch4fig3.jpg; 1.39M)

Figure 4. Severe tubular necrosis with deposition of fibrous connective tissue 10 weeks post-exposure.
(ch4fig4.jpg; 1.26M)
Phagocytosis of tubular basement membrane is also evidenced by the presence of basement membrane fragments inside inflammatory cells of the interstitium adjacent to degenerative tubules.

Figure 5. Development of a fibroepithelial crescent through inflammatory cell proliferation adjacent to Bowman's capsule of the glomerulus (10 weeks post-exposure).
(ch4fig5.jpg; 780 K)

Figure 6. Glomerular basement membrane thickening and splitting 7 weeks post-exposure.
(ch4fig6.jpg; 1.14 M)

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General Summary

As alluded to by a number of previous investigators, results of this dissertation suggest that the immunological aspects of bacterial kidney disease (BKD) appear to be quite complex and multifaceted. Involvement of immunomodulatory effects related to the extracellular protein (ECP) are evidenced by these chapters. *In vivo* alterations in leukocyte numbers in peripheral blood related to ECP exposure and challenge with *Y. ruckeri* included an increase in peripherally circulating small lymphocytes and inhibition of an increase in monocytes post-challenge. Interpretation of these findings is somewhat difficult, given current limitations in the understanding of fish (salmonid) hematology. It would seem logical that elevation of lymphocyte numbers represents immunostimulation while the lack of change in the monocyte population may constitute an immunosuppressive change. Still, it is uncertain whether these changes represent deviations from the "normal" ranges for hematological parameters (i.e. represent a true lymphocytosis and/or impediment of monocytosis). These findings, in conjunction with the *in vitro* ECP-related suppression of phagocyte respiratory burst activity, suggest an immunomodulatory effect upon cell-mediated immunity. As described in these investigations, cell-mediated immunomodulation does not appear to alter phagocytosis capabilities as no evidence of ECP-related effect upon this parameter was noted. The extent to which any immunomodulation involves humoral immunity (and associated lymphocytes) is uncertain as no differentiation of Ig+ and Ig- lymphocytes was performed. Otherwise, no evidence of humoral immunomodulation, as indicated via the microagglutination titer data, was apparent.

Exogenous facilitation of BKD development related to environmental variables was negligible in these results. This finding may be an indication that vertical transmission of *R. salmoninarum* lends itself to the development of immunological tolerance and thereby minimizes the effects of exogenous parameters which influence immunocompetence. Alternatively, the results may be a factor of an experimental design which failed to reflect the impact of environmental stress. If the former is truly the case, this notion has serious implications for BKD control and the necessity for continued emphasis on management techniques which prevent or minimize vertical transmission of the pathogen.

The role of immunological hypersensitivity in BKD pathogenesis is supported by these results, although there remains some question as to the type or types of hypersensitivity involved. Immune-complex mediated hypersensitivity (Type III) is consistent with many of the findings related to the observed glomerulopathology in this and previous investigations. Granulomatous cell-mediated hypersensitivity (Type IV) is also consistent with the BKD pathology described here and elsewhere, involving intracellular localization of antigen within macrophages and subsequent inflammatory responses characterized by macrophage and lymphocyte infiltration, collagen deposition, and granuloma formation with focal necrosis. Moreover, results of the investigation suggest the possibility of an antibody-mediated hypersensitivity (Type II) involving a specific humoral response against an autologous tissue, namely, tubular basement membrane. Tubular necrosis with subsequent exposure of the basement membrane coinciding with what appears to be phagocytosed tubular basement membrane in interstitial macrophages are consistent with the autologous tissue destruction encountered in Type II hypersensitivity. Given the nature of findings in BKD-related literature, it is likely that Type III and IV hypersensitivities are involved in BKD pathogenesis; further investigation is warranted to confirm the involvement of a Type II hypersensitivity and the consistency of Type III hypersensitivity.

These characterizations of the salmonid immune response to BKD have significant bearing upon BKD control. For instance, the likelihood of an immune-complex mediated hypersensivity and the potentially immunosuppressive effects of the *R. salmoninarum* ECP suggest that antigenic stimulation (particularly ECP-related antigen) of humoral immunity in vaccine development would be difficult to develop. Previous literature again suggests that cell-mediated immunity presents a more favorable approach for BKD control. Indeed, other pathogens such as *Mycobacterium* spp., *Brucella* spp., and *Listeria monocytogenes* which share similar characteristics related to pathogenesis (i.e. facultatively intracellular location, granulomatous disease involving Type IV hypersensitivity) have been controlled through the stimulation of cell-mediated immunity. Specifically, the development of vaccines has involved the use of live, attenuated organisms. Certainly, this approach invites a great deal of caution when applied to BKD, as the potential for the vaccine strain to regain virulence and subsequently disseminate in an aquatic environment must be considered.

There are a number of avenues for future research regarding the immunology of BKD which seem worthy of pursuit. The potential for development of a vaccine which would stimulate cell-mediated immunity, possibly incorporating a live, avirulent strain of *R. salmoninarum*, warrants further investigation. Other considerations include: the immunomodulatory role of the ECP with reference to its specific antigenic constituents (p57 and others); immunomodulatory effects directed against parameters of cell-mediated immunity, particularly against the monocyte cell line; differences in the immune response depending upon route of infection (i.e. vertical versus horizontal transmission of the pathogen); and differences in the immunological responses among species and strains of salmonids with different levels of susceptibility to BKD.

Vita

Christine Lynn Densmore was born May 2nd, 1964 in Frostburg, Maryland. She grew up in Denton, Maryland on the Delmarva peninsula where her parents, Jack and Linda Densmore, currently reside.

Christine earned a B.S. degree with a major in biology and minor in history from Gettysburg College in 1986. In 1990, she received the D.V.M. degree from the Virginia-Maryland Regional College of Veterinary Medicine. Following three years as a veterinary associate in small animal practice, Christine returned to the VMRCVM at Virginia Tech to pursue a Ph.D. in veterinary medical science with an emphasis upon aquatic animal medicine. Through a Cooperative Education Agreement, Christine has been fortunate to spend a significant portion of her time as a graduate student at the National Fish Health Research Laboratory in Kearneysville, West Virginia working on projects concerning diseases of cultured and feral fish.

Apart from the laboratory, Christine enjoys outdoor activities such as baseball and a variety of water sports, researching the medical history of the Civil War, and spending time with her eight month old son, Casey.