STRESS PROTEINS, PHAGOCYTES, AND PATHOLOGY IN COHO SALMON WITH BACTERIAL KIDNEY DISEASE.

by

ROBERT BRUCE FORSYTH

B.Sc., The University of British Columbia, 1985

M.Sc., The University of British Columbia, 1989

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Department of Animal Science (Agricultural Sciences)

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Bacterial kidney disease (BKD) is an important disease of wild and cultivated salmonid fish in Canada and around the world. It is known to be caused by a slow growing Gram positive bacterium, *Renibacterium salmoninarum,* which grows as an intracellular pathogen of phagocytic cells. The disease can not currently be prevented and BKD cannot be cured with antibiotic treatment. Stress is a major factor in the susceptibility of fish to BKD and the disease can itself be considered a chronic stressor. The pathology of the disease involves a chronic and progressive spread of the pathogen throughout the organs with an accompanying chronic inflammation and accumulation of fish leukocytes at the site of infection. The inflammatory response and the phagocytes present at the sites of infection are not able to eliminate the pathogen but are thought to contribute to the tissue damage caused by BKD.

Stress proteins (SP) are a set of proteins synthesized by cells *in vitro* and *in vivo* as a result of exposure of the cells to stressors, such as toxic substances or heat. They can be used to monitor the effects of a variety of stressors. Up to now most of the research on SP has been focussed on induction of SP by physical or chemical stressors which act directly on the cells or tissues in culture. The fact that BKD can act as a chronic stressor upon the tissues of infected fish makes it an attractive system to study the induction of SP in whole animals by physiological processes such as tissue damage caused by phagocytes and the oxidants they produce.

I am the first to report SP induction in a fish disease and this work is one of only two known examples of SP induction in fish during pathophysiological processes (Forsyth *et*

al., 1997; Janz *et al.*, 1997). Using an enzyme linked immunosorbent assay (ELISA) for HSP-70 and immunofluorescent staining of tissue sections, I found that in the tissue of coho salmon (*Oncorhynchus kisutch*) with active BKD, host HSP-70 was induced in the vicinity of bacterial microcolonies. This localized SP expression may have important implications for the pathology of BKD and possibly for diseases caused by other Gram positive bacteria and intracellular pathogens. The presence of elevated HSP-70 levels in these diseased fish also raises the practical concern that disease in natural populations might interfere with the use of SP levels as an indicator of environmental stress. Investigators studying SP expression should be careful to examine the sampled animals for signs of infectious disease as a potential confounding factor.

I found no compelling evidence to support the hypothesis that this SP induction resulted from the action of phagocytes at the sites of infection. Culture experiments with rainbow trout (*Oncorhynchus mykiss*) phagocytes, with hepatocytes and with mixtures of those cells support the idea that salmonid phagocytes may have a weak stress response, at least when cultured *in vitro*. The hepatocyte cultures had a strong SP response to heat shock, but in mixed cultures I saw no evidence for SP induction by phagocyte oxidants or direct induction by *R. salmoninarum*. I conclude that other less obvious mechanisms should be considered and that the process of SP induction in BKD may be more complicated than can easily be modeled in a simple culture system. I suggest some possible improvements to *in vitro* and *in vivo* culture systems to facilitate the study of the interactions between the bacteria and the host cells at sites of infection in fish with BKD.

In this course of the experiments with BKD in coho salmon I also made two incidental observations which may be important. The first is that crowding may be useful in challenge

experiments as a means to reduce the variability of fish in their susceptibility to BKD. The second is that *R. salmoninarum* might grow in two different modes, and that the bacterium may switch to a more virulent form when a sufficient density is reached; a quorum sensing system may control virulence in *R. salmoninarum.*

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"Of making many books there is no end, and much study wearies the body."

Ecclesiastes 12:12b (NIV)

Chapter I General Introduction

1.1 Overview

Bacterial kidney disease (BKD) is an economically important disease of salmonid fish that causes direct losses of farmed salmon in Canada which can be estimated at more than $20 million each year. The effects of environmental stressors and the stress response can make fish more susceptible to a number of diseases including BKD. Environmental stressors can compromise the health and productivity of both wild and cultured fish (Barton & Iwama, 1991; Wedemeyer, 1996a). Diseases and disease agents are themselves stressors and could potentially cause stress responses in fish. One versatile and convenient way of monitoring the effects of a variety of stressors is measurement of stress proteins.

Stress proteins (SP) are a set of proteins synthesized by cells *in vitro* or *in vivo* as a result of direct exposure to stressors such as toxic substances or heat. Most, but not all SP, can be induced by heat shock and the first SP found were named heat shock proteins (HSP) because they were discovered in heat shocked tissue. Stress proteins would perhaps be more accurately called cellular stress proteins since other proteins such as acute phase proteins are synthesized in response to stress in the whole animal and therefore could also be called stress proteins. To avoid confusion, however, I use the term stress induced proteins in the few instances where I mean the whole set of proteins induced *in vivo* as a result of stress. Throughout this thesis, I use the term stress proteins in the conventional way as a general term referring collectively to members of the heat shock protein families and to other SP that are not HSP but are induced in response to stressors which are directly experienced by cells. I use the term heat shock protein when referring to specific proteins

which are HSP. I refer to all of the individual SP by their ordinary names (i.e. HSP-70, HSP-60, HSP-30) rather than renaming them with the SP prefix (SP70, SP60, SP30). I use the abbreviations HSP and SP for both the singular and plural forms.

Up to now most of the research on SP has been focussed on induction of SP by physical or chemical stressors which act directly on the cells or tissues. In whole animals however, SP can also be induced as a result of physiological or pathophysiological processes triggered by an initial stressor.

The distinction between induction of SP by means of a physiological response rather than by the direct action of a stressor upon the cells may seem like nit picking but I believe that it is important. Researchers working with whole animals need to be cautious about assuming that a stressor will cause the same pattern of response, or SP expression in the cells of a living animal as that stressor causes in isolated tissues or cells in culture. For example, when a fish or other ectothermic animal is subjected to a heat shock, all of the cells within that animal experience a heat shock. However, heating an animal could also have effects at higher levels of organization. The heat shock could affect individual cells, affect tissues, and also affect the whole organism in a systemic manner.

The extreme example of effects at the organismal level is that a heat shock could cause the death of the animal by disrupting some critical physiological process even if the elevated temperature of the animal would not directly kill cells in culture. It seems that membrane-based processes and especially synaptic neurotransmission are the most critical of heat sensitive processes in animals with nervous systems (Somero *et al.*, 1996). The disruption of nervous function could cause rapid behavioural changes or death. Sub-lethal changes in

the physiology of the animal such as production of hormones, as well as changes in oxygen levels, nutrients, or metabolites circulating in the blood, could result from the effects of heat-induced stress and influence cells throughout the animal. Such systemic changes might even cause the induction of specific proteins in individual cells.

One specific physiological mechanism which might cause SP induction is the generation of oxidants by phagocytes as part of an animal's natural defenses against pathogens. Initial exposure to a stressor such as a pathogen, for instance, could trigger an inflammatory reaction leading to the production of oxidants. These oxidants could then act as a direct stressor to all of the host cells nearby, as well as to the pathogen (Kantengwa & Polla, 1993; Nishimura *et al.*, 1997). There is a growing body of knowledge relating to the physiological induction of SP in human diseases and in other mammals (Holbrook & Udelsman, 1994; Dillmann & Mestril, 1995; Wick *et al.*, 1995; Polla *et al.*, 1998b). Many of the researchers studying SP in bacterial diseases have focused on: the effects of SP induction in the bacteria, the potential for the use of bacterial SP in vaccines; and in the role of bacterial SP in stimulating autoimmune and inflammatory diseases such as reactive arthritis (Kaufmann *et al.*, 1991; Silva, 1995; Suzue & Young, 1996; Gaston, 1998; Multhoff *et al.*, 1998). There is very little knowledge about induction of host SP in bacterial diseases (Young & Mehlert, 1990). I have been able to find only a few published reports of SP expression by infected host tissue (Emoto et al., 1992; Emoto et al., 1993; Khanolkar-Young et al., 1994; Kol et al., 1998).

I am the first to report SP induction in a fish disease and this work is one of only two known examples of SP induction in fish during pathophysiological processes (Forsyth *et al.*, 1997; Janz *et al.*, 1997). This work opens up new directions of study for our

understanding of BKD and of SP responses in fish but also for our understanding of the role of host SP in the pathology of diseases other than BKD.

In summary, there are several features that make SP expression in fish with BKD an attractive area to study, and in many regards fish are perhaps the ideal vertebrates in which to study SP. They are affected by the temperature and chemical composition of the water in which they live. For many species of fish, body temperature varies with the water temperature over a wide range. Coho salmon (*Oncorhynchus kisutch*) and other salmon, for example, can live at temperatures from around 0 °C to over 20 °C (Wedemeyer, 1996b). Information about SP in healthy and diseased fish may be of practical value in our use and management of wild and cultured fish. A clearer understanding of SP responses in fish is also likely to cast light on SP responses in other vertebrates, including humans. Finally, understanding the role of host SP in the pathology of BKD is likely to yield insights into the pathology of bacterial diseases other than BKD.

1.2 Hypotheses and overview of thesis organization

Induction of SP by physiological processes in fish was the focus of this research. As an example of physiological induction of SP, I expected that SP would be induced in BKD as a result of phagocyte function. In order to examine this question I tested the following series of major hypotheses.

1. **SP are induced in fish with BKD. (This is an example of induction of SP by a physiological response to a stressor.)** H_0:HSP-70 is not induced in fish with BKD.

2. **SP induction in fish with BKD is associated with the sites of infection and phagocyte infiltration in tissue.** H_0:There is no association of HSP-70 with sites of infection in BKD.

3. **SP can be induced *in vitro* as a result of phagocyte function and oxidant production.** H_0: HSP-70 is not induced by isolated phagocytes *in vitro*.

This thesis is organized in five main chapters. There are also three technical appendices with important methodological details and technical data relating to the development of the main experimental techniques.

In this General Introduction I outline the rationale for studying SP in fish with BKD and some background information which provides the framework for understanding the results. The three experimental chapters (II, III, and IV) correspond roughly to the three major hypotheses, respectively. In Chapter II, I introduce BKD in more detail and discuss the patterns of mortality, disease, and elevated SP levels which I observed in coho salmon with BKD. In Chapter III, I discuss the pattern of the microscopic distribution of host HSP-70 and its association with microcolonies of bacteria in the tissue of fish with BKD. In Chapter IV, I discuss the results of the *in vitro* experiments which I conducted with fish cells in culture and with mixtures of hepatocytes, phagocytes and bacteria in order to model some of the interactions which could occur in the tissue of fish with BKD.

In each of the experimental chapters I restrict the Discussion mainly to exploring what possible mechanisms are consistent with the results. In the General Discussion (Chapter V)

I present some conclusions from this work and some practical and conceptual implications of the results for research on BKD and other bacterial diseases. During the course of this thesis work, other groups have published findings which have strengthened the conceptual foundation for my hypotheses. I discuss the significance of my results in the context of some of these recent advances in our understanding of SP and the pathology of bacterial diseases.

1.3 Reasons for studying bacterial kidney disease

There are several features of BKD which make it an attractive fish disease to study. It has a serious economic impact on public and private salmonid culture. It is resistant to treatment. It is potentially a good model for gram positive intracellular pathogenesis. Finally, it provides an opportunity to study physiological induction of SP by a relevant stressor in fish.

1.3.1 BKD has a serious economic impact.

In Canada and in many countries around the world, BKD is an important and currently incurable disease of cultured salmonid fish for both commercial aquaculture and government enhancement programs (Evelyn, 1993; Evenden *et al.*, 1993; Fryer & Lannan, 1993).

In farmed fish, BKD can cause losses on an individual farm of up to 80% in Pacific salmon species and up to 40% in Atlantic salmon (Evenden *et al.*, 1993). The landed value of farmed fish in Canada for 1996 was approximately $172 million for Pacific salmon and $276 million for Atlantic salmon for a total production of about $450 million (Kent *et al.*, 1998). In a recent field study of farmed Atlantic salmon on Canada's Atlantic coast, BKD caused loses of 5% of the fish in the sea pens (Salonius, 1999). BKD causes heavier loses in Pacific salmon and the loses occur when the fish are nearly ready for harvest. The direct losses due to BKD in the fish farming industry of Canada can be conservatively estimated as being at least 20 to 30 million dollars of fish per year. We do not know the BKD-associated costs to Canadian salmon farmers in lost revenue, increased capitalization costs, and increased expenses associated with the farm practices needed to manage the disease. It

is also difficult to know what are the costs to wild fish stocks of releasing infected or diseased fish from government-run hatcheries (Sanders *et al.*, 1992; Elliott *et al.*, 1997).

Bacterial kidney disease affects wild salmonid stocks throughout the North Pacific and in Western Europe and affects all of the major species of farmed salmonids (Evelyn, 1988; Evelyn, 1993; Evenden *et al.*, 1993; Fryer & Lannan, 1993). In farmed fish the disease may be maintained by carriers in the farmed populations (Benediktsdottir *et al.*, 1991; Brown *et al.*, 1994) but the populations of infected wild fish around the world may also act as reservoirs of infection for domesticated fish (Mitchum & Sherman, 1981). On fish farms in North America, Europe and Chile, BKD causes disease in Pacific salmon (coho and chinook), Atlantic salmon, and rainbow trout (Evenden *et al.*, 1993; Fryer & Lannan, 1993).

1.3.2 Insufficient knowledge of BKD and lack of treatment.

In spite of the importance of BKD, we still do not understand the disease well enough to have a preventative vaccine, or a drug treatment which cures the disease. Since we cannot prevent fish from becoming chronically infected with *Renibacterium salmoninarum* (Rs), the fish culturist can at best minimize the impact of the disease through careful management and treatment to reduce the prevalence and severity of infection. To a limited extent erythromycin injection can be used to reduce mortality from BKD and to prevent transmission (Brown *et al.*, 1990; Peters & Moffitt, 1996). Also, the susceptibility of salmon to BKD and the severity of the disease are known to be increased by stress (Barton & Iwama, 1991; Mazur *et al.*, 1993; Wedemeyer, 1996a). Management practices which reduce crowding and handling reduced the losses from stress-related diseases including

BKD. Careful screening and hygiene can reduce the incidence of infection in cultured fish. Screening brood stock to reduce exposure of the eggs is one effective measure (Brown *et al.*, 1994; Lee & Evelyn, 1994; Elliott *et al.*, 1995). Despite these preventive measures BKD remains one of the most difficult and costly disease problems in cultured salmonid fish.

1.3.3 BKD as a model for diseases of Gram positive intracellular pathogens.

In addition to being costly, BKD is scientifically intriguing as an example of a chronic disease involving a slowly-growing Gram positive intracellular bacterial infection. The intracellular nature of the pathogen may serve to limit the access of antibacterial drugs and the fish's own natural defenses. This may explain in part the poor results of drug therapy and vaccination. Several antibiotic drugs kill or inhibit Rs *in vitro* (Bandin *et al.*, 1991) but have little therapeutic value in treating BKD. Antibiotic therapy with erythromycin is effective in reducing and delaying disease, but it does not eliminate the pathogen and the disease can eventually recur (Peters & Moffitt, 1996). Similarly, vaccines which have succeeded in producing strong antibody responses do not consistently prevent the disease (reviewed in Evenden *et al.*, 1993). In order to eliminate the disease, Rs must either be prevented from growing inside host cells, or new measures must be found which can kill Rs while it is inside the host cells.

A better understanding of the pathology of BKD may reveal vulnerabilities in the virulence strategy of Rs which can be exploited to design better treatments. Other diseases caused by Gram positive pathogens and intracellular pathogens may have analogous mechanisms and vulnerabilities. Understanding BKD, and the pathology it causes in the host tissue, may

increase our understanding of Gram positive pathogens which persist inside host cells of other animals including humans.

Gram positive pathogens cause many serious diseases in humans and terrestrial domestic animals. There is recent evidence (Wesson *et al.*, 1998) that intracellular persistence of Gram positive bacteria may be far more important and common than was previously recognized. Facultative intracellular pathogens are capable of growing outside of host cells but also invade and colonize host cells. Intracellular invasion and persistence is known to be important in many diseases caused by Gram negative bacteria as well as in infections by the Gram positive *Listeria monocytogenes* and by *Mycobacteria*, both of which are well known as intracellular pathogens (Finlay & Falkow, 1997). Until recently, facultative intracellular pathogenesis has been thought to be relatively unimportant for the most common Gram positive pathogens. However, even *Staphylococci*, which are the most prevalent of the Gram positive pathogens in humans, are capable of living inside host cells (Vann & Proctor, 1987). This intracellular persistence may be an additional virulence mechanism in *Staphylococcal* diseases. Rather than just causing host cell lysis and escaping, *Staphylococcus aureus* has a specific mechanism to induce apoptosis and remain inside epithelial cells (Bayles *et al.*, 1998; Wesson *et al.*, 1998). This recently discovered ability to subvert the host cell and favour the survival and growth of the bacteria inside cells whose main function is not phagocytosis suggests that intracellular persistence in at least part of the infectious process may be important in diseases caused by *S. aureus* and perhaps other Gram positive bacteria that previously were thought not to be intracellular pathogens.

The small Gram positive pathogen Rs is not a *mycobacterium*, but BKD has some similarities to mycobacterial diseases. One striking similarity is that the host responds to

the infection with an infiltration of leukocytes into the sites of infection. However, the large numbers of phagocytic cells generally are unable to clear the pathogen, and can even become infected and serve as host cells (Bandin *et al.*, 1993; Gutenberger *et al.*, 1997). There are also some similarities, such as a tendency to enter the CNS (Speare *et al.*, 1991) and the ability of the pathogen Rs to escape from the phagocytic vacuole into the cytoplasm (Gutenberger *et al.*, 1997), shared with another Gram positive intracellular pathogen *Listeria monocytogenes*. *L. monocytogenes* escapes the phagocytic vacuole by means of an haemolysin (listeriolysin) and an haemolysin has been reported in Rs (Grayson *et al.*, 1995b). This toxin could potentially be involved in escape from the phagocytic vacuole. It is worth pointing out that, unlike *Listeria*, but like pathogenic *Mycobacteria*, Rs also has the ability to persist inside modified vacuole structures (McIntosh *et al.*, 1997).

Consistent with the possibility that Rs may have two modes of intracellular persistence is the fact that BKD seems to occur in two phases. For much of the course of infection Rs accumulates in the tissue of the fish but causes little obvious disease or tissue damage and few mortalities. After the infection has reached some critical point, many mortalities occur in a short time span. This shift may be the result of rising water temperature, stress or some unknown factor. The shift also may be an inherent virulence strategy of Rs. The bacteria may at first grow in the tissue, perhaps within intracellular vacuole structures, and cause relatively little damage. Later as the disease advances Rs may switch to producing toxins which allow it to break out of phagocytic vacuoles and so may cause more tissue damage.

If Rs has the ability to both escape from the phagocytic vacuole (Gutenberger *et al.*, 1997) and to live within modified vacuoles in the host cells (McIntosh *et al.*, 1997), as the

published *in vitro* data suggests, then it is a very versatile intracellular pathogen which is worthy of much greater attention than it has received.

1.3.4 BKD as a model to study physiological SP induction.

Beside the fact that BKD is an important disease of salmon and a potentially valuable animal model system for studying intracellular pathogenesis, one of the main reasons why I chose to study SP in fish with BKD is that the disease characteristically is associated with a chronic inflammatory pathology (Bruno, 1986b). Since SP can be induced in phagocytes during the process of phagocytosis (Polla & Kantengwa, 1991), I hypothesized that SP might be induced in the inflamed tissue of diseased fish. Thus, BKD also presents an opportunity to study the possibility that there is a physiological induction of SP expression in the inflamed tissue of diseased fish rather than in some contrived model of inflammation.

1.4 Stress and stress proteins.

1.4.1 Stressors and stress responses in fish.

Stress is known to increase the susceptibility of fish to BKD and other diseases. Although stress is a familiar word there is often no clear definition in mind when the word is used. Indeed there are several reasonable definitions for stress (Barton & Iwama, 1991) which are used in different contexts. Captive and wild fish can be exposed to adverse conditions (stressors) with which they must cope to survive and successfully reproduce. Stress is the general term for the responses of an animal to threatening changes in the external environment or internal conditions. A useful definition for stress is: "a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that in either

case, the chances of survival are significantly reduced." (Brett, 1958 cited in Barton & Iwama, 1991).

Stress in fish has been recently reviewed in detail (Barton & Iwama, 1991). Stressors may be chronic or acute and range from such factors as biotic (e.g. pathogens, predators) or abiotic changes in the water (e.g. hypoxia, particulate concentration, pollution) to such factors as aquaculture practices (e.g. crowding, handling, etc.). Stress involves a series of modifications in behavior, biochemistry and tissue structure that are thought to provide resources needed to escape the threat and repair damage. Hans Selye discovered that part of the stress response, which he named the General Adaption Syndrome (GAS; Selye, 1950), is caused by stress hormones (such as corticosteroids and catecholamines) and is largely independent of what the stressor is. This kind of stress hormone response occurs in fish and causes changes in physiology which are thought to help the fish survive the harmful effects of the stressor; however, stress itself can also be harmful (Barton & Iwama, 1991). Severe or prolonged stress can cause mortalities and compromise growth, immunity and reproduction (Adams, 1990). At least part of the negative effects of stress in fish, such as impairment of immune function, appeared to result from chronically elevated cortisol (Pickering & Pottinger, 1989) or increased catecholamine levels (Flory & Bayne, 1991) and are therefore due to the stress response itself rather than the specific stressor.

1.4.2 Stress proteins and stress inducible proteins.

An animal can respond to stressors in several ways instead of or in addition to the increased levels of stress hormones and the resulting metabolic changes. One important class of response is the production or synthesis of stress proteins and other stress induced proteins.

In stressed animals, stress induced proteins can accumulate to concentrations higher than are present in the relatively unstressed state. Increased levels can be due to increased conversion from precursors or increased synthesis of a subset of proteins. In fish, this category of proteins includes pre-acute phase proteins (Demers & Bayne, 1997; Ruis & Bayne, 1997), acute phase proteins (Winkelhake *et al.*, 1983; Fletcher, 1986) , and SP which I discuss in more detail below. Stress induced proteins are potentially useful because in addition to any functional significance they may have in the stressed and unstressed state, their presence at concentrations above their normal constitutive levels could be used as an indicator of stress.

Stress proteins are a set of proteins for which synthesis increases in response to heat shock or a variety of chemical stressors in species from bacteria to man (reviewed by Welch *et al.*, 1991; Sanders, 1993; Feder *et al.*, 1995; Morimoto *et al.*, 1997) and in fish (Iwama *et al.*, 1998). Cells, *in vitro* and *in vivo*, produce SP in response to stressors which disturb the internal conditions of the cell (Subjeck & Shyy, 1986; Lindquist & Craig, 1988; Hightower, 1991). The most numerous and best studied class of SP are the heat shock proteins (HSP). They are called heat shock proteins because they are induced by heat shock and were initially discovered as a new set of puffs on the salivary gland polytene chromosomes of heat shocked Drosophila (Ritossa, 1962). The heat shock proteins are in general highly conserved and are organized into families of homologous proteins which are named based on their approximate molecular weights. For example, HSP-90, HSP-70, HSP-60 and the small HSP including HSP-30 are among the major HSP in vertebrates.

In addition to being induced by heat, HSP are induced in cells of many species, including fish, in response to many other chemical and physical stressors (Lindquist & Craig, 1988;

Feder *et al.*, 1995; Iwama *et al.*, 1998). Furthermore, some stressors such as glucose starvation, hypoxia, or oxidative damage induce additional SP which are not induced by heat (Subjeck & Shyy, 1986). The mechanisms of induction have not been completely clarified, but expression of HSP is known to results when damaged proteins are present in the cell (Hightower, 1991) and also when the cell is damaged in a number of ways (Iwama *et al.*, 1998). Any severe disturbance of the internal conditions of the cell is likely to damage proteins and other vital cellular components and induce HSP.

Potentially, SP synthesis could be induced in fish in several ways. The internal conditions of the cells could be disturbed directly by stressors which cannot be regulated in fish or which overwhelm the regulatory capacity. Elevated water temperature, for example, causes synthesis of HSP in various tissues of living fish (Dyer *et al.*, 1991). Stressors other than heat could induce SP less directly. For instance, systemic disturbances of physiology such as acidosis or changes in plasma ion concentrations could result from mal-adaptive effects of the stress response (Barton & Iwama, 1991) and these disturbances could stress cells throughout the animal. Finally, cellular SP synthesis could be stimulated as a result of some more localized physiological process such as the release of reactive oxygen species (ROS) from phagocytic cells at the site of an inflammatory response. These oxidants can induce SP synthesis (Winyard *et al.*, 1992). Biologically active signaling molecules which can be released and can act locally such as interferon gamma, IL-6,TNF-α, and prostaglandins A and J can also induce SP synthesis in mammalian cells (Koga *et al.*, 1989; Santoro *et al.*, 1989; Koller *et al.*, 1993).

It is perhaps important that in whole animals, stressors can simultaneously trigger several separate responses. For example, heat shock of whole fish is experienced directly by the

cells and induces SP. Heat shock is also experienced as a threat by the fish and induces a hormonal stress response (Mazur, 1996). In a similar way, toxic heavy metals such as cadmium can induce both specific proteins like metallothioneins and general SP such as HSP-70 (Heikkila *et al.*, 1982); In living fish cadmium can also induce the stress hormone cortisol (Tort *et al.*, 1996). Since each stressor will have a set of responses which it induces, monitoring of a variety stress responses can give information about the type and severity of the stressors that a population of fish has experienced. This information may allow the stressors to be identified and controlled and thus improve the health, survival or productivity of the population.

1.4.3 Biological functions of stress proteins.

Although the biochemical functions of SP are not the main focus of this thesis it is important to point out that a great deal is known about their functions and their value to cells under stressful and normal conditions. The biochemical functions and regulation of stress proteins have been studied so extensively that a vast body of literature exists. As Feder and Hoffmann (1999) point out, there are even too many excellent reviews to cite. This introductory overview of SP functions is drawn mainly from information in very good recent reviews by Fink (1999), by Buchner (1999), and by Beissinger and Buchner (1998) which discuss some of the important biochemical functions of stress proteins.

The term stress protein covers a wide variety of individual proteins which may have many subtle functions at the cellular level. However, the main functions of the various members of the most important stress protein families are in facilitating proper folding of proteins and in regulating the molecular interactions and functions of specific proteins. Many stress proteins including HSP-60, HSP-70, HSP-90 and the small HSP/α-crystallin family have been shown to function as "chaperones" by specifically binding to partially unfolded proteins and thereby favouring appropriate folding and protein-protein interactions by preventing inappropriate interactions such as aggregation (Fink, 1999). Members of several heat shock protein families including HSP-60, HSP-70 and HSP-90 function as components of biochemical protein folding machines. There are other known functions and probably other important but so far unknown functions performed by the myriad of different members of even these main HSP families. However, the central roles of HSP as chaperones and in protein folding have been studied extensively in biochemical model systems and are the best understood functions of the HSP.

The folding intermediates of newly synthesized proteins and the partially unfolded structures of denatured proteins contain regions with exposed hydrophobic amino acids. These exposed hydrophobic areas are prone to bind with hydrophobic areas on other proteins and form nonfunctional aggregates. Chaperones bind to proteins with exposed hydrophobic structures, prevent aggregation, and also assist in the folding (or refolding) process so that unfolded intermediates do not accumulate. The chaperones do not have a strong affinity for most proteins once they are properly folded (or refolded).

Under normal physiological conditions, members of the HSP families are needed in living cells for functions including the proper folding of many newly synthesized proteins, assembly of proteins into protein complexes and into specific subcellular locations, and targeting of badly denatured proteins for degradation by the ubiquitin-proteasome pathway. The functions as chaperones and in protein folding and degradation are also thought to help prevent and repair damage caused by heat shock or other cellular stressors. These functions of the HSP are also believed to help prevent the death of stressed cells.

The chaperone and protein folding functions are best understood for HSP-60, HSP-70, and HSP-90 which form the main components of three protein folding machines which actively (consuming ATP) facilitate the refolding of proteins. These protein complexes also contain accessory proteins which are required for full function. The main aspects of the functioning of these three protein folding machines are summarized briefly below.

The HSP-90 and HSP-70 machines can act independently *in vitro* but can also work in concert. It is likely that the chaperones and protein folding machines cooperate within

living cells and interaction of multiple chaperone systems with the same substrate molecules is probably important *in vivo.*

The HSP-60 machine of bacteria is the most studied of chaperone complexes. It contains HSP-60 (GroEL) and a smaller accessory protein (GroES). Eukaryotic plastids such as mitochondria and chloroplasts have closely related GroEL homologues but eukaryotic cells also possess cytoplasmic HSP-60 machines containing subunits homologous to T-complex polypeptide 1 (TCP-1). These TCP-1 ring complexes are hetero-oligomers of subunits which are distinct members of the HSP-60 family. The HSP-60 in the plastids share much more sequence identity with GroEL than do the TCP-1 homologues. The TCP-1 ring complexes have structures similar to the GroEL complex and are thought to function in a similar manner but do not require a GroES homologue to function *in vitro.*

The GroEL complex of *E. coli* contains a double ring structure of 14 GroEL molecules in two stacked rings with hydrophobic central cavities which are capable of binding partially unfolded proteins. By interacting with collapsed hydrophobic structures and stabilizing the unfolded state, this hydrophobic pocket may also function to unfold proteins that are folded incorrectly. The functional complex which is ready to bind a substrate protein has a disc shaped lid composed of seven subunits of GroES on one end of the stack and one ADP molecule bound to each of the seven GroEL subunits in that ring. The ring which is closed off by the attached GroES lid is called the cis-ring while the opposite (open) end of the stack is the trans-ring. The presence of the GroES lid causes a shift in the structure of the cis-ring so that it forms a larger and more hydrophilic central cavity. This cavity is thought to provide a suitable polar environment to stabilize the native state of unfolded proteins and thereby favour refolding. The GroEL/GroES complex can accommodate proteins up to

about 55 kD. The presence of GroES also increases nucleotide binding by the GroEL in the trans-ring.

In a recent model accounting for the sequential reactions of the GroEL complex, Weissman *et al.*(1995) proposed that the partially folded substrate protein binds initially to hydrophobic pocket of the trans-ring of a bullet shaped 14GroEL:7ADP:7GroES complex. The ADP can be displaced by binding of ATP. In the absence of bound ATP the trans-ring is in the high affinity state for binding of substrate proteins. Binding of ATP to the cis-ring causes release of GroES and ADP. A new GroES lid and ATP bind to the ring containing the bound protein (which is now the cis-ring). This may involve a football shaped (cis-cis) transitional intermediate in which a GroES lid is present at both ends of the GroEL complex. Binding of GroES and ATP causes the central pocket of the cis-ring to change conformation and become a large hydrophilic pocket. This conformational change allows more space for refolding and reduces the affinity of the pocket walls for binding the substrate protein. Although free to rotate and refold the substrate protein remains trapped inside the pocket by the GroES lid covering the entrance. Hydrolysis of the ATP in the cis-ring and binding of ATP in the trans-ring causes a conformational change which releases the GroES lid so that the protein can leave the cavity. The released substrate protein may then complete the folding process or if it still contains exposed hydrophobic regions it may bind to a GroES complex (or another chaperone) for another cycle of folding.

HSP-70 (DnaK in bacteria) has also been thoroughly studied. The full function of HSP-70 requires a J-protein (DnaJ in bacteria; HSP-40 or other specialized J-proteins in eukaryotes). The complete HSP-70 complex is much less complicated than the HSP-60 machine. It contains a single subunit of HSP-70 and HSP-40. The constitutive form (HSC70) also binds to an accessory protein called Hip (HSC70 interacting protein) which stabilizes the complex in the high substrate affinity (ADP bound) state. HSP-70 binds to short, linear, hydrophobic peptide sequences within the structure of partially unfolded proteins. It is generally believed that the substrate initially binds to HSP-70 containing bound ATP. The ATP bound conformation of HSP-70 has a low affinity for substrate proteins. The J protein (HSP-40) may bind at the same time as the substrate and may even act as a chaperone itself to deliver the substrate protein. Binding of the J protein to the complex stimulates ATP hydrolysis to ADP and the resulting conformational change increases the affinity of HSP-70 for the substrate. The J protein, the ADP, and the phosphate are then released and a fresh ATP binds to the HSP-70. ATP binding causes the rapid release of the substrate protein because the HSP-70 shifts to the conformation with low affinity for the protein substrate. Hydrolysis of the ATP occurs later and may occur after a new substrate has bound if there is an abundance of substrate proteins. The substrate that has been released may refold correctly or it may bind to the HSP-70 complex or another chaperone system for another cycle of folding.

The HSP-90 machine has also been studied in considerable detail. Unlike HSP-60 and HSP-70, the functions of the HSP-90 homologues in eubacteria appear to be quite different from the functions of HSP-90 in eukaryotes. In *E. coli* HSP-90 (HtpG) is not even required for growth under stress conditions whereas mutant yeast with no HSP-90 are not viable at any temperature. The dominant (cytoplasmic) HSP-90 proteins of eukaryotes are very similar and their genes can rescue yeast strains with lethal HSP-90 deletions. The functioning of eukaryotic HSP-90 has been studied to the greatest extent using yeast and human HSP-90, but the complex seems to be conserved among eukaryotes since the same components are also present in the HSP-90 machines of plants.

In mammals, two major and one minor form of HSP-90 have been identified in the cytoplasm and one form has been identified in the endoplasmic reticulum. HSP-90 occurs as a dimer, either free or in a complex. The HSP-90 complex is not as large and elaborate as the HSP-60 complex but it has more known components than the HSP-60 or HSP-70 machines. In fact, the fully functional HSP-90 machine contains HSP-70. The HSP-90 protein has two separate domains which have chaperone activity *in vitro.* Since HSP-90 is a very abundant protein, free HSP-90 probably helps to prevent aggregation *in vivo.* The structures of the N-terminal (ATP dependent) domains of yeast and human HSP-90 have been determined using X-ray crystallography.

It is generally believed that the main function of the HSP-90 machine is to bind and stabilize a limited subset of proteins which function in receptor and signaling pathways. It prevents aggregation and holds the substrates in a form which can be folded properly or which can interact functionally with some other molecule or structure in the cell. HSP-90 is involved in stabilizing steroid hormone receptors and many protein kinases.

The function of the complete HSP-90 machine has been studied most extensively in its role of stabilizing steroid hormone receptors (SHR). A complex containing HSP-90 is required for SHR activity. In the absence of the steroid hormone, the receptor has an empty hydrophobic pocket. HSP-90 is thought to prevent this pocket from undergoing hydrophobic collapse to a nonfunctional state. The functional HSP-90 machine assembles when a dimer of HSP-90, and the functional HSP-70 machine (HSC70, HSP-40 and Hip) are united via an accessory protein Hop. The substrate (SHR) probably binds to HSP-90 along with the HSP-70 complex and may be presented to the HSP-90 in a partially unfolded state by the HSP-70 machine. The HSP-70 machine then dissociates from the complex and is replaced by a large immunophilin (such as one of the FK506 binding proteins) and an accessory protein called p23 in an ATP dependent step. This mature complex is active and the SHR can dissociate and bind to its hormone ligand or dissociate and reenter the binding cycle with HSP-90 and HSP-70. Upon binding to the hormone, the receptor dimerizes and moves to the nucleus where it binds to specific steroid response elements to exert its regulatory function.

1.4.4 Protective effects of stress proteins.

One of the most compelling reason why there is so much current interest in the biology of SP is that they can protect living cells and tissue. Both mild heat shock which induces SP and some SP themselves protect cells and tissues from being killed by wide range of otherwise damaging stressors including a subsequent more severe heat shock (Morimoto & Santoro, 1998). This has applications in medicine. For instance mild heat pretreatment can dramatically reduce the damage to heart tissue which occurs after it is deprived of oxygen (Dillmann & Mestril, 1995). Cellular protection by SP has a wide variety of other potential applications and benefits in medicine (Ribeiro *et al.*, 1995).

The most direct example of protection is the phenomenon of induced thermotolerance in which cells pretreated with a mild heat shock become highly resistant to killing by subsequent heat shock (Parsell *et al.*, 1993). There are two major modes by which cells die: necrosis and apoptosis (Whyllie, 1981). Necrosis occurs when a cell is severely damaged so that it can no longer make sufficient cellular ATP and other high energy substrates to repair itself. It involves breakdown of the cell membrane and other structures by cellular enzymes and release of the cytoplasmic contents and other cellular components into the surrounding tissue. Apoptosis is an active energy dependent process which occurs when the cell is stressed by environmental factors in pathological conditions or by biochemical signals such as removal of growth factors in normal physiology and development. It is sometimes called programmed cell death. Apoptosis involves an orderly series of events which culminate in the breakdown of the cell into membrane bounded apoptotic bodies which are removed by phagocytosis without release of the cells contents (Hart et al., 1996; Samali & Orrenius, 1998). Without pretreatment, cells which

receive a heat shock can undergo apoptosis or necrosis depending on the severity of the shock.

Cells can be protected by a mild heat shock or genetic over expression of cloned HSP-70 not only from apoptosis induced by a subsequent heat shock but also from apoptosis induced by stressors other than heat such as the toxic cytokine tumour necrosis factor-α (TNFα) and the membrane-derived second messenger ceramide (Mosser *et al.*, 1997; Buzzard *et al.*, 1998). Ceramide is released by hydrolysis of sphingomyelin and is a central mediator of stress induced apoptosis (Verheij *et al.*, 1996).

In fish, SP are thought to serve a role in thermotolerance, and a mild heat shock prior to a subsequent more severe heat shock can protect the ability of fish cells to perform complex cellular functions such as the transport of sulphate across polarized renal tubule cells in cultures (Brown *et al.*, 1992). There is good evidence that SP can also protect whole fish from heat since genetic variability in the speed of HSP-70 induction and in the level of constitutive expression correlate with the ability of desert fish (Poeciliopsis hybrids) to survive rapidly increasing water temperatures (Dilorio et al., 1996). The protective affects of SP in fish may also extend to stressors other than heat, since a mild heat shock has been reported to increase the resistance of salmon smolts to the osmotic shock of transfer to sea water (DuBeau *et al.*, 1998). Feder and Hoffmann (1998) have recently prepared an excellent review of the role of HSP in evolutionary and physiological ecology of organisms (including fish) facing natural stressors.

It is clear that not all of the protective effects or thermotolerance, induced by mild heat shock, can be explained by expression of SP. Heat treatment causes a greater

thermotolerance than expression of HSP-70 alone. Furthermore, mild heat treatment of cells has effects on other cellular components beside SP. The composition of cell membranes for instance can be modified in response to heat and have an important influence on thermotolerance (Gracey, 1996). However, SP themselves have been shown to protect cells against many important kinds of stressors (Morimoto *et al.*, 1997). Another area where the protective effects of SP may be important is in the possible protection of host cells during bacterial infection. One of the consequences of infection is that there may be cellular necrosis or apoptosis in the infected tissue (Barsig & Kaufmann, 1997). Cell death may be triggered by the direct effects of the pathogen or by the host's response to the pathogen. By releasing cytoplasmic contents and other cellular components cell death can also contribute to stimulating the inflammatory response. Necrosis which involves the disintegration of the dead cells and phagocytosis of the cellular debris tends to cause more stimulation of the inflammatory response than does apoptosis. Apoptosis involves breakdown of the cell into membrane bounded apoptotic bodies which are removed by phagocytosis without release of the cell's contents (Hart *et al.*, 1996; Samali & Orrenius, 1998). Increased levels of SP can protect cells from dying but might also prevent cells from dying earlier by apoptosis so that they might eventually die later by necrosis. Thus SP could result in a shifting from one mechanism of death to another. Favouring of necrosis by preventing apoptosis has been proposed as a possible negative effect of HSP-70 in inflammatory diseases (Polla *et al.*, 1998a). By increasing the intensity of the inflammatory response death by necrosis might lead to more killing of bacteria. Alternatively, increased inflammation might only cause greater tissue damage without actually killing an intracellular pathogen like Rs which lives in phagocytes.

1.4.5 Use of stress proteins to detect the effects of stressors.

Monitoring stress and reducing exposure of fish to stressors is one approach to protecting and improving the health of cultured and wild fish. Since SP can be induced by many stressors, SP expression has been proposed as a marker of environmental stress in aquatic organisms (Sanders, 1993; Sanders & Martin, 1993; Dunlap & Matsumura, 1997; Vijayan *et al.*, 1997; Vijayan *et al.*, 1998). The measurement of SP has several features that make this a very useful way of detecting the effects of a broad range of stressors before these stressors reach lethal levels. Firstly, measurable SP induction occurs slowly enough that it is not affected by the stress of capturing the fish and HSP-70 does not seem to be affected by moderate handling stress (Vijayan *et al.*, 1997). Secondly, SP can remain elevated for weeks after exposure and so provide a longer period of time for detection of the effects of a stressor (Mazur, 1996). Thirdly, SP are induced as a result of disturbance of the internal biochemistry of cells and as such are indicators of the effects of a stressor rather than mere exposure. Induction of HSP-70, for instance, occurs in fish exposed to contaminant concentrations just below the lethal levels. Exposure to lower contaminant concentrations which may not disrupt the animals physiology and biochemistry does not seem to cause induction (Vijayan *et al.*, 1998). In contrast, although it has been widely used as an indicator of environmental contamination, induction of cytochrome P-450 oxygenases indicates exposure to some aryl hydrocarbon pollutant rather than indicating that the pollutant has caused harm.

1.4.6 Physiological induction of SP in whole animals.

There are two conceptually different ways that SP induction can occur in a whole animal. The first and most obvious way is that a stressor can act as it does on cells in culture to

directly damage cells and induce a response in those cells. The second way is that a stressor can trigger a physiological response in the animal, the physiological changes may damage cells either locally or systemically, and this may induce a response in those cells even if the initial stressor had no direct damaging effect on the cells.

Most of the research on SP has employed simple forms of living material, such as single cell organisms, cells, or tissues in culture. As a result, relatively little is known about the induction of SP in whole animals by physiological processes. Until now, there has been almost no published research in fish into the possibility of SP induction by physiological processes (Janz *et al.*, 1997). This is in spite of the reports of SP induction in mammals as the result of important physiological processes such as the hormonal stress response to surgery and confinement (Holbrook & Udelsman, 1994), and the pathology of atherosclerosis (Kleindienst *et al.*, 1993), arthritis (Hermann *et al.*, 1991) and interstitial lung disease (Polla *et al.*, 1993).

The reductionist focus on SP in cell cultures and single celled organisms is probably also responsible for the relative lack of interest in physiological responses caused or influenced by SP expression in cells or tissues of whole animals. The important role of SP in protecting against tissue damage is easy to understand, relatively easy to study, and well supported by experimental evidence (Ribeiro *et al.*, 1995). However, there is no good reason for believing that the benefits of SP are limited to this kind of direct effect nor for that matter that induction of SP is always beneficial to the whole animal.

Just as a stressor can trigger a physiological response in a whole animal and indirectly induce SP, a stressor could also induce SP expression in cells or tissue and indirectly cause

a physiological response in the whole animal which would not have been triggered directly by the stressor which caused the SP induction. One area where indirect effects of SP induction are thought to be important is in the stimulation of immune and inflammatory responses (Multhoff *et al.*, 1998; Polla *et al.*, 1998b). This stimulation may not always benefit the animal since such responses to SP may lead to autoimmune reactivity (Gaston, 1998) and SP may contribute to inflammatory disease processes such as asthma (Polla *et al.*, 1998a). The idea that elevated SP levels could be detrimental raises an important distinction between protection of a host cell and protection of the host. Individual cells may be protected against damage by expressing SP but this SP might simultaneously trigger or favour pathophysiological processes which are detrimental to the whole animal. Furthermore, as I discuss in more detail in the final chapter, there are also situations in which the direct cytoprotective effects of SP may be detrimental to the animal when the death of individual cells is needed.

1.4.7 Phagocyte oxidants and physiological SP induction.

One physiological process in mammals which has been implicated in SP induction is phagocyte oxidant production at sites of localized inflammation (Polla & Kantengwa, 1991; Polla & Cossarizza, 1996). Inflammation, in mammals, is the production of redness, swelling, heat, and pain at the site of injury or infection (Weissmann, 1992). The inflammatory system, which produces this reaction, is essential to prevent invasion by pathogens. The process of inflammation is inseparably intertwined with the mechanisms of nonspecific immunity (phagocytic cells, lytic enzymes, and complement activation). A variety of mediators produced at the inflammatory site including eicosanoids, modified phospholipids and complement fragments, C3a and C5a, attract and stimulate phagocytic

cells. Stimulated phagocytic cells release additional mediators, lytic enzyme, and oxidants. The phagocytic cells function to engulf and kill pathogens. The toxic materials, such as oxidants, which the phagocytes use for this defensive killing can also damage host cells and induce SP.

In vitro, the addition of oxidant chemicals such as hydrogen peroxide can cause induction of SP in eukaryotic cells (Morimoto *et al.*, 1997). Furthermore, under the appropriate conditions, SP can be induced in phagocytes by their own oxidant production during the process of phagocytosis (Polla *et al.*, 1995) and in the pathogen which is being engulfed (Kantengwa & Polla, 1993). Phagocytosis of pathogens does not always induce SP in the phagocytes and simply producing superoxide and peroxide is not sufficient to induce SP in phagocytes (Clerget & Polla, 1990). It has been argued that conditions which lead to production of more reactive oxidants, such as hydroxyl radicals are needed for SP to be induced by oxidants (Barazzone *et al.*, 1996; Polla *et al.*, 1998b). The Fentin reaction is one physiological mechanisms that produces hydroxyl radicals from hydrogen peroxide in the presence of iron (Winyard *et al.*, 1992). The concentration of available iron is normally very low but the Fentin reaction may be important in some pathological situations. Another physiological mechanism by which hydroxyl radicals could be formed is the reaction of superoxide with nitric oxide to produce peroxynitrite which decomposes at physiological pH to generate hydroxyl radicals. Peroxynitrite is probably important physiologically since phagocytic cells have enzymatic pathways to produce large quantities of superoxide and when activated by cytokines some phagocytes produce inducible nitric oxide synthase (iNOS) an enzyme which produces nitric oxide using L-arginine as a substrate. It is not clear whether peroxynitrite is important in fish, but purified salmon and

trout neutrophils produce large quantities of superoxide (Hamdani *et al.*, 1998). Fish probably produce NO since a gene homologous to mammalian iNOS has been cloned from rainbow trout and is induced by stimulation with bacteria (Secombe *et al.*, 1998). An homologous gene is also present in a goldfish macrophage cell line (Laing *et al.*, 1996).

The induction of SP by oxidants in a living animal is somewhat more complicated than the induction of SP by oxidants *in vitro.* Living animals have physiological mechanisms which serve to limit damage caused by oxidants and by inflammatory processes. Some mechanisms involve removing the oxidants so that they can act locally but are prevented from spreading through the body. Other mechanisms act by providing negative feed back to limit the extent of activation of the system and to initiate processes of repair.

1.4.8 Potential for physiological SP induction in BKD.

Stress is known to increase BKD in cultured salmon, as stated above. However, as a stressor in its own rite, BKD could also cause stress in fish. In particular, the chronic nature of the disease and the presence of large numbers of phagocytes and other leukocytes at the sites of infection provides the opportunity to look for physiological induction of SP in a relevant *in vivo* situation.

If we regard the initial stressor in BKD as being the infectious agent Rs, then this stressor could potentially induce SP directly or as a result of triggering a physiological response. Contact of cells with Rs could conceivably cause stress to those cells and induce SP expression. This seems reasonably plausible since Rs invades host cells. Such direct SP induction would be analogous to SP induction by a chemical or physical stressor acting on cells in culture. Alternatively, the presence of Rs might trigger some pathophysiological

process which could cause SP induction in cells which are neither infected nor directly in contact with the pathogen. These two means of induction are conceptually distinct but, as I stated above, a stressor such as heat (or Rs) could act both as a direct stressor to individual cells and by triggering a physiological process which might lead to additional SP induction.

Chapter II Bacterial kidney disease[1]

2.1 Introduction

2.1.1 Bacterial kidney disease and physiological induction of SP.

The experiments studying coho salmon with BKD form the core of the work for this thesis and provide the point of reference for the other experiments with cultured cells. In this chapter and in the next I present and discuss the experiments using coho salmon with BKD.

I set out to study BKD as a relevant biological model in which to look for physiological induction of SP (as I discuss briefly in the Introduction and expand on below). In order to get a consistent population of diseased fish under controlled conditions I used a laboratory challenge procedure to induce BKD in coho salmon (*Oncorhynchus kisutch*) injected with *Renibacterium salmoninarum* (Rs).

As hypothesized, I found elevated levels of host HSP-70 in the tissue of coho with BKD. The pattern of mortality and disease was consistent with other published studies of experimentally induced BKD so the results of this study can be interpreted in the context of those other studies. The mortality data also reveals evidence that a shift in the growth or virulence of Rs may occur at high bacterial density. Although it has not been previously suggested, a density dependent sensor may regulate virulence in Rs.

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[1] A paper containing some of the data in this chapter and part of the Discussion was published in:
R.B. Forsyth, E.P.M. Candido, S.L. Babich and G.K. Iwama. 1997. J. Aquat. Anim. Health. 9:18-25.

2.1.2 The nature of the disease.

Bacterial kidney disease is a serious disease problem of both wild and farmed salmonid fish (reviewed by Fryer & Sanders, 1981; Evelyn, 1993; Evenden *et al.*, 1993; Fryer & Lannan, 1993). Bacterial kidney disease is highly prevalent in many wild stocks of salmon in the North Pacific (Evelyn, 1988). The pathogen is enzootic in wild and farmed salmon in British Columbia and is widely distributed in cultured salmonids in many countries throughout the temperate world (Evelyn, 1988; Evenden *et al.*, 1993; Brown *et al.*, 1994).

Bacterial kidney disease is caused by *Renibacterium salmoninarum* which is a slowly growing, short (approximately 0.5 to 1 micron), gram positive rod shaped bacterium which sometimes appears to be diplococci. In culture, Rs requires cysteine in the medium and grows very poorly at temperatures above 20°C. In natural infections, the disease has a long latent period. The first deaths occur in juvenile fish from about 1-3 months after exposure in a hatchery setting (Wood 1974 cited in Fryer & Sanders, 1981). The latent period can apparently be 2-3 years or longer since many fish infected as juveniles do not develop disease until much later in life and many adult Pacific salmon returning to spawn are still asymptomatic carriers (Brown *et al.*, 1994). Bacterial kidney disease is frequently a chronic disease and many mature salmonid fish have advanced BKD when they return to spawn (Fryer & Sanders, 1981).

The bacteria are able to persist and grow inside phagocytes (Gutenberger *et al.*, 1997) and other fish cells (McIntosh *et al.*, 1997). As the bacteria multiply in the tissue of the fish, granulomatous lesions frequently form (Bruno, 1986b), but the infiltrating phagocytes are

not able to eliminate the bacteria. These inflammatory lesions may be present in virtually any tissue but are most characteristic in the kidney. The exact mechanisms of pathology have not been determined but there is evidence that immune complex deposition in the kidneys and infiltration of inflammatory cells may contribute to kidney damage in fish with BKD (Sami *et al.*, 1992). There is also evidence that when growing inside cells, Rs produces virulence factors (McIntosh *et al.*, 1997), and these might contribute to tissue destruction.

As the disease progresses the kidney frequently becomes swollen and necrotic. Ion regulation may fail and in fresh water abdominal distention results from a build up of peritoneal fluid. Since the disease is chronic, infected fish could persist in a population for long periods of time. This chronic nature also makes it possible to test the hypothesis that SP expression is induced in inflamed tissue of fish with BKD.

2.2 Materials and methods

Fish and rearing conditions —— Coho salmon *Oncorhynchus kisutch* (Capilano river stock) were reared at The University of British Columbia in dechlorinated Vancouver municipal water. All fish were cared for in accordance with recognized principles for the care of laboratory animals (Canadian Council on Animal Care 1993). For final sampling, fish were killed humanely by gentle dip netting into an aerated solution of 200 mg/L tricaine methane sulphonate (TMS Syndel Labs) buffered with 400 mg/L $NaHCO_3$. For physical measurement and injection, fish were anaesthetized in an aerated solution of 50 mg/L TMS and 100 mg/L $NaHCO_3$.

For the BKD infection experiments, fish with a mean weight of 26.5 ± 7.05 g (SD) were netted and randomly transferred to the experimental tanks. To allow repeated sampling of the groups for the main experiment, six groups of 40 fish were confined in 20-L plastic chambers floating in six separate 200-L oval fiberglass tanks. Each of the 200-L tanks also received groups of 100 fish as described below; These fish could swim freely in the tank throughout the course of the experiment. The 3 control tanks were separated from the 3 disease challenge tanks by barriers of clear polyethylene sheet (6 mil) to prevent crossinfection. The flow rate to each tank was initially 5 L/min and was raised to 10 L/min on day 29 of the experiment. The cylindrical chambers had circles of plastic mesh (1-mm mesh size, area 550 cm^2) covering the bottom and had two 33-cm^2 mesh covered ports on the sides. Individual chambers were aerated to promote mixing between the water of the chamber and that of the tank. Over the course of the experiment the mean dissolved

oxygen concentration was 8.3 ± 0.83 mg/L (SD) and the mean water temperature, 12.4 ± 2.33 °C (SD). The fish were acclimated for 1week before experimental injection. The fish that were free to swim about resumed feeding and were fed salmon pellets (EWOS Canada) throughout the experiment, but the fish confined in the chambers did not resume feeding throughout the experimental period.

Experimental induction of bacterial kidney disease —— Infection was established by intraperitoneal (ip) injection with *R. salmoninarum* using the same strain (isolate 384) as Murray et al. (1992). The bacteria were cultured at 15 °C for 28 d on solid medium (Selective Kidney Disease Medium: Austin *et al.*, 1983) with charcoal (0.1% w/v) substituted for serum (Daly & Stevenson, 1985). Confluent bacterial cultures were scraped from the agar surface and resuspended in cold peptone saline (0.9% w/v NaCl, 0.1% w/v peptone) to an optical density (540 nm) of 1.2. Bacterial suspensions were held on ice and homogenized just before use by 10 strokes in a Dounce-type homogenizer with a Teflon™ pestle. For the main experimental groups (in the chambers), the homogenized suspension was further diluted 1/400 with saline for injection and plate counts; three groups of 40 fish received ip injections of 0.1 mL of the diluted suspension containing 3 X 10^4 colony forming units/mL determined by a modified drop plate counting method (Evelyn *et al.*, 1989). Three groups of 40 control fish received ip injections of 0.1 mL of saline. For the free swimming fish, 60 non-injected fish were left in each tank to serve as sentinels for horizontal transfer of BKD. Four groups of 10 fish in each tank were injected with 0.1 mL

of saline or of various dilutions of bacterial suspension and returned to their original control or disease challenge tank. Three groups in each tank were injected on the same day as the main experimental group (in the chambers). In the disease challenge tanks, one group was injected with the same dilution of bacteria (1/400) and the other two groups were injected with suspensions of 100 fold more or 100 fold fewer bacteria (dilutions of 1/4 or 1/40000 respectively). Because no deaths had occurred a fourth group of 10 in each tank was injected 54 d later with saline or with undiluted bacterial suspension 1 X 10^9 colony forming units/mL.

Disease progression and sampling —— Seven or eight fish were sampled from each tank in the main experimental group 1, 4, and 9 weeks after injection and killed humanely with buffered TMS (200 mg/L). Blood samples were taken into heparinized capillary tubes from the severed caudal peduncle of each fish for plasma and measurement of haemoglobin concentration. The fish were dissected to observe signs of disease, and tissue samples of liver, head kidney, kidney, gill, heart and spleen were removed and frozen immediately in plastic bags by pressing under dry ice. Separated plasma was transferred to polypropylene tubes, frozen on dry ice, and stored at -20°C or below. Tissue samples were stored frozen at -60 °C or below. Haemoglobin concentration was measured as cyanomethemoglobin using modified Drabkin's reagent and a standard containing human haemoglobin (Sigma kit 525-A). Protein concentration in lysates and plasma was measured using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). All reported protein values are in BSA equivalents.

Plasma chloride concentration was measured by titration with a Buchler chloridometer (model 4425000).

The fish were monitored for mortality for 120 d. The time to death for each fish was assigned as the middle of the interval between the time it was observed alive and the time it was found dead. When more than one fish died in an interval, times to death were distributed evenly throughout the interval. Two fish which died within 1 d following injection are excluded from the analysis. The calculation of cumulative fractional mortality was complicated because some fish died before the final sample of fish was taken. Prior to the final sampling, cumulative fractional mortality was calculated as $F_t = M_t / N$, where M_t is the number of fish which had died by any time t and N is the total number of fish. After final sampling, it was calculated as $F_t = F_s + (M_t - M_s)(1-F_s)/N_s$, where s is the time at which sampling occurred and N_s is the number of surviving fish after sampling.

Preparation of lysates for measurement of stress protein —— Negative control lysate was prepared from healthy coho liver removed aseptically and frozen immediately. Positive control lysate was prepared from coho salmon liver cells, stressed *in vitro* with sodium arsenite. Healthy coho liver was removed aseptically, cut into 3-mm pieces, incubated at 15 °C with 50 mM sodium arsenite for 5 h, centrifuged 10 min at 300 g, resuspended in fresh medium (Eagle's MEM, 10% v/v fetal calf serum, 15 mM HEPES, 100 U/mL penicillin, 100 mg/L streptomycin, 10 mg/L gentamicin sulphate), allowed to recover for 19 h at 15 °C, centrifuged 10 min at 300 g and stored frozen at -60 °C or below.

Tissue samples were lysed and homogenized by grinding with a rotating Teflon™ pestle in 10 volumes of ice cold lysis buffer (50 mM 2-amino-2-hydroxymethyl-1,3 propanediol (Tris), 20 mM NaCl, 1% v/v polyethylene glycol *p*-isooctylphenyl ether (Triton X-100), 0.5% sodium deoxycholate, 5 mM EDTA, pH 7.5) containing protease inhibitors (0.5 mg/L leupeptin, 1 mg/L aprotinin, 1 μM pepstatin). Phenylmethylsulphonyl-fluoride (PMSF 100 mM in isopropanol) was added immediately before and after homogenization to a total concentration of 2 mM. The tissue lysates were cleared in a micro-centrifuge at 20,000 g for 15 min at 0 °C, aliquoted and stored frozen at -20 °C or below.

Bacterial lysate was prepared for use in Western blotting to test the antibody for crossreactivity with antigens from the pathogen. Bacteria were scraped from solid medium as described above, washed once with sterile distilled water, and centrifuged for 5 min at 12000 g. The pellet (20 mg wet weight) was resuspended with an equal mass of glass powder in 15 volumes of lysis buffer (10% w/v sodium dodecyl sulphate (SDS), 250 mM Tris, pH 6.8) with protease inhibitors as above. The suspension was sonicated (20 bursts of 0.5 sec at 80 dB) with the tube cooled in ice water. The resulting lysate was cleared at 12000 g for 10 min and the supernatant stored frozen at -20 °C or below.

Antibody production ——Polyclonal antibodies against HSP-70 and HSP-30 were produced by collaborators in Dr. Peter Candido's laboratory at UBC. For production of HSP-70, RTG-2 cells (a fibroblast like cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonads ATCC CCL 55) were cultured in roller bottles in Eagle's MEM with 10% v/v fetal bovine serum. Before reaching confluence, cells were induced with 50 μM sodium

arsenite overnight (15-24 h), allowed to recover in fresh medium for 2-3 h, harvested by trypsinization, washed in phosphate buffered saline and stored at -70 °C. For preparation of rainbow trout HSP-70, frozen RTG-2 cell pellets were homogenized using a stainless steel Dounce homogenizer in a lysis buffer consisting of 10 mM Tris-acetate pH 7.5, 10 mM NaCl, 0.1 mM EDTA to which was added PMSF to 120 μM immediately before use. The lysate was centrifuged at 12,000 g for 15 min at 4 °C, and the HSP-70 was purified from the resulting supernatant by ATP-agarose affinity chromatography essentially as described by Welch and Feramisco (1985). Recombinant chinook salmon HSP-30 was expressed in *E. coli* carrying cDNA cloned by Dr. L. Weber, University of Reno, Reno Nevada. New Zealand White rabbits were initially immunized with purified HSP-70 or HSP-30 emulsified in complete Freund's adjuvant, and boosted with the antigen in Freund's incomplete adjuvant.

ELISA and Western blot —— The primary antibody was polyclonal rabbit antibody raised against rainbow trout HSP-70 as described above. For a few experiments I used antibody raised against chinook salmon HSP-30 as described above or two commercially available antibodies against HSP-60 (StressGen SPA 804, SPA 805). The secondary antibody, alkaline phosphatase conjugated goat anti-rabbit IgG (GAR-AP), was from GIBC0-BRL (9815SA). The antibodies were diluted with 2% w/v skim milk powder in TBS-T (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5). After binding of proteins, ELISA plates and nitrocellulose were blocked for 1 h with 2% w/v skim milk powder in TBS-T and were washed three times with TBS-T between each subsequent assay step.

I used an ELISA procedure to measure fish HSP-70 in tissue samples. The method is based on the buffers and reagents recommended by Kemeny (1991). Appendix I gives a detailed description of the ELISA and its development, performance, and precision. Briefly, aliquots of lysate were re-dissolved with an equal volume of 0.1 N NaOH and diluted to 30 mg/L in coating buffer (50 mM sodium carbonate-bicarbonate, pH 9.6). Wells of ELISA plates (Dynatech Immulon-4™) were coated with 50 µL of diluted lysate overnight at 4 °C. After blocking, the plates were incubated at room temperature (20 °C) for 1 h with rabbit anti HSP-70 (100 µL/well, 1/1000) followed by 1 h with GAR-AP (100 µL/well 1/6000). The plates were incubated at room temperature with 100 µL/well of substrate (1 g/L disodium p-nitrophenol-phosphate, 10% w/v diethanolamine, pH 9.5) until the absorbance of the highest standard was between 0.5 and 1.0. Absorbance was measured at 405 nm with a microplate reader (Molecular Devices THERMO$_{max}$™). The total amounts of protein bound to each well were assumed to be equal and to be representative of the proteins present in the lysate. A standard curve was constructed using a positive control liver lysate which was found to contain 6.9 times more HSP-70 than normal liver. Positive control lysate and normal coho liver lysate were mixed to produce a series of standards containing 30 mg/L protein in coating buffer of which between 0 and 80 % was contributed by the positive control lysate and the balance contributed by the normal control lysate. The standard curve was linear over the assay range. A linear regression of the standards against the signal produced in the ELISA was used to estimate the HSP-70 content of each sample in relative units as a percentage of the HSP-70 content of the

positive control. The values reported are the average of two estimates from separate assays.

For Western blots, proteins were separated by SDS PAGE (Laemmli, 1970) using the acrylamide concentrations described by Blattler *et al.* (1972). Separated proteins were transferred to nitrocellulose (BIO-RAD 0.2 μm pore size) at 13V for 30 min using alkaline buffer (Bjerrum & Hinnerfeldt, 1987 : 48 mM Tris, 39 mM glycine, 20 % v/v methanol, 0.0375 % w/v SDS, pH 9.2) and a semi-dry transfer apparatus (BIO-RAD ™Trans-Blot). For isoelectric focusing (IEF), samples prepared as for SDS-PAGE were diluted 1/10 with IEF sample buffer (Garrels, 1979). The acrylamide gels for IEF were prepared as described by O'Farrell (1975), using BIO-RAD carrier ampholytes (1.2 % w/v Bio-Lyte™ pH 5-8, 0.8 % w/v Bio-Lyte™ pH 3-10), and cast as slab gels. The samples were loaded at the cathode end and the gels were run at 4 Watts for 0.5 h and then at 750 V for 3.5 h. To determine the shape of the pH gradient, gel slices were soaked in freshly degassed 10 mM KCl and the pH measured. Proteins were transferred from IEF slab gels to nitrocellulose (Liu & Li, 1992) at 250 mA for 2 h using acidic buffer (0.7M acetic acid, 20% methanol). After blocking the nitrocellulose blots, HSP-70 was detected by incubating for 1 h with rabbit anti HSP-70, 1 h with GAR-AP and 1 to 15 min with substrate (330 mg/L nitroblue tetrazolium, 167 mg/L 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt) in alkaline buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) as described by Blake et al. (1984).

Analyses —— All statistical tests were performed using Sigma Stat™ (Jandel Scientific, Corte Madera, CA). Using Sigma Stat™ logistic regressions were performed by an iterative (least squares of residuals) method without logarithmic transformation of the data. Calculations and Kaplan-Meier product limit estimation were performed on spreadsheets in Microsoft Excel™. Comparisons were made between pairs of groups by t-test, and between three or more groups by ANOVA with multiple pair-wise testing by Dunn's method. Differences were considered significant where $P_{\alpha} < 0.05$. The P_{α} values (observed significance) between groups were calculated by t-test. When the assumptions of the parametric tests were violated, the nonparametric analogue (rank sum test or ANOVA on ranks) was used, and this is noted in the text when it occurs. Correlation was measured by Pearson's product moment and differences in proportions were tested using Fisher's exact test. To calculate rank ordering and to calculate the mean and median survival times long-term survivors were arbitrarily assigned as dying on day 120 (the end of the experiment). I have included a note in the figure captions where these assigned survival times were included in calculations of the summary values reported. The raw data was used for graphic presentation and in all other analyses.

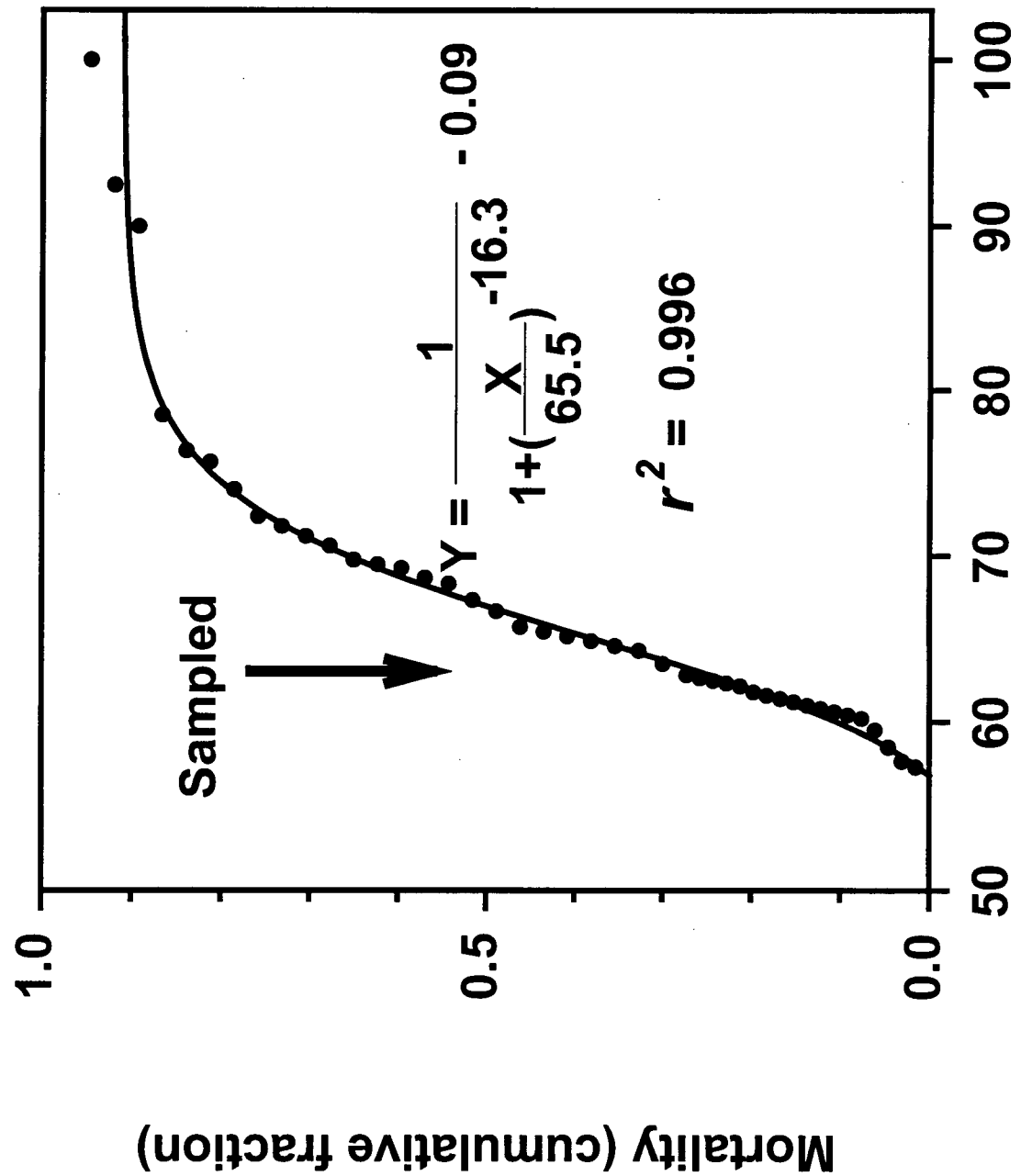
2.3 Results and Discussion

2.3.1 Experimental induction of BKD by interperitoneal injection of Rs.

The two most reproducible and reliable ways that BKD can be induced experimentally are interperitoneal injection of Rs (Bell *et al.*, 1984) or the use of infected donors (cohabiting) in the same tank as the fish to be infected (Murray *et al.*, 1992). I set up the experimental challenge to obtain groups of fish infected by both of these methods. Only the peritoneal injection method yielded sufficient diseased fish to allow sampling of enough affected fish at one time. However, several groups of donor fish were injected with different doses of Rs, and although the donor fish were not sampled, the pattern of mortality in these groups confirmed that the main experimental group had received a reasonable dose of Rs.

In order to have a reliable and meaningful challenge, I needed to expose the main group to a moderate dose which would allow for a more natural response to the infection, but which would successfully establish a persistent infection in most of the fish. Normally, in a challenge in fresh water, obvious signs of disease appear only days before the death of the fish when the fish are injected with relatively high doses of Rs (Starliper *et al.*, 1997). The dose given to the main group successfully caused a high overall disease incidence, but allowed a reasonable time window for sampling. The dose used to induce BKD in the main study group was at the lower end of the range in which disease was reliably induced in most of the fish within a reasonable time. The shape of the mortality curve (Figure 2-1) shows that nearly all of the fish in the main group eventually succumbed to the disease. Groups given larger doses died too quickly to allow appropriate sampling. At low doses there

Figure 2-1. Cumulative fractional mortality from BKD in the main experimental group of coho salmon (N= 45 fish) after injection with *R. salmoninarum.* The line indicates the nonlinear regression expressed by the formula. The arrow indicates the point at which the fish were last sampled.


Mortality (cumulative fraction)
1.0
0.5
0.0
50
60
70
80
90
100
Time after injection (Days)
Sampled
Y = 1 / (1+(X/65.5)^-16.3) - 0.09
r^2 = 0.996

appeared to be evidence of undesirable variability between the replicate groups reared in separate tanks (tank effects). Tank effects can cause severe problems for interpretation and reproducibility of disease challenge experiments and should be avoided when possible by rearing all groups in the same tanks or by minimizing the differences between the conditions in separate tanks. Furthermore, groups of fish infected with a dose lower than the main group did not develop disease quickly and failed to accumulate enough affected fish to allow observations of affected and unaffected fish in the same sample.

2.3.2 Pattern of mortality in coho with BKD.

Although a detailed study of the mortality in fish with BKD is not the topic of this thesis, the mortality data confirm that the dose and method of the challenge experiment was reasonable and produced a pattern of disease which is comparable to other published studies. I also want to point out some details in the pattern of disease which may have value for future experiments. Finally, the mortality data suggests that Rs may switch modes of growth or virulence and cause disease once a critical level of bacteria has been reached.

2.3.2.1 Shape and analysis of the mortality curves.

Bacterial kidney disease developed in the coho salmon exposed by injection with Rs with a pattern similar to that reported for intraperitoneally injected sockeye salmon *Oncorhynchus nerka* (Bell *et al.*, 1984) and chinook salmon *Oncorhynchus tshawytscha* (Murray *et al.*, 1992). The times to death in the three replicate groups of the main experimental group were not significantly different from one another (ANOVA on ranks $P_{\alpha}=0.736$). There

were also no tank effects in the high dose groups so the replicates for each of these groups were pooled and the patterns of mortality are analyzed and discussed below. The mortality data from the lower dose groups was not pooled or analyzed in detail because there was considerable variability (tank effect) among the replicates.

In the main experimental group the first death in the exposed group was observed 58 d after injection. The cumulative fractional mortality reached 0.946 by 101 d but did not rise further by the end of the experiment at 120 d (Figure 2-1). Most of the remaining fish died shortly after the final sample was taken but two of the 27 fish remaining after sampling survived to the end of the experiment. Only one saline-injected control fish died (day 27, cumulative fractional mortality = 0.010). This fish had no clinical signs of BKD, nor did any other control fish.

The mortality curves had a sigmoid shape which was apparent in the main study group (Figure 2-1) and also apparent in the mortality data from other studies of experimentally induced BKD (Murray *et al.*, 1992). In the main study group the median time to death was 67.0 d, and the cumulative fractional mortality data fit very closely ($r^2 = 0.996$) a sigmoid curve with a function of the form $\mathbf{F_t = 1/(1 + (t/c)^{-b}) - d}$ where **t** is the time after injection in days. This is a simplification of a four parameter logistic function of the form

$\mathbf{F_t = (a-d)/(1 + (t/c)^{-b}) - d}$. In this equation the constant **c** is an estimate of the centre of the mortality curve. The constant **b** is the exponential slope at the centre of the mortality curve. The two parameters **a** and **d** reflect the curvature at the ends. On a log-odds scale

(Logit $L_t = F_t /(1- F_t)$ the central portion of the curve (Figure 2-2) can be fit to a two parameter logistic function of the form **$F_t = 1/(1 +(t/c)^{-b}$** for which log-odds is a linear function of the logarithm of time **($L_t = b\ln(c) - b\ln(t)$ or $F_t = 1/(1 +e^{-(b\ln(c) - b\ln(t))})$**. This central portion is nearly linear and can also be fit to the more convenient simple logistic regression line $L_t = B_0 + B_1 t$ $F_t = 1/(1 +e^{-(B_0+B_1 t)})$. Both forms allow a good estimate of the time at which half of the fish have died **($F_t = 0.5$ at $t = c = -B_0/B_1$)**. This amounts to an estimate of the median survival time based on a regression of all the data.

The parameters from these logistic regression lines are a more meaningful way to summarize mortality data than the median or mean survival time for the following reasons. Both the median and the mean are more difficult to calculate when some individuals are withdrawn part way through the experiment. The median is determined by the value of one or two observations and is highly susceptible to sampling effects. The mean is overly influenced by extreme values and is subject to bias by censoring (not all fish die). Where there was sufficient data I used logistic regression analysis to summarize the mortality curves in the groups of fish injected with different doses of Rs (Table 2-1; Figure 2-2). The number of fish observed in each set is large enough that there is good agreement between the conventional median and the value estimated from the logistic regressions. However, this method would be useful for small sets of data which are more susceptible to the fluctuations in the mean and median described above.

Figure 2-2. Cumulative fractional mortality from BKD in coho salmon after injection with various doses of *R. salmoninarum.* The stepped lines are the fraction dead in that group at any point. The doted lines indicate the logistic regressions of the untransformed data. The fish were injected with: undiluted Rs suspension in the very high dose group (N=30 fish), 1/4 diluted suspension in the high dose group (N=30 fish), and 1/400 diluted suspension in the medium dose group (main experimental group ; N=45 fish).

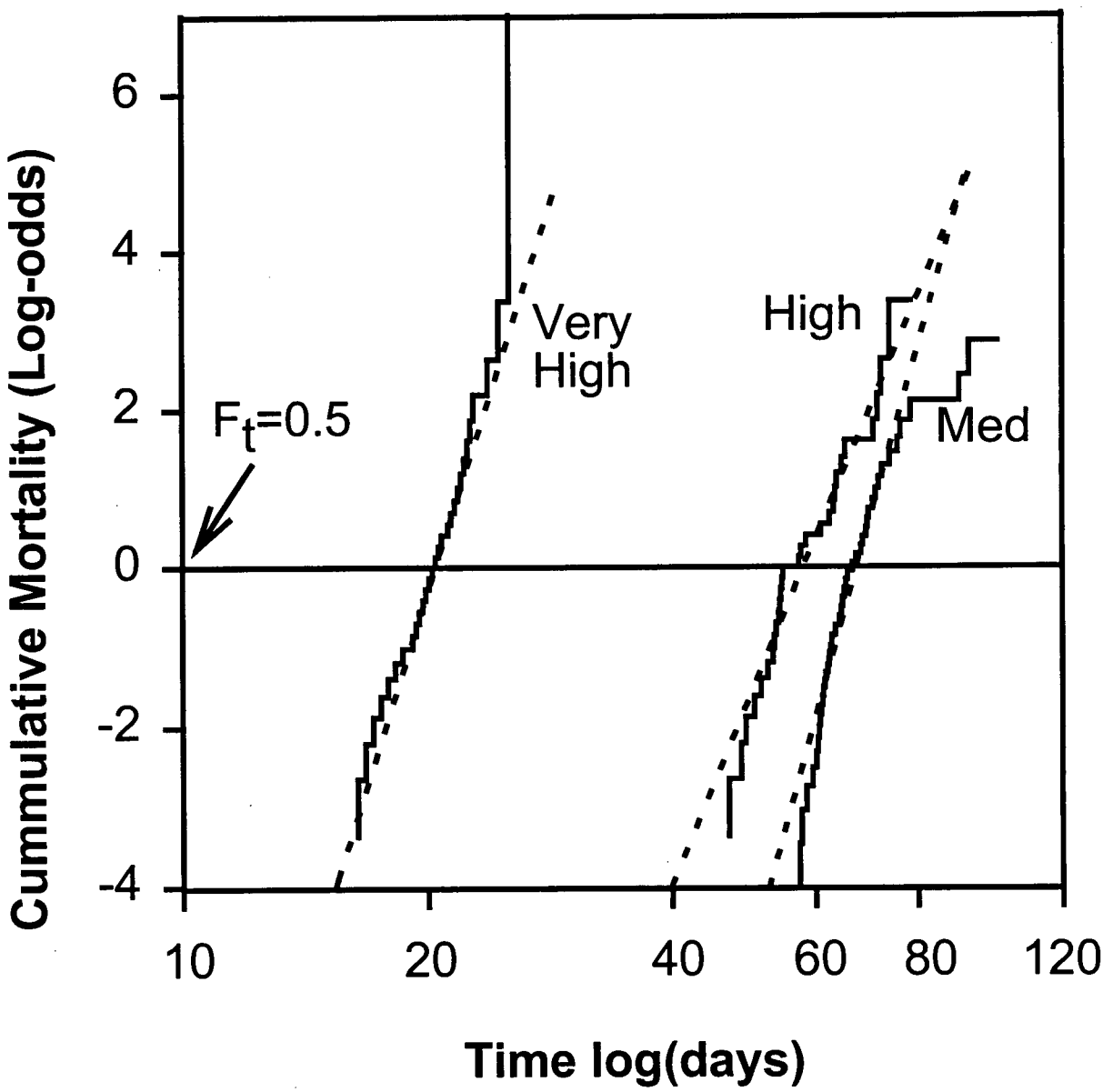

Cummulative Mortality (Log-odds)
6
4
2
0
-2
-4
F_t=0.5
Very
High
High
Med
10
20
40
60
80
120
Time log(days)

Table 2-1. Estimates of median mortality from BKD in coho salmon after injection with various doses of *R. salmoninarum.* The times are expressed in days. The interval shown with the median is the time period spanning the deaths of the middle half of the fish (25%, 75%). Cumulative mortality is the fraction of the fish which had died by the end of the experiment. The other values are the estimate (±SEE). The estimated parameters marked with an asterisk are significantly different from the values for all other groups (ANOVA; Pairs compared by Student-Newman-Keuls Method; $P_{\alpha} < 0.05$)

	Observed Survival Time		Logistic Regression		Cumulative
Dose injected	Mean [a]	Median	Time (F_t=0.5)	Slope (b)	Mortality
Very High (undiluted)	20.72 +/- 0.4613	20.5 (18.9, 22.1)	20.45 * +/- 0.0290	-14.01 * +/-0.3129	30/30
High (1/4 diluted)	61.23 [b] +/- 2.474	57.3 (53.4, 64.2)	58.04 * +/- 0.272	-10.68 * +/-0.567	29/30
Medium (1/400 diluted)	71.69 [b] +/- 2.291	67.0 (62.6, 72.2)	67.40 * +/- 0.149	-16.19 * +/-0.579	43/45 + 21 withdrawn

[a] Kaplan-Meier

[b] Set contains censored data (fish surviving at 120 d) which are excluded for calculation of the mean.

$F_t = 1/(1 + e^{-(bln(c) - bln(t))})$

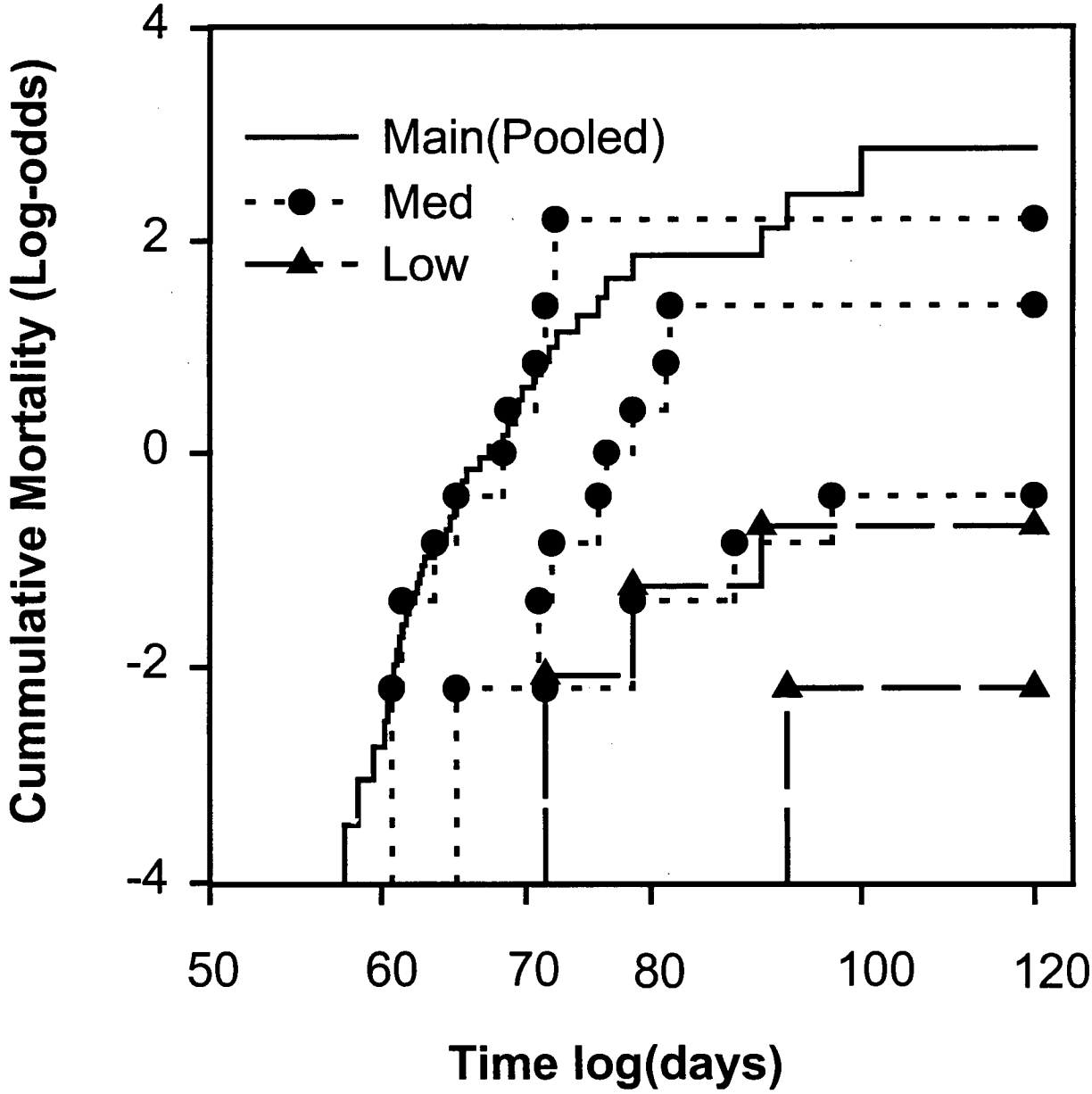
Table 2-2. Cumulative fractional mortality from BKD in coho salmon after injection with various doses of *R. salmoninarum*.
Cumulative mortality is the fraction of the fish which had died by the end of the experiment. The observed significance (Pα) is stated where the proportions are not significantly different. Groups labeled NT were not tested.

Dose injected	Mortality		Comparison of Proportions	
	First death (Days after Infection)	Cumulative Mortality	Different From Main group ($P\alpha < 0.05$)	Different From Free Medium group ($P\alpha < 0.05$)
Very High (undiluted)	19	30/30	No ($P\alpha = 0.514$)	Yes
High (1/4)	48	29/30	No ($P\alpha = 1.000$)	Yes
Medium (1/400) (main group) Pooled	58	43/45 + 21 withdrawn	No (Same Group)	Yes
Medium (1/400) (free group) Pooled	62	21/30	Yes	No (Same Group)
Replicates	66	8/10	No ($P\alpha = 0.147$)	NT
	73	4/10	Yes	NT
	62	9/10	No ($P\alpha = 0.459$)	NT
Low (1/40000) Pooled	73	4/27	Yes	Yes
Replicates	73	3/9	Yes	NT
	none	0/8	Yes	NT
	94	1/10	Yes	NT

2.3.2.2 Stress and tank effects in low dose groups.

As stated above the higher dose groups were free of tank effects; however, in the lower dose group there was significant variation in the times to death of the replicate groups. The variation in the lower dose groups may reflect differences in the resistance of different groups due to individual variability or subtle differences in the conditions in each tank. At higher doses this host resistance was probably overwhelmed so that no tank effects were present. It is worth noting, however, that tank effects were apparent in the groups which were given the same dose as the main group (Figure 2-3) but which were free to swim around in the tanks. Stress and crowding are known to increase the prevalence of BKD in naturally infected populations of fish (Mazur *et al.*, 1993) and increased density is a highly reliable stressor in fish. It is therefore plausible that the differences between the main group and the other three replicate groups given the same dose are a result of differences in stress due to the conditions in which they were held. The absence of tank effects in the main group could be interpreted as the presence of a maximal tank effect (due to confinement stress) in all three replicates whereas the groups that were free to swim about had some variability. Thus the mortality data shows evidence of two kinds of saturation effects. At high doses all variation between the replicate groups is swamped so that no tank effect differences are apparent. At moderate doses, the effects of stress may have saturated any variation in the susceptibility between the groups held in more stressful conditions while groups which received the same dose but were held under less stressful condition showed variability between replicates.

Figure 2-3. Variations in mortality from BKD after injection with lower doses of *R. salmoninarum.* The stepped lines are the fraction dead in that group at any point. The line for the main group are pooled data of three replicate groups (symbols are omitted for clarity). The other lines are individual replicate groups. One of the low dose groups had no mortalities so there are only two lines for that dose. The fish were injected with 1/400 diluted Rs suspension in the medium dose groups (N= 10,10,10 fish) and the main experimental group (N=45 fish). The fish were injected with 1/40,000 diluted suspension in the low dose groups (N= 9,10,8 fish).


Cummulative Mortality (Log-odds)
4
2
0
-2
-4
Main(Pooled)
Med
Low
50
60
70
80
100
120
Time log(days)

If the crowding effect in the main group amounted to a saturation of the effect of stress on susceptibility to BKD, this suggests a simple and expedient solution to a problem which plagues these kind of experiments. Tank effects can cause severe problems for interpretation and reproducibility of disease challenge experiments. It seems to be very difficult in practice to maintain identical conditions in replicate tanks throughout the course of an experiment. One likely source of variability is exposure to intermittent stressors in the environment (noise, movement, light). Usually researchers try to minimize these stressors but it would be much easier to ensure that all groups were exposed to stressors so as to saturate their response. Although such conditions would not be physiological for wild fish, simple crowding might be a cheap and reproducible way of attaining a consistent baseline of stress. Such an approach might be useful in vaccine trials for instance, because it would also provide a more demanding test of the ability of the vaccine to prevent disease in cultured fish when rearing conditions are not ideal for reducing stress.

2.3.2.3 Variation in incubation time with the dose of Rs.

The mortality data from this experiment showed a potentially meaningful pattern which suggests that there are two distinct phases to the development of BKD. The fish in this study were injected with various doses of Rs in order to have fish which would develop disease over an extended period of time and therefore provide a continuing source of infection. The dose mainly influences the speed at which disease develops after infection, or incubation time. In groups given the three highest doses the first deaths occurred earlier with increasing dose (Table 2-2) and the time for half of the fish to die was significantly shorter for the two higher dose groups than for the main group (medium dose; Table 2-1).

In contrast, the shapes of the mortality curves for these three groups are very similar once the fish started to die and in the higher dose groups there was no clear influence of increasing dose on the death rate or the number of fish which died. There were significant differences between the slopes of the logistic regression line for these groups but there was no trend related to dose and in fact the steepest slope occurred in the group (medium dose) which received the lowest of the three doses. Over this large range of doses (400 fold) there were also no significant difference between the final cumulative mortality observed (Table 2-2; contingency tables Fisher).

In the lower dose groups there appeared to be differences in the proportions of fish surviving to the end of the experiment (Table 2-2). This should be interpreted conservatively because more deaths might have occurred in these groups if the experiment had been continued. Furthermore, pooling of the final cumulative mortality data may not be valid for the low dose groups since there appeared to be tank effects in the overall pattern of mortality in the replicates for those two groups. However, there were no statistically significant differences in the final cumulative mortality between the replicates for any group (tank effects), but the power of testing small numbers in contingency tables is low so the pooled data for the two lowest dose groups should be interpreted conservatively. When the individual replicate groups were analyzed separately, the final cumulative mortality of each of the low dose groups and one of the three medium dose groups were significantly different from that of the pooled data from the main group (Table 2-2)

It is usual in bacterial diseases for the dose of bacteria to affect the speed and extent of mortality at the end of the experiment. The pattern in BKD is unusual in that there was a marked parallel nature to the mortality curves for the different dose groups. There was a

long incubation time which depended on the dose. After the initial death occurred the fish died in a very similar pattern for the three highest doses (Figure 2-2). Once the incubation period had elapsed the course of the disease for these dose groups was nearly the same. This suggests that there may be a simple relationship (such as a growth rate) between the dose injected and the incubation time before obvious disease occurs. This relationship may be apparent for BKD because the growth rate for the bacteria is slow.

My mortality data (Figure 2-2) and other published reports show that groups of fish injected with various doses of Rs had similar mortality curves except that fish given higher initial infectious doses required less time to reach the point at which disease and death occurred. In natural infections in the wild and on fish farms the situation is more complicated since the population may contain fish infected at different times and occurrence of mass mortality may be triggered by environmental factors such as rising water temperature, stress, sexual maturation or some unknown factor (Fryer & Lannan, 1993). However, there may still be an underlying pattern requiring some minimum level of bacterial infection to accumulate before overt disease can occur.

The parallel nature of the mortality curves of different dose groups has been noted in data from other published studies of experimentally induced BKD, but no explanation has been proposed (Iwama, 1980; Murray *et al.*, 1992; Starliper *et al.*, 1997). It is quite possible that the abrupt change in the pattern of disease results from a collapse in the host defenses once the bacteria reaches a critical level which overwhelms the host. However, as I mentioned briefly in the Introduction to this chapter the pattern of mortality in BKD is also consistent with the idea that Rs might grow in two different modes and that a switch occurs between the modes once enough bacteria have accumulated in the fish. The number of bacteria in

each fish is relatively small after infection, and since Rs grows slowly it may take a long incubation period for the infection to reach numbers sufficient to cause overt disease and to allow transmission to other fish. During this initial phase it might be a selective advantage for this pathogen not to cause disease because it needs the host for its continued growth and does not grow in the environment.

Initially the level of Rs increases in the tissue of the fish but may not cause much disease or tissue damage. At first the bacteria spread rather innocuously along the capsule of organs (Bruno, 1986b) whereas in latter stages of disease there are large numbers of bacteria and there can be extensive tissue necrosis and even visible areas of liquefied necrotic tissue (Evelyn, 1993). The increased tissue damage in the second phase might be caused by a shift to production by Rs of one or more of the potential virulence factors which have been reported such as an haemolysin (Grayson *et al.*, 1995b) or a protease (Griffiths & Lynch, 1991). Alternatively, it is possible that the tissue damage is caused by a host response to the pathogen which leads to the progressive release of damaging enzymes from host cells.

One speculative explanation of the timing of mortality and the possible shift to a diseased state is that the bacteria may exist at first in a relatively non cytotoxic form, perhaps within vacuole structures, and then as their numbers reach a critical level, switch to a more aggressive and cytotoxic mode in which it escapes into the cytoplasm of the host cell. As I stated in the main Introduction, intracellular pathogens generally use one of these two mechanisms to escape being killed by phagocytes (Finlay & Falkow, 1997). Some pathogens like *L. monocytogenes* dissolve the membrane of the phagocytic vacuole and escape into the cytoplasm as Rs seems to do in phagocytes (Gutenberger *et al.*, 1997). As Rs has been shown to do in cell cultures (McIntosh *et al.*, 1997), other pathogens like the

Salmonella spp can survive inside a vacuole within the host cell. There is no reason why Rs could not use both strategies or even switch between the two different strategies.

If this kind of shift does occur at a particular density of bacteria, it suggests the possibility that a "quorum sensing mechanism" (Kleerebezem *et al.*, 1997) is operating which allows a shift in expression of quorum-regulated genes when a critical density (quorum) of bacteria has been reached. Quorum sensing systems have been found in many species of both Gram negative and Gram positive bacteria and are the subject of several recent reviews (Swift *et al.*, 1996; Kleerebezem *et al.*, 1997; Lazazzera & Grossman, 1998). Quorum sensing involves the production of small signaling molecules which can then be detected by a sensor protein. So far these signal molecules have been found to be either acylhomoserine lactones or short peptides. The local concentration of each small signaling molecule increases along with the concentration and number of bacteria producing it until the concentration is sufficient to activate the sensor protein. These systems allow the bacteria to regulate expression of some genes so that they are expressed once the population density is high enough (Swift *et al.*, 1996).

There is, in fact, published evidence that some kind of quorum sensing mechanism exists in Rs, although quorum sensing has not been previously proposed as an explanation for the phenomenon. When grown in culture, Rs produces a metabolite of an unknown nature which stimulates the rapid growth of the bacteria in culture. Addition of spent medium containing this substance as a supplement allows much more rapid growth of Rs and efficient isolation of Rs from infected tissue (Evelyn *et al.*, 1990). Observing the effects of culture supernatants (conditioned medium) on the phenotype of bacteria is a usual way of detecting the signal molecules of quorum sensing systems (Mcclean *et al.*, 1997). The

growth enhancement of Rs by culture supernatants has been generally interpreted in the literature as a nutritional (cross feeding) phenomenon, but it could also be interpreted as the production of a molecule which when present at a sufficient concentration acts as a signal for some quorum sensing pathway which shifts the bacteria into rapid growth.

If a quorum sensing system exists in Rs it could help explain the variations in the virulence of Rs grown under different culture conditions (McIntosh *et al.*, 1997). Such a mechanism might also operate *in vivo* to allow Rs to shift from a slowly growing non cytotoxic form to a rapidly growing more aggressively cytotoxic form when the load of bacteria is high enough. The required concentration of the quorum-signal could have evolved over time so that the switch occurs when there are enough Rs to overwhelm the host defenses.

2.3.3 Pattern of disease in coho with BKD.

The pattern of mortality is usually reported in studies of fish with BKD. This provides a reasonable basis for comparing the level of disease in the experimental fish with that of other studies. To give a stronger basis for assessing the extent of the disease in the fish sampled, I also examined some basic clinical indicators of disease. I needed to have an idea of what physiological changes were present in the diseased fish in order to interpret the results. The level of disruption of vital physiological functions gives a reproducible indication of the stage of the disease process in these fish so this information will also allow future comparison with other experiments where changes in physiological indicators are measured.

Anemia, depressed plasma protein concentrations, and compromised ionic regulation are typical of BKD in fresh water and correlate with disease (Bruno, 1986a; Bruno & Munro, 1986; Iwama *et al.*, 1986). The diseased fish in my experiments also showed sign of these physiological changes. In the fish sampled from the main group at week 9 (day 63), in the middle of the mortality curve, 17 of 21 exposed fish (injected with Rs) had clinical signs of disease (Table 2-3). The other four fish had no signs of disease. Most of the diseased fish were anemic with low plasma protein and plasma chloride concentrations. The 14 anemic fish completely account for the significantly lower haemoglobin levels (Table 2-3) of the exposed group relative to the control group (ranks sum $P_\alpha < 0.001$). The mean haemoglobin concentration of the remaining seven exposed fish (9.37 g/dL ± 0.370 SE) was not significantly different ($P_\alpha = 0.958$) from that of the control group. There was no significant difference at week 1 ($P_\alpha = 0.338$) or week 4 ($P_\alpha = 0.781$) between the haemoglobin levels of the exposed and control fish (data not shown). Of the exposed fish, only diseased fish were anemic and only anemic fish had depressed plasma chloride or protein levels (Table 2-3). When all of these data for controls and exposed fish at week 9 were analyzed as a single set, there was highly significant correlation between visible signs of disease and concentrations of haemoglobin, plasma chloride and plasma protein ($P_\alpha < 0.001$ for all combinations). Disease correlated negatively with haemoglobin ($r = -0.772$), plasma chloride ($r = -0.626$) and plasma protein ($r = -0.673$). The depression of plasma chloride in the diseased fish suggests that ion regulation was beginning to fail. The severe anaemia and compromised ion regulation indicate an advanced disease state in this subset of the fish (Bruno, 1986a; Bruno & Munro, 1986).

Table 2-3. Disease and systemic effects of BKD in coho salmon sampled 9 weeks after exposure. Haemoglobin, plasma chloride and plasma protein values are means (±SE); The asterisk indicates significant difference from the control group ($P_\alpha < 0.05$) and the fraction indicates fish with values more than 2 SD below the mean for the controls.

	N	Diseased[a]	Haemoglobin (g/dL)	Plasma Chloride (meq/L)	Plasma Protein (g/dL)[b]
Control	23	0/23	9.35 ± 0.177	130.5 ± 0.644	2.79 ± 0.083
			1/23	0/23	1/23
Exposed	21	17/21	*6.29 ± 0.566	*116.5 ± 3.95	*1.93 ± 0.228
			14/21	10/19	10/19
Not clinically Diseased	4	0/4	9.40 ± 0.402	130.0 ± 1.08	2.96 ± 0.212
			0/4	0/4	0/4
Diseased	17	17/17	*5.56 ± 0.558	*112.9 ± 4.57	*1.65 ± 0.236
			14/17	10/15	10/15
Anemic	14	14/14	*4.75 ± 0.398	*110.8 ± 5.04	*1.40 ± 0.165
			14/14	10/13	10/13

[a]Fraction of fish which had obvious signs of disease (peritoneal fluid or soft swollen kidneys).
[b]BSA equivalents

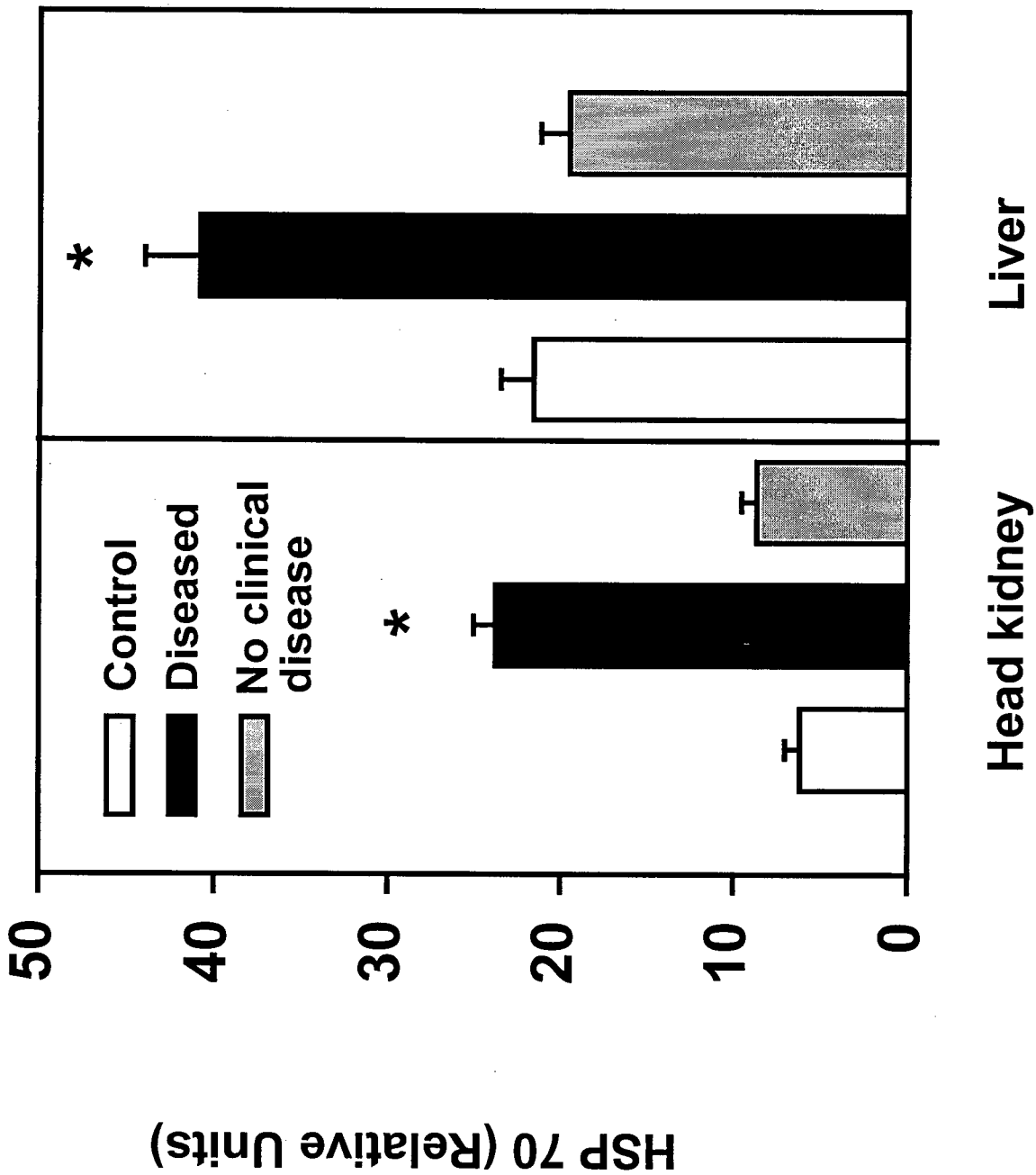
2.3.4 Stress proteins in coho with BKD.

The levels of stress protein-70 (HSP-70), also known as heat shock protein-70 (HSP-70), was measured by an enzyme linked immunosorbent assay in kidney and liver from coho salmon *Oncorhynchus kisutch* with bacterial kidney disease (BKD), experimentally induced by injection with Rs. Fish with BKD had more HSP-70 in both kidney and liver than did the control fish. There was no detectable HSP-30 on Western blots of tissue from diseased or control fish. The HSP-70 measured was derived from the host tissue, not from the pathogen. In fish which had been exposed to pathogen but had no obvious signs of disease, HSP-70 was not significantly elevated.

2.3.4.1 ELISA of SP –70 levels in coho tissues.

As an indicator of whether SP were increased in the tissue of coho salmon with BKD, I developed an ELISA for HSP-70 which is described in Appendix I. I measured the HSP-70 content of tissue from control and diseased fish in this ELISA on a linear scale in arbitrary units relative to a positive control. Fish with BKD had significantly higher HSP-70 levels in both liver and head-kidney than the control fish (Figure 2-4). HSP-70 levels in tissues from fish without clinicai disease were not significantly different from controls ($P_\alpha = 0.511$ for liver, $P_\alpha = 0.108$ for head-kidney). All of the clinically diseased fish analyzed for SP were anemic and had large numbers of Rs detectable as short Gram positive rods in smears of liver or kidney tissue. No bacteria were detected in tissue smears from the fish without clinical disease.

Figure 2-4. Stress protein (HSP-70) in head kidney and liver of coho salmon with BKD. Samples from diseased fish (N=3 fish) were compared by ELISA with sham injected controls (N=6 fish) and exposed fish that had no signs of disease (N=3 fish). The positive control = 100 arbitrary units. Bars are one standard deviation. Groups marked with an asterisk are significantly different from the control group ($P_{\alpha} < 0.05$).

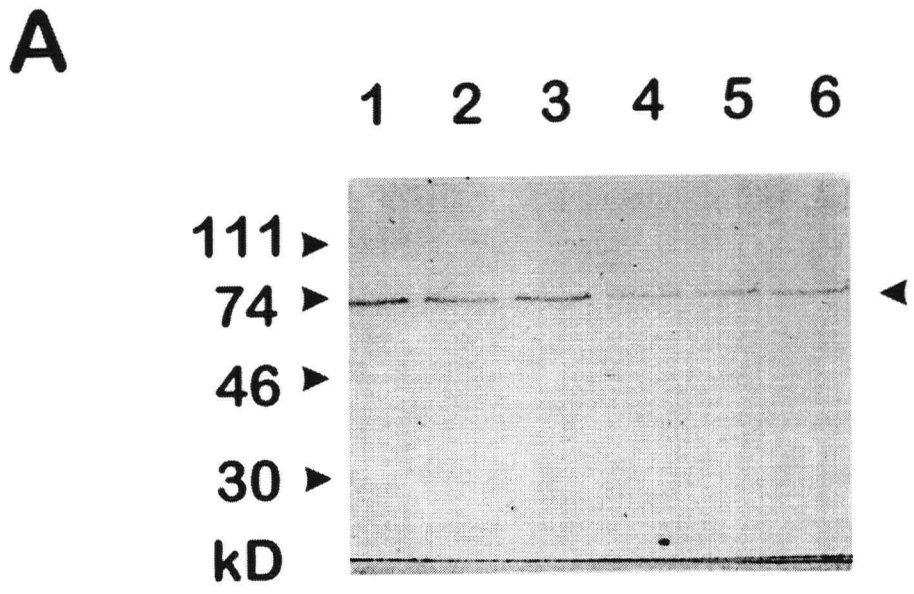

HSP 70 (Relative Units)
50
40
30
20
10
0
Control
Diseased
No clinical disease
*
*
Head kidney
Liver

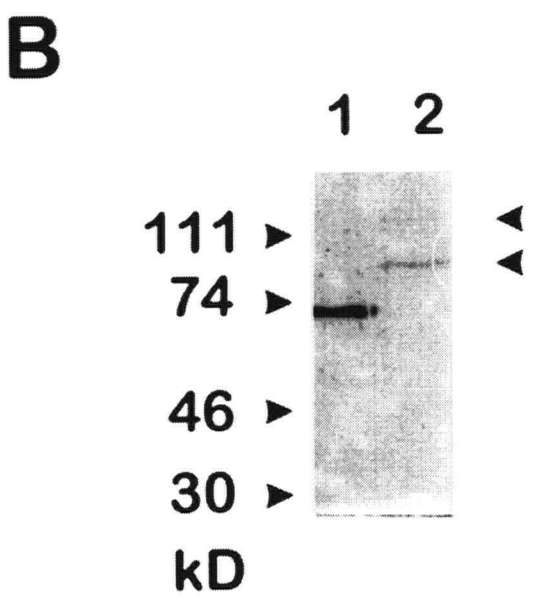
2.3.4.2 HSP-70 is from the host not from the pathogen.

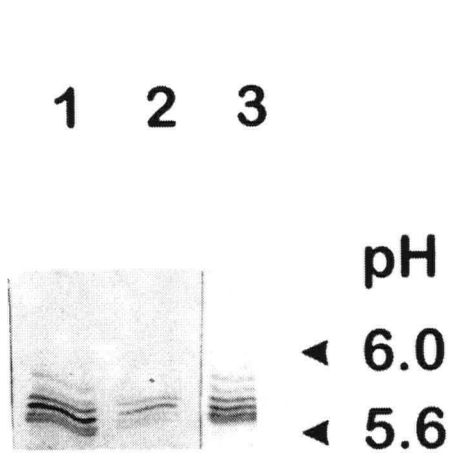
In fish with BKD there can be very large numbers of bacteria in the tissue along with antigens produced by the bacteria (Rockey *et al.*, 1991). For this reason I wanted to make sure that the HSP-70 in diseased fish was from the host cells and not simply due to the bacteria that are present in the diseased tissue. To confirm that the antigen detected was fish HSP-70 and not a bacterial protein, I performed Western blots of the tissue lysates which had elevated SP in the ELISA (Figure 2-5). The antibody reacted with a single major band of about 72 kD in liver lysates from fish with BKD or from fish without signs of disease (Figure 2-5A). In lysates of head kidney there were additional lower molecular weight bands which may be proteolytic fragments of HSP-70 (not shown).

I also analyzed extracts from Rs in order to rule out the possibility that the antibody against HSP-70 was cross reacting with material from the pathogen. No detectable HSP-70 reactivity could be seen in extracts prepared by grinding an equal mass of packed Rs under the same conditions used to prepare the tissue lysates (not shown). However, very little protein is released from Rs under those conditions so a bacterial lysate was prepared by sonication. The antibody did not react strongly with any protein band in lysate of *R. salmoninarum*. Prolonged incubation with substrate revealed two extremely faint bands (Figure 2-5B) at about 110 and 90 kD in lysates of *R. salmoninarum.* These cross reactive bands could not account for the signal observed in tissue lysates from fish with BKD since the stronger 90 kD band had an intensity less than 10% of the 70 KD band in an equal amount of protein from the liver of a fish with BKD. Even in heavily diseased fish, only a very small fraction of the protein in tissue lysates could be contributed by the pathogen (Rockey *et al.*, 1991).

Figure 2-5. Western blots of HSP-70 in lysates of coho salmon tissue and *R. salmoninarum* separated by SDS-PAGE and isoelectric focusing. The samples were separated by SDS-PAGE in A and B, and by isoelectric focusing in C. In A, lanes 1-3 are liver from fish with BKD and lanes 4-6 are liver from fish with no signs of disease (30 μg/lane). In B, lane 1 is liver from a fish with BKD and lane 2 is lysate of *R. salmoninarum* (40 μg/lane). In C, lane 1 is liver from a fish with BKD (40 μg), lane 2 is liver from a control fish and lane 3 is positive control (arsenite induced) liver cells (40 μg/lane). Lane 3 was incubated with substrate for 1 min whereas lanes 1 and 2 were incubated for 15 min.


A
1 2 3 4 5 6
111
74
46
30
kD


B
1 2
111
74
46
30
kD


C
1 2 3
pH
6.0
5.6

As an additional confirmation of the identity of the host HSP-70 I used Western blots of proteins resolved to their isoelectric points (pH at which a molecule has no net charge) by isoelectric focusing (IEF). When the proteins from livers of fish with BKD were separated on slab gel IEF (Figure 2-5C), the antibody reacted with six major bands between about pH 5.5 and pH 6.0. All six bands appeared to correspond with the six major bands present in the positive control liver (arsenite treated) and to four major and two minor bands present in control livers. Thus, the measured HSP-70 appears to be from the host tissue rather than the pathogen since the antibody does not cross react significantly with the pathogen and since all of the immunoreactive bands in the diseased liver lysates correspond to bands in control liver lysates separated on IEF and SDS-PAGE.

2.3.4.3 Other stress proteins.

Since induction of a SP response can lead to simultaneous increases in several different SP, I wanted to determine if there were some other SP which might also be elevated in the tissue from diseased fish. I used Western blotting to detect HSP-30 and HSP-60 since no ELISA was available for these proteins. On Western blots of HSP-30 in tissue from control and infected fish I found no detectable HSP-30 in tissue from control fish or from infected fish with or without BKD. The absence of detectable HSP-30 was not the result of problems with the antibody since the arsenite induced positive control lysates had high concentrations of HSP-30 (Figure 2-6). In these positive control lysates and in heat shocked fish (Norris *et al.*, 1997) HSP-30 is strongly induced along with HSP-70 so the absence of any measurable induction in fish with BKD may suggest that a different induction pathway is involved.

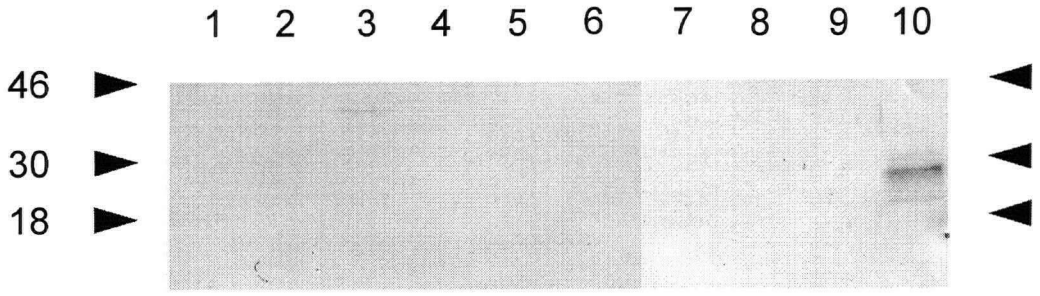


Figure 2-6. Western blot of HSP-30 in coho salmon tissue separated by SDS-PAGE. Lanes 1-3 are lysates of liver from fish with BKD; Lanes 4-6 are liver from exposed fish that had no signs of disease; Lanes 7-9 are liver from control fish (30μg/lane). Lane 10 is positive control arsenite induced liver lysate (6μg/lane).

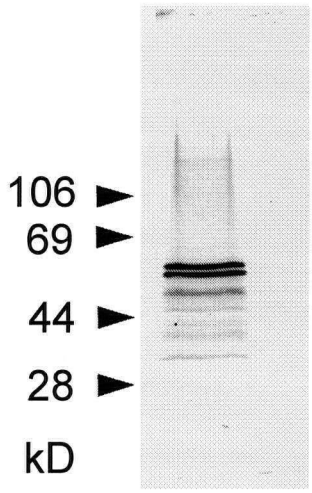


Figure 2-7. Western blot of *R. salmoninarum* HSP-60 separated by SDS-PAGE. The lane shown was loaded with sonicated Rs lysate (30μg/lane) and probed with polyclonal anti HSP-60 (SPA-804).

Heat shock and a variety of other stressors induce HSP-60 in addition to HSP-70 and the antibodies which I used react with HSP-60 of fish. However, HSP-60 is highly conserved between bacteria and vertebrates and unlike the antibody against HSP-70 the antibodies which I used to measure HSP-60 both cross react strongly with HSP-60 in lysates of Rs (Figure 2-7). The presence of this strong cross reaction to the bacteria would have made it difficult to interpret whether HSP-60 in tissue from diseased fish was from the host or the pathogen and I did not measure HSP-60 in tissue from fish with BKD.

Incidentally, this observation of cross reactivity of the antibodies with HSP-60 from Rs may be useful. Although I have not attempted to quantify HSP-60 (GroEL homologue) in Rs, the strength of the reaction on Western blot suggest that it is (as would be expected) a relatively abundant antigen. HSP-60 has been previously reported in Rs as the cause of a cross reactivity problem in some antisera against the main surface antigen of Rs (p57) which has molecular weight similar to that of HSP-60 (Brown *et al.*, 1995; Wood *et al.*, 1995). HSP-60 might also be a useful target antigen and I believe that it would be fruitful to pursue isolation of HSP-60 from Rs in the future. The fact that the commercially available antibodies react with HSP-60 from Rs should make it easier to identify and purify.

2.3.4.4 Conclusions.

I set out to look for elevated levels of SP in fish with BKD because I thought SP might be induced by the action of phagocytic cells in the inflamed tissue of diseased fish. From the results of the ELISA and Western blotting experiments I can conclude that induction of HSP-70 in fish with BKD may have resulted from the inflammatory pathology of the

disease or may have resulted from some other aspect of pathology. Abnormal ion levels, possible hypoxia due to low haemoglobin levels or cellular injury caused directly by the intracellular Rs are all reasonable alternate explanations. Although data which I show below rules it out, the increase in HSP-70 as a proportion of protein could have been caused by selective degradation of other proteins rather than an induction of SP synthesis.

Regardless of what caused elevation of HSP-70 in BKD, it raises the practical concern that disease could interfere with the use of SP levels as an indicator of environmental stress in populations which have unknown health status.

2.4 Next steps to investigate the implications of SP in BKD

The presence of elevated host SP levels in fish with BKD also indicates that SP could have an impact on the pathology of BKD, but further research will be needed to determine what role it might play. In a wide variety of diseases in humans and other mammals there is evidence that the induction of SP plays a role in the pathology of the disease. The SP of pathogens are often highly immunogenic antigens and immunoreactivity directed at SP can confer a high level of resistance in animal models of disease caused by *Mycobacteria*, *Yersinia* and other pathogens. SP may also act as adjuvants and play a role in inducing protective immunoreactivity to other antigens (Suzue & Young, 1996; Multhoff *et al.*, 1998). In diseases which have some bacterial component to the pathology the highly conserved nature of the major SP can lead to immune cells against SP of the pathogen cross reacting with the SP of the host (Handley *et al.*, 1996). In autoimmune diseases there is evidence that immune reactions to bacterial and host SP may cause inflammation and injury

which induces SP in host cells and continues the stimulation of the immune reaction (Gaston, 1997; Gaston, 1998). There is also evidence that regulatory T-cells which recognize host SP normally produce cytokines which control rather than cause inflammation and autoimmunity (Van Eden *et al.*, 1996). It is still a subject of controversy whether the continued presence of infection is required in human diseases such as reactive arthritis or whether the initial triggering infection is eliminated and the resulting cycle of autoimmunity is self perpetuating (Braun *et al.*, 1997).

One of the main reasons why I chose to study SP in fish with BKD is that the disease has a highly inflammatory pathology. There has even been speculation that immune reaction to the pathogen and deposition of immune complexes may play a role in causing disease pathology (Sami *et al.*, 1992). The presence of elevated SP in fish with BKD does not by itself indicate a link between the inflammatory pathology of BKD and induction of SP. However, the elevated levels of host SP clearly raises the possibility that SP could play a role in the pathology of BKD.

The possible significance and implications of the elevated SP levels which I found in BKD would be easier to discuss intelligently with more information about what might cause it and where and when SP was induced in the tissue. As next steps in this thesis it seemed desirable to determine what the pattern of SP distribution was and what causes could be favoured or ruled out by additional experimental data. In the next two chapters I investigate the distribution of HSP-70 in the tissue of fish with BKD and seek to explore some of the possible causes of SP induction in BKD using mixtures of cells in culture.

In future studies of fish with BKD, measurement of SP over the course of time could determine at what stage SP induction occurs. Measurement of the levels of other SP and their mRNA could confirm whether HSP-70 is induced in BKD as part of a general SP response or if only HSP-70 is elevated. It would also be worthwhile to know whether HSP-70 increases in other common infectious diseases of salmon such as furunculosis (*Aeromonas salmonicida*) or vibriosis (*Vibrio* spp.).

Chapter III HSP-70 expression in BKD: What could cause it?

3.1 Introduction

Since the work presented in the previous chapter showed that SP was elevated in BKD but not what specific mechanisms caused the SP response, I chose to next pursue where in the tissue SP expression occurred so as to determine what cells and mechanisms could have been involved. In this chapter I discuss the relationships between the microscopic patterns of HSP-70 expression, bacterial colonization, and structural morphology of the cells and tissue in frozen sections of liver from fish with BKD. I conducted the experiments in this chapter in order to determine what possible mechanisms could have caused increased HSP-70 levels in the tissue of fish with BKD.

In order to explore the possible mechanisms of SP expression I examined the histological pattern of SP expression. There have been several paper reporting the histopathology of infected tissue from fish with BKD (Young & Chapman, 1978; Bruno, 1986b; Speare *et al.*, 1993; Flano *et al.*, 1996a; Flano *et al.*, 1996b; Speare, 1997). None of these researchers studied the distribution or expression of HSP-70 or any other SP.

All of the studies of the pathology of BKD support the same general pattern of disease progression and chronic infection in many different organs. *Renibacteria* in the tissue tend to be inside macrophages or other host cells (Flano *et al.*, 1996a; Flano *et al.*, 1996b). Over time there is an increasing distribution of bacteria throughout the tissue and increasing damage to the organs involved. Initially, the bacteria are present along the capsule of the organ. Later, the bacteria can be seen spread throughout the parenchyma of the organs and in the latter stages of disease areas of necrosis can form with large numbers of bacteria and

infiltrating leukocytes. In extreme cases these zones of necrosis in the tissue can form macroscopic abscesses filled with pus (Evelyn, 1993; Bruno, 1986b).

In addition to the localized changes in the tissue which occur in BKD, there are also changes in systemic physiology such as anemia and impairment of ion and water regulation. Each of the many factors in the pathology of BKD which could potentially cause SP induction would lead to an expected pattern of induction. I expected that by examining the distribution I could get some indication as to whether expression was relatively uniform or if it was associated with certain regions or cell types. In this way I sought to rule out some of the possible mechanisms and gather evidence for whether the induction was associated with any specific aspect of pathology. In particular I wanted to test the second hypothesis. **SP induction in fish with BKD is associated with the sites of infection and phagocyte infiltration in tissue.** If phagocyte function is involved in SP induction in BKD I would expect that SP expression would be clustered in discrete areas where there were phagocytes in the tissue and perhaps in the phagocytes themselves.

3.2 Materials and Methods

Preparation of sections — Frozen tissue from the main experimental group of fish described in Chapter II were used to prepare frozen tissue sections for staining and microscopic examination. Tissue pieces were held frozen on dry ice and mounted in Histo Prep™ (Fisher Scientific) tissue embedding medium on aluminum stubs cooled with dry ice. The mounted specimens were allowed to equilibrate for 15 min in the cryostat at –18°C before cutting sections. Frozen sections 5-8 μ M thick were cut using a rotary microtome in a Reichardt cryostat. Ribbons of two or three serial sections at a time were

picked up and thaw mounted onto room temperature slides previously coated with poly-L-lysine (Sigma P-8920). Mounted sections were allowed to air dry for at least 1 h at room temperature (20 to 23°C). Tissues to be compared were picked at different locations on the same slides. The dry slides were placed in slide boxes wrapped in aluminum foil as a vapour barrier and stored frozen until needed. Immediately before use the slides were warmed to room temperature, removed from the slide box and fixed for 5 min in acetone held at –20°C. The slides were then fixed for 10 min in 3% paraformaldehyde in phosphate buffered saline (PBS) and washed three times for 10 min each in PBS. The slides were then incubated with freshly prepared sodium borohydride in PBS for 15 min to reduce autofluorescence and washed two times for 10 min each in PBS. The backs of the slides were then wiped dry and the excess liquid was drained from around the sections and carefully blotted dry using wedges of filter paper. This blotting procedure was repeated after all subsequent washing steps in the fluorescent staining procedures. Some slides were stained with hematoxylin and eosin to examine the morphology of cells within the tissue.

Fluorescent antibody staining procedure —— I used an indirect staining technique using rabbit primary antibody (anti-HSP-70) and Texas Red (TR) labeled secondary antibody (goat anti-rabbit IgG) in conjunction with direct staining of the bacteria with fluorescein isothiocyanate (FITC) labeled rabbit anti-Rs antibodies.

All reagents used in processing the slides, including the washes were, in PBS containing 0.05% Tween-20 and 0.1% BSA (TPBS-BSA) at 37°C. The slides were held in moist chambers for all incubations and were not allowed to dry at any step. Slides were blocked by incubating for 3 h in 10% nonimmune goat serum (NGS). The excess fluid was

removed and the slides were incubated with either 1/500 diluted rabbit anti HSP-70, or an equivalent amount of nonimmune rabbit IgG (NRIgG Sigma I-8140) in 5% NGS, or 5% NGS with no primary antibody for 3 h at 37°C and then stored in the fridge overnight. The slides were drained, washed three times for 10 min each by immersion in a glass slide jar and blotted. All subsequent washes were performed the same way. The slides were incubated with 1/200 diluted TR-conjugated goat anti rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; Cat# 111-075-045) in 5% NGS for 3 h, washed and blotted. The slides were incubated with 1/100 diluted FITC-conjugated rabbit anti Rs (Microtek International, Saanichton, BC, Canada; lot# RS8-0116C) in 5% NGS and 10% non immune rabbit serum for 3 h, washed and blotted. To detect DNA, some slides were stained with Hoechst 33258 (bisBenzimide; Sigma B-2883) by diluting the dye to 10 mg/L in the first of the three final washes. After the final washes each slide was thoroughly blotted and covered with mounting medium (8 mL glycerol; 2 mL 1M $NaHCO_3$; pH 9.5; 250 mg diazobicyclo-octane) and the cover slip was sealed by ringing with coloured nail polish. The antibody concentrations used were established by titration experiments to determine workable conditions and dilutions for all antibody reagents. Replicate slides were incubated with serial dilutions of anti-HSP-70, NRIgG and the fluorescent antibodies in a series of trial experiments until there was no noticeable difference in the background between the NRIgG treated slides and the control slides incubated without primary antibody.

Microscopes, photography and composite images —— All fluorescence microscopy was performed using a Zeiss Axioscop or a Zeiss Axiophot microscope with epifluorescence illumination and phase contrast optics. All photographic images were taken using a 35 mm

camera with automatic exposure metering (Zeiss MC-100). Most images were taken on black and white film (Kodak T_{Max} 400 pushed to 1600 ASA) and developed using Kodak T_{Max} developer in a manual processing tank according to the manufactures direction. Each color channel and the phase contrast image was captured as a separate black and white image without adjusting the microscopes position. In this way a series of images of the same field were produced in registry. Some images were captured directly on high speed color film (Kodak EPH pushed to 1600 ASA) using multiple exposures of the same microscope field on different color channels to create two or three color composite images. Composite images were also created digitally by scanning individual images of the same field and overlaying the separate color channels using Adobe PhotoShop™.

Systematic scanning of fields — In order to determine the proportion of HSP-70 staining areas that were associated with Rs, microscopic fields were scanned and scored manually. While watching the fluorescence on the red channel the stage was moved back and forth and advanced at the end of each sweep so as to avoid overlapping fields. The area of damaged tissue at the edge of each section was avoided and 20 separate fields were scored on each slide. Areas which were brightly fluorescent on the red channel were examined on the green channel to determine if there was any Rs staining within three cell diameters of the centre of the feature and whether the feature was HSP-70 (red) or autofluorescence (red and green = yellow).

Intensity measurement of grayscale — The intensity of the fluorescence of HSP-70 staining in BKD liver was compared with that of liver tissue from heat shocked fish by scanning the gray scale intensity of photographic prints. Comparison was made only between images

captured on the same roll of film and printed, scanned and analyzed using identical conditions. The printing time was chosen so that there was a gradation of the gray scale through the intensity range of the features being measured. Using Adobe PhotoShop™ a grid of 1 pixel wide lines was superimposed on the image to allow different areas to be analyzed separately. The flood fill mode of Sigma Scan Image™ was used to automatically select and measure the average intensity of areas of bright fluorescence.

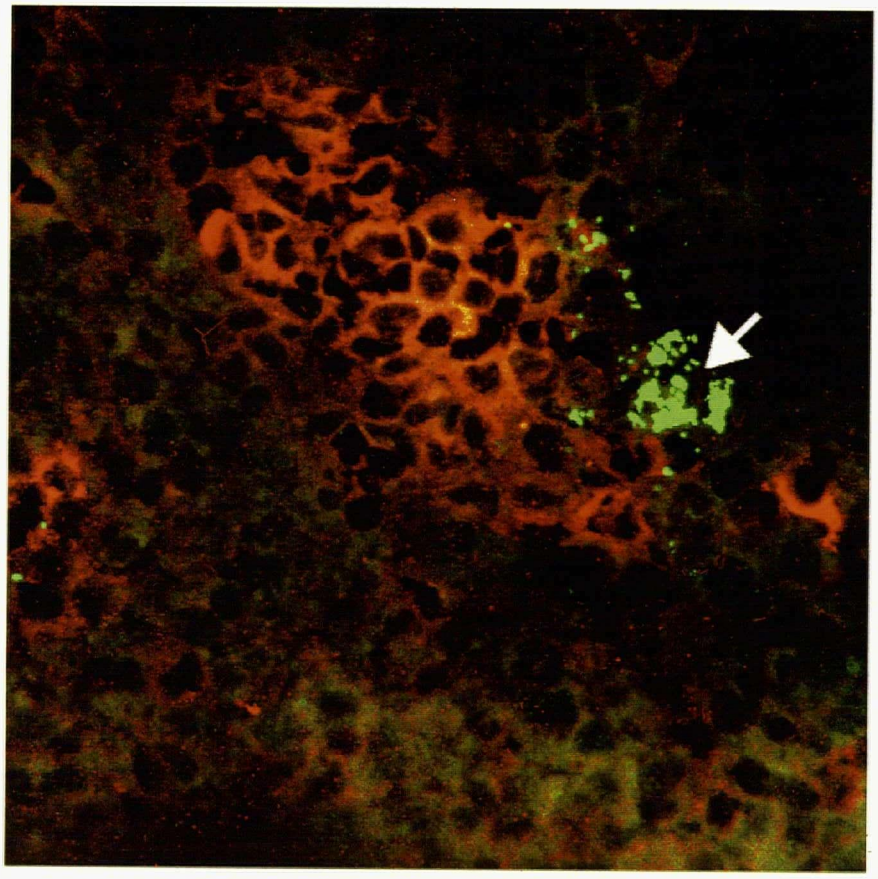
3.3 Results

3.3.1 Histopathology and expression of HSP-70.

I investigated the distribution of HSP-70 within sections cut from frozen tissue using a two colour Fluorescent Antibody Technique (FAT). In sections from the livers of fish with BKD, I found localized areas of bright (red) staining of HSP-70 and bright (green) staining of individual bacteria and colonies of Rs (Figure 3-1). To approximate the magnitude of the response in the liver of coho with BKD, I compared the intensity of HSP-70 staining on scanned grayscale images with that of liver from heat shocked coho. On a brightness scale ranging from 0 to 255 the regions of most intense staining in BKD liver (median = 126) were similar in intensity (P_{α}= 0.5105 ; Rank Sum) to the regions of most intense staining in liver of heat shocked coho (median = 130). The most intense staining in heat shocked liver was observed 6 h after a 30 min heat shock at a water temperature increased by 10°C. There was no appreciable HSP-70 staining in either sections from control fish without BKD or sections from fish with BKD which were stained using negative control non-immune rabbit Ig (NRIg) instead of anti-HSP-70 antibodies.

On all of the sections examined there was a general distribution of various intensities of yellow autofluorescent material. This autofluorescence was present even on the control slides which were stained with NRIgG and on slides not treated with any antibody reagents (Figure 3-2). The autofluorescence was concentrated in the cytoplasm of the cells such that it provided a convenient negative stain to show the position and size of the cell nuclei. The structure of the tissue could be seen by observing the distribution of the yellow autofluorescence.

Figure 3-1. Immunofluorescence staining with anti-HSP-70 and anti-*R. salmoninarum* in BKD liver.
The top panel (A) is a digital composite of the red (anti-HSP-70) and green (anti-Rs) channels adjusted to show the position of the bacteria and the variation in intensity of the HSP-70 staining and yellow autofluorescence. The arrow indicates a large cluster of Rs. The bottom panel (B) is a phase contrast image of the same field.



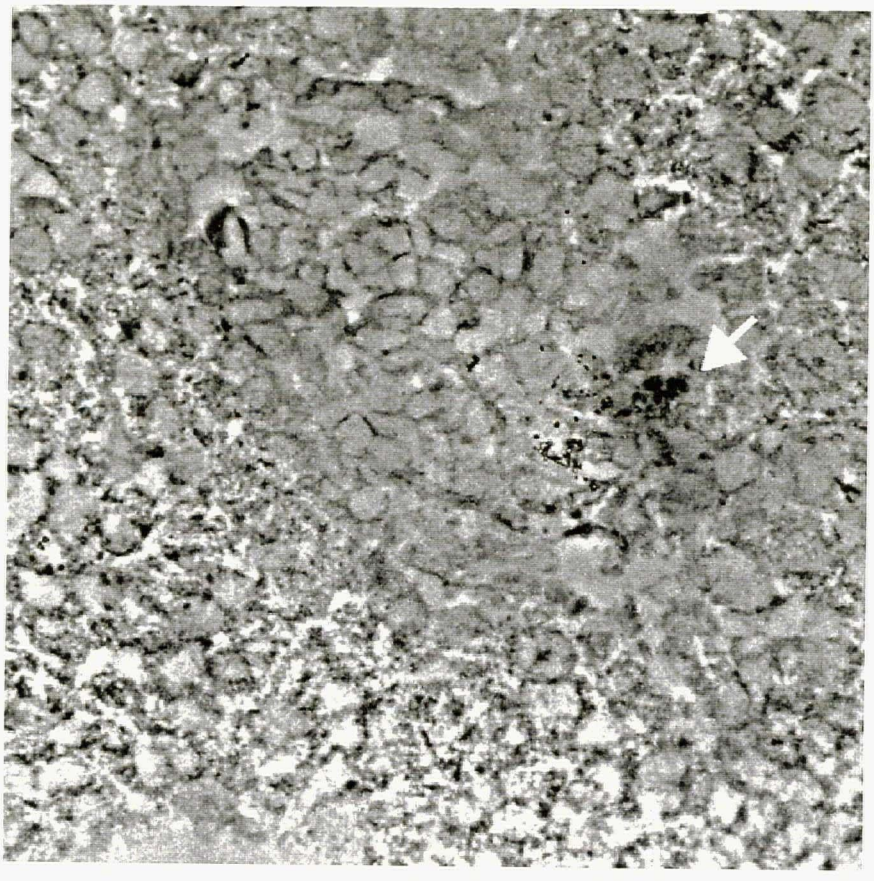
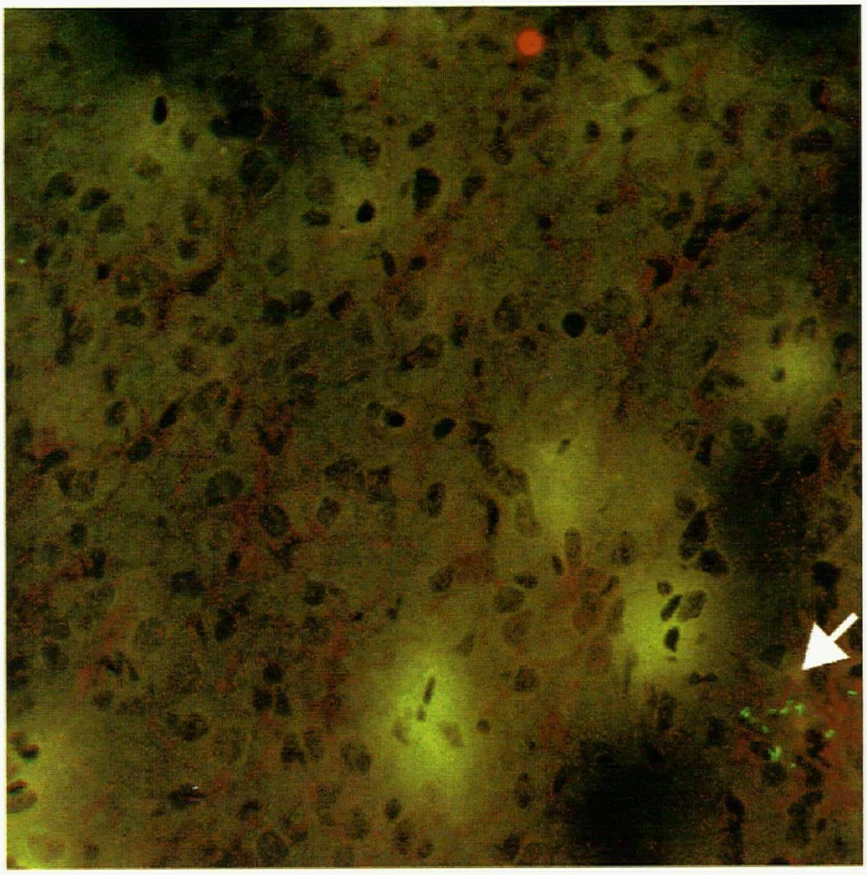
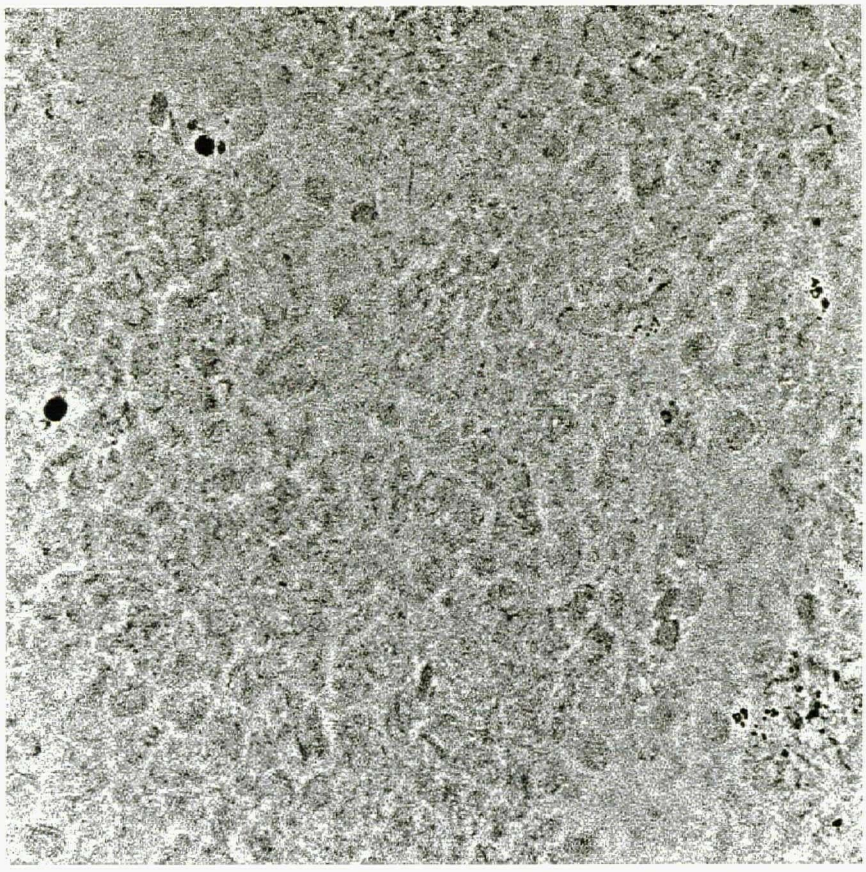


Figure 3-2. Nonspecific immunofluorescence staining and *R. salmoninarum* in BKD liver. The top panel (A) is a digital composite of the red (nonspecific normal rabbit IgG) and green (anti-Rs) channels. The arrow indicates a large cluster of Rs.
The bottom panel (B) is a phase contrast image of the same field.





In the sections of liver the structure was reasonably well preserved. Individual cells, nuclei and variations in the intensity of autofluorescence of cytoplasmic contents can be discerned on the fluorescence images (Figure 3-1, 3-2, 3-3). Frozen sections from head kidney tissue were not as mechanically stable as sections of liver tissue and could not be examined reliably. During the antibody incubation steps much of the kidney tissue detached from the slides and was lost (not shown). The intensity of yellow autofluorescence was lower in areas of necrosis where there were large numbers of bacteria and the tissue structure was breaking down. The loss of autofluorescence corresponds with the loss of structure on phase contrast images (Figure 3-3 B).

The position and shape of the nuclei could also be seen on sections on which the DNA was stained (blue) with Hoechst 33258 (Figure 3-3 B and 3-4 B). The apparent size of the nuclei varied throughout the tissue and the nuclei of parenchymal hepatocytes were roughly spherical to oblong in areas away from bacterial colonies or necrosis. There was no obvious distinction in the size of the nuclei in the large areas of necrosis although there were fewer nuclei and some nuclei may have been somewhat irregular and packed together in necrotic areas (Figure 3-3 B). Discrete areas of DNA staining were still present even in the areas of necrosis where autofluorescent cytoplasmic material was largely absent (Figure 3-3 A). This is consistent with the characteristic membrane rupture and loss of cytoplasmic material which occurs in necrotic cells while the degenerating nucleus remains relatively intact. Major changes in the size of nuclei do not generally occur in necrosis (Whyllie, 1981) so the appearance of the nuclei is consistent with these areas being necrotic.

Figure 3-3. Tissue structure and decreased autofluorescence in necrotic tissue. Tissue section from a necrotic area in the liver of a fish with BKD. The top panel (A) is a digital composite of the red (anti-HSP-70) and green (anti-Rs) channels. Notice the large numbers of Rs spread throughout the necrotic area. The bottom panel (B) is the same field showing nuclei stained (blue) for DNA double exposed with the phase contrast image.

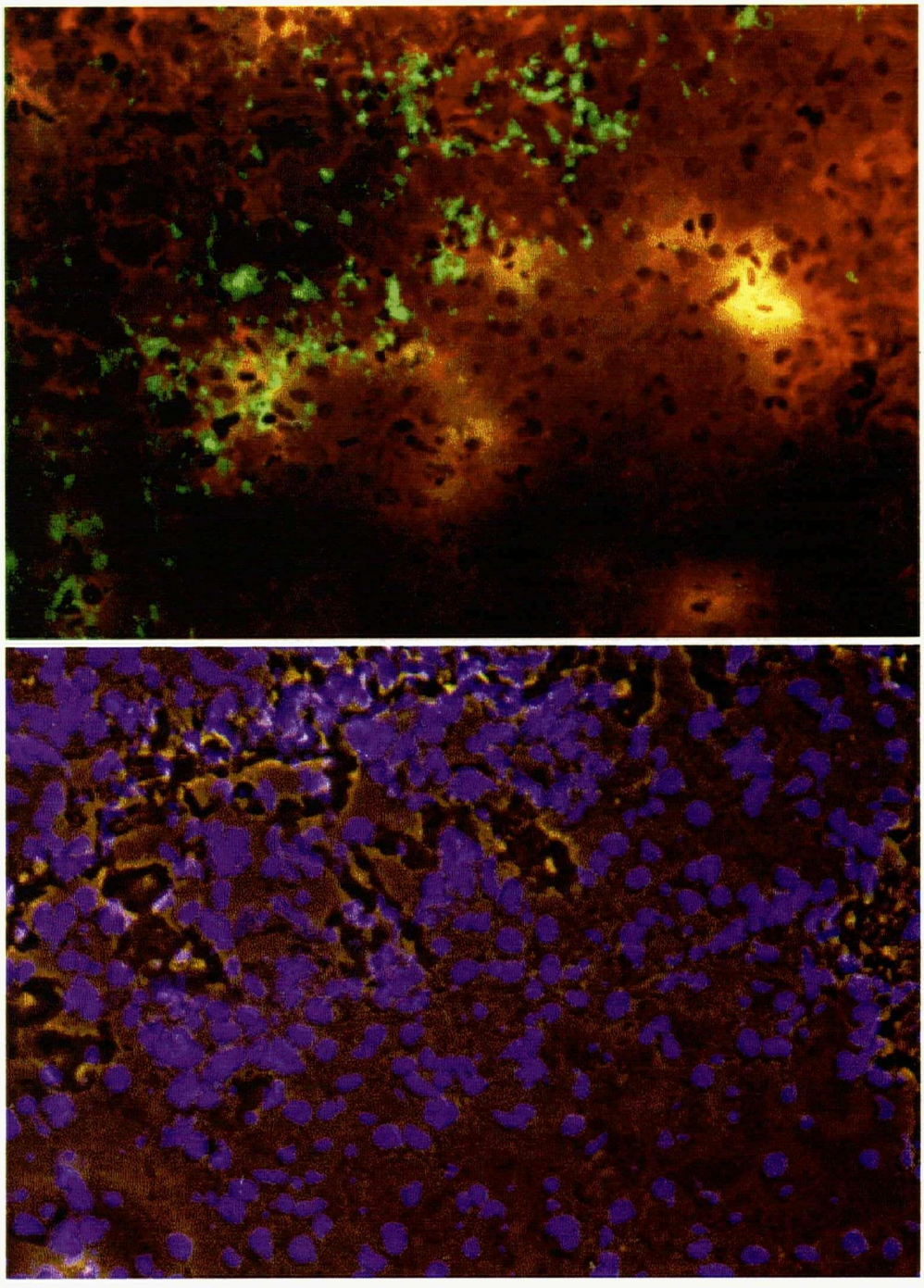
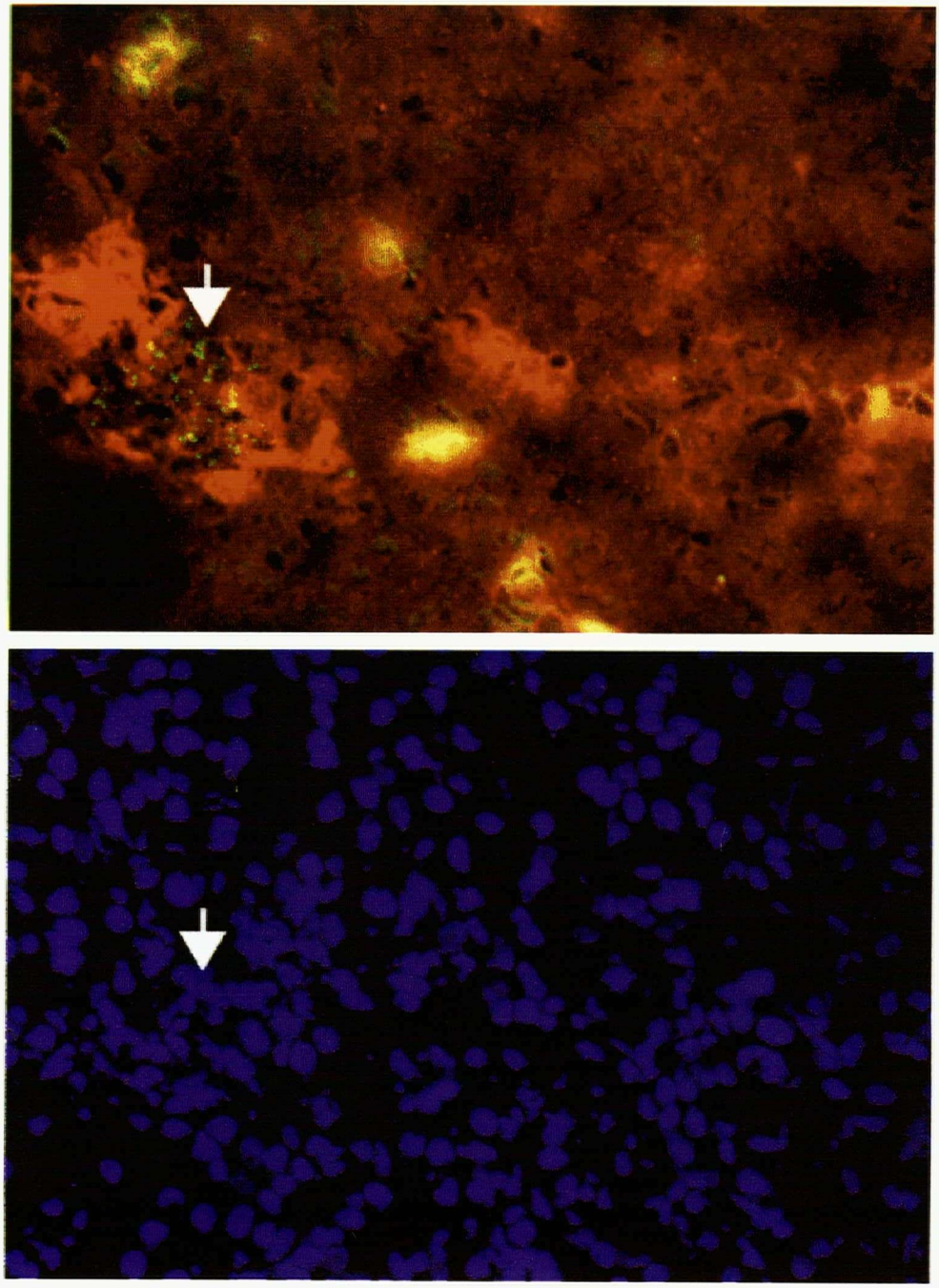


Figure 3-4. Nuclei and tissue structure near bacterial colonies.
Tissue section from an area containing a bacterial colony in the liver of a fish with BKD. The top panel (A) is a digital composite of the red (anti-HSP-70) and green (anti-Rs) channels. The arrow indicates a large cluster of Rs.
The bottom panel (B) is the same field showing nuclei stained (blue) for DNA.



3.3.2 Association of host HSP-70 with sites of infection.

In liver tissue from fish with BKD, HSP-70 immunofluorescence was concentrated in focal areas which were associated with colonies of Rs (green) in the tissue (Figure 3-1). Similar colonies of Rs were not associated with any appreciable nonspecific (red) staining with NRIgG (Figure 3-2). In order to determine whether this apparent association of HSP-70 with Rs colonies was coincidental I systematically scanned slides and scored the various features on tissue sections of BKD liver stained using anti-Rs and either anti-HSP-70 or NRIgG. In BKD-affected liver tissue all (14/14) of the observed areas of HSP-70 staining were associated with Rs (Table 3-1).

Using the areas of bright autofluorescence (yellow) as an internal control there was a highly significant ($P<10^{-6}$) correlation (R=0.7638) between HSP-70 staining in the liver tissue and the presence of Rs colonies within three cell diameters of the centre of the fluorescence. In contrast, there was no significant correlation (P=0.6180) of Rs with nonspecific staining of BKD affected liver by the NRIgG control (Table 3-2).

Since each slide could not be stained with both HSP-70 and NRIgG I used the association of Rs with the yellow autofluorescent features on each slide (Column 2; Table 3-1 and 3-2) to test whether the areas of tissue examined on the different slides were comparable. There was no significant difference between the frequency of association of Rs with areas of bright autofluorescence on slides of BKD liver stained with either anti-HSP-70 or NRIgG (Fisher method for contingency; P=0.734). This indicates that the distribution of colonies of Rs was comparable.

Table 3-1. Correlation between HSP-70 staining and *R. salmoninarum* colonies in liver from a fish with BKD.
The tissue was stained using anti-Rs to detect the bacteria and with anti-HSP-70 to detect areas with high HSP-70. The number in each cell is the number of features observed which have the specified combination of fluorescent staining. Each bright red or yellow feature observed was scored for whether or not there were green stained Rs within three cell diameters of the centre of the feature. The coded variables were assigned the binary values shown for each row and column. The asterisk indicates a significant correlation between the coded variables (Pearson; $P< 0.05$).

	Red HSP-70 = 1	Yellow Autofluorescence = 0
Rs (green) = 1	* 14	4
No Rs = 0	0	12

Table 3-2. Absence of correlation between nonspecific staining and *R. salmoninarum* colonies in liver from a fish with BKD.
The tissue was stained using anti-Rs to detect the bacteria and with normal rabbit IgG to detect nonspecific binding of antibody to the tissue. The number in each cell is the number of features observed with the specified combination of fluorescent staining. Each red or yellow feature observed was scored for whether or not there were green stained Rs within three cell diameters of the centre of the feature. The coded variables were assigned the binary values shown for each row and column. There was no significant correlation between the coded variables (Pearson; P = 0.6180).

	Red Nonspecific = 1	Yellow Autofluorescence = 0
Rs (green) = 1	0	8
No Rs = 0	1	30

3.3.3 Host cells associated with Rs colonies.

It is not clear what cell types were present at the infected sites in the liver of fish with BKD. I obtained an antibody against rainbow trout neutrophils but it did not cross react to any coho head kidney leukocytes on control slides (not shown). The appearance of the cells associated with bacterial colonies was clearly different from those in the surrounding tissue, but the preservation and resolution of structure was not high enough in the frozen sections to distinguish on the basis of morphology whether the cells associated with the bacteria are parenchymal hepatocytes or some other cell such as phagocytes or epithelial cells.

There are, however, some features of the cells associated with bacterial microcolonies that suggest that they may be phagocytes or hepatocytes which are damaged or dying. Although I observed bright HSP-70 staining in the cells surrounding colonies of Rs, the cells which had Rs associated with them were not as bright. In fact, both HSP-70 staining and autofluorescence appeared to be lower in the cells with Rs. Lower fluorescence in these cells may have resulted from loss or degradation of cytoplasm due to cellular death. Furthermore, when stained for DNA with Hoechst 33258 the nuclei in the cells associated with small Rs colonies appeared irregular in shape and somewhat smaller and packed together compared to the roughly spherical or oblong nuclei present in the surrounding parenchymal cells (Figure 3-4). The difference in the shape of the nuclei might indicate that these cells are phagocytes or that the nuclei have condensed and degenerated due to cell death. In diseased liver tissue there were some areas with many bacteria which appeared to be necrotic (Figure 3-3) and some of the cells associated with the small colonies of Rs may also have been necrotic. However, I can not make any final conclusion about the nature or identity of the cells associated with Rs.

3.4 Discussion

The fact that HSP-70 is expressed in discrete areas adjacent to colonies of Rs within the tissue of fish with BKD discredits some of the more general mechanisms by which SP might have been induced. If one dose not invoke some unknown mechanism by which tissue in some areas happened to be more sensitive to SP induction, the focal pattern of SP distribution is inconsistent with mechanisms such as stress caused by disruption of systemic control of the physiological conditions within the fish. Induction of SP in BKD must involve a locally acting mechanism. The pattern of focal expression also raises the possibility that the SP which are expressed near the bacterial colonies might have an important role in the pathology of disease rather than merely being the result of the pathology. I discuss some of the possible impacts of SP on disease pathology in the General Discussion chapter.

3.4.1 Locally acting mechanisms of SP induction.

The induction of SP in the vicinity of microcolonies of Rs could have resulted from several different local mechanisms triggered by the presence of Rs within the tissue. This explanation requires the least additional postulates and in the rest of this Discussion section I outline some possible mechanisms by which Rs could have caused the pattern of SP induction which I observed in the surrounding cells.

Before discussing those plausible mechanisms it should be acknowledged that the association of SP with colonies of Rs could also be explained by postulating the existence of some unknown factor which directly or indirectly caused both SP induction at discrete sites within the tissue and Rs colonization at those same sites. It is conceivable that a site

of some previous injury, which could have induced SP, might be more prone to infection. Such a model would not explain why all of the areas of SP induction were associated with bacteria. Thus, it is unlikely but possible that Rs preferentially colonized sites that already had elevated SP rather than actually inducing SP at the infected sites.

3.4.2 Intracellular bacterial infection and SP induction.

In principle, the bacteria which are intracellular pathogens, could have induced SP just by being inside the host cells. The presence of the pathogen might interfere with the cell's metabolism or biochemistry in nonspecific ways and cause a stress response. For example, modified or denatured proteins have been reported to result in activation of the cellular stress response and SP induction (Ananthan *et al.*, 1986; Goldberg *et al.*, 1997). Proteins and even genetic material from intracellular bacteria can be transferred into the host cell (Darji *et al.*, 1997). Rs has been reported to escape into the cytoplasm of host cells (Gutenberger *et al.*, 1997) and so could export proteins directly into the cytoplasm. The major extracellular protein of Rs (p57) is a fimbrial protein (Dubreuil *et al.*, 1990) which tends to form aggregates and is expressed in large amounts both *in vitro* and *in vivo* (Rockey *et al.*, 1991). Aggregates of p57 in the host cell cytoplasm might be perceived by the regulatory mechanism within the cell as being denatured proteins and so might induce a stress response.

Direct induction by intracellular bacteria might be relevant for the cells which appeared to contain Rs even though these cells didn't seem to have as much SP expression as the surrounding uninfected cells. The area of low SP staining in cells with Rs might have resulted from the degradation or loss of cytoplasmic material rather than an absence of SP,

as I discuss in more detail below. This mechanism does not explain the induction of SP in the surrounding cells which were not in direct contact with Rs. Induction of SP in BKD must involve other mechanisms beside direct induction by intracellular bacteria.

3.4.3 Toxins and SP induction in BKD.

Another way that Rs could have caused SP induction is by producing or causing the release from the host cells of some toxic material or enzyme which could cause stress to the surrounding cells. Bacterial toxins can cause induction of SP in mammalian cells (Koller *et al.*, 1993) and Rs is known to produce some mildly toxic proteins such as an haemagglutinin, an extracellular protease and an haemolysin (Daly & Stevenson, 1990; Griffiths & Lynch, 1991; Grayson *et al.*, 1995a; Grayson *et al.*, 1995b). These substances could potentially induce SP. Even if substances released from the bacteria or the dying infected cells did not induce SP, such substances might have triggered host reactions in the tissue which could have lead to localized SP induction. For instance, the bacteria or the resulting inflammatory response might have caused clotting and local disruption of the microcirculation. The resulting ischemia and anoxia could lead to SP induction, apoptosis and necrosis. A toxin produced at the site of infection could diffuse through the surrounding tissue and cause a stress response in cells that were not in direct contact with bacteria. At high concentrations such toxins might kill the cells nearest the bacteria and so such mechanisms might explain the fact that SP was lower in cells associated with Rs than in the surrounding tissue where a clear induction had occurred.

3.4.4 Phagocytes and SP induction in BKD.

A final way that Rs could have caused SP induction is that phagocytes drawn to the site of infection and stimulated by the bacteria could have caused SP induction, as I discussed in the general Introduction. Infiltrating phagocytes can release enzymes and toxic ROI which can damage or kill cells in the area. In cells that were not killed by the actions of phagocytes sublethal damage could have induced SP.

HSP-70 induction was clearly associated with the sites of infection, but I did not directly demonstrate whether there were phagocytes at those sites. Although I could not determine that the cells associated with Rs were phagocytes, published studies of the histopathology of BKD have found that, especially at the later stages of infection, there are infiltrating phagocytes and leukocytes at the sites of infection and that Rs is often present inside phagocytes (Young & Chapman, 1978; Flano *et al.*, 1996a; Flano *et al.*, 1996b). Since the tissue samples were taken from fish at the later stages of infection, phagocytes were almost certainly present at most if not all of the sites of infection and some of the cells associated with the bacteria were probably phagocytes.

I used several approaches to look at the structure of the tissue and the cells associated with microcolonies of Rs. The irregular shape of the nuclei in the cells with Rs might be an indication that these cells are a different cell type (phagocytes for instance) than the surrounding parenchymal hepatocytes. Alternatively, the differences in the nuclei might be an indication that the cells are dying. The nuclei of dying cells become condensed during the process of apoptosis and the nuclear chromatin becomes marginated in the early stages of necrosis (Whyllie, 1981). The lower intensity of fluorescence in the cells with bacteria

could be an indication that these cells are necrotic, that there has been a loss or degradation of cytoplasmic material, or that these cells are less capable of synthesizing HSP-70.

3.4.5 Lower SP levels in the host cells with Rs.

Whether or not the cells associated with Rs were phagocytes these cells were different in some way from the surrounding cells because they appeared to have lower levels of both HSP-70 staining and autofluorescence. These cells were probably being exposed to a stressor at least as severe as that experienced by the cells surrounding them. The lower levels of SP might have resulted from either lower levels of induction or from degradation of SP. If SP was induced in these cells to the same extent as the surrounding tissue autolytic enzymes or protease from the bacteria might have degraded the HSP-70 so that it lost immunoreactivity or was lost during the processing of the sections. Such loss or degradation of cytoplasmic material could also explain the lower autofluorescence in these cells. If SP induction was actually lower in these cells, they may have been less capable of synthesizing HSP-70 than the surrounding cells. The cells might have been dead or dying and so not able to make HSP-70, or the cells might have been a different cell type with a weaker stress response than hepatocytes.

A weaker SP response in the cells associated with bacteria would be consistent with the idea that these cells were phagocytes. The presence of bacteria in phagocytes might not induce a stress response since infection of phagocytes by intracellular pathogens does not always induce SP in the phagocytes. Polla's group have reported that phagocytosis of *Pseudomonas aeruginosa* did not induce a stress response in the phagocytes even though strong oxidant production was stimulated (Barazzone *et al.*, 1996). Furthermore, merely

engulfing particles and producing oxidants is not a sufficient stressor to cause induction of SP in human neutrophils (Polla *et al.*, 1995) so perhaps SP are not routinely induced in phagocytes by the oxidant that they produce. The results with phagocytes in culture in the next chapter suggest that fish phagocytes may also not have a very strong SP response compared to hepatocytes.

On the other hand, if the cells with Rs did express SP which was subsequently degraded this might be explained by the fact that when Rs is grown in infected fish cell cultures Rs forms bacterial filled cystic areas within the cytoplasm of the cells. In electron micrographs these appear empty (except for bacteria) and are devoid of normal cytoplasmic organelles and structures (McIntosh *et al.*, 1997). Rs is known to produce an extracellular protease which has an unknown function in virulence (Griffiths & Lynch, 1991; Evenden *et al.*, 1993). Perhaps this protease could be involved in degrading host cytoplasmic proteins and thus contribute to the apparent loss of cytoplasmic structures in these cystic areas containing Rs. Alternatively, the cells with Rs and low SP staining may have been in the early stages of necrosis since there were other areas where there were large numbers of bacteria and extensive necrosis. Areas of tissue necrosis are an usual feature in advanced stages of BKD and the loss of cytoplasmic material occurs early in the process of necrosis (Whyllie, 1981). It is also possible that the areas of SP expression around the microcolonies of bacteria may have been related to necrosis of the cells with Rs. Whatever is leading to SP expression may ultimately also cause necrosis of these cells. Thus the areas of SP expression surrounding the microcolonies of Rs may have been destined to progress to larger areas of necrosis.

3.5 Conclusions

The discrete focal pattern of SP induction which occurred in BKD must have had some locally acting cause, or it would have been more widely distributed and relatively uniform. The pattern cannot be explained by systemic disturbances such as anemia or failure of ionic regulation. If the pattern of SP expression had been more or less uniform throughout the diseased tissue it could have been adequately explained by the fact that the fish were sick. Failure of the vital systems could reasonably be expected to have complex effects on all of the cells within the fish and these would be difficult to interpret in an informative way. However, since SP was induced in discrete areas and in close proximity to bacterial colonies all of the potential mechanisms are experimentally testable and may yield important information about the pathology of BKD.

My results generally support the second hypothesis. **(SP induction in fish with BKD is associated with the sites of infection and phagocyte infiltration in tissue.)** HSP-70 induction was clearly associated with the sites of infection, and phagocytes were almost certainly present at the sites of infection.

The focal pattern of SP expression is consistent with the idea that phagocytes might be involved but there are other consistent explanations as well. All of the most likely explanations for SP induction in BKD involve the postulate that the presence of the bacteria causes SP induction either directly or indirectly. There are three basic mechanism which are highly plausible: 1.) As mentioned, the killing pathways of phagocytic cells might have been triggered by the bacteria and induced SP in themselves or in the adjacent cells; 2.) The presence of the bacteria or secreted bacterial proteins within the cytoplasm of infected cells may have induced SP; and 3.) Some toxin from the bacteria or toxic material

released from infected or dying cells might have induced SP in the surrounding cells or triggered a local reaction such as clotting which in turn induced SP.

All of these plausible mechanisms could be tested experimentally and such experimentation is likely to produce useful information about the interactions between bacteria and host cells in BKD and in other bacterial diseases. It would be desirable to test some of these mechanisms under conditions that could be controlled and manipulated. The first two plausible mechanisms can be investigated to some extent using cells in culture. It seemed appropriate as a next step in this thesis to investigate what cells at the sites of infection might induce SP. Using mixtures of cells in the final series of experiments I hoped to further elucidate the possible mechanisms of SP induction in BKD and whether phagocytes could be implicated.

Chapter IV HSP-70 expression in cultured cells

4.1 Introduction

Since the work presented in the previous chapter showed that SP induction is localized to the areas near the sites of infection, some discrete locally acting mechanism must be involved. Although this pattern eliminates a large number of possible explanations and is consistent with the possibility that phagocytes might be involved in SP induction in BKD, there was no conclusive evidence for the presence of phagocytic cells at the sites of HSP-70 expression. As a final approach to investigating the cause of SP induction in BKD I chose to investigate some possible local interactions between the hepatocytes, phagocytes and bacteria which are present in the liver of fish with BKD. In this chapter I discuss a series of experiments with isolated rainbow trout phagocytes and liver cells (hepatocytes) in culture. I conducted these experiments in order to determine whether SP expression could be induced by phagocytes or oxidants produced by phagocytes and to explore some of the possible mechanisms for SP induction in BKD without the complicating factors present in the diseased fish.

The original reason for studying SP in BKD was the expectation that SP expression would be associated with phagocytes and the inflammatory pathology of the disease. The pattern of expression which I observed in BKD is consistent with the possibility that phagocytes were involved. If phagocytes caused SP induction in BKD I would expect the resulting SP to be distributed in the kind of focal pattern which I observed in liver tissue from fish with BKD (see Chapter III). There are, however, other possible explanations that would be consistent with this pattern. In exploring some of these possible explanations for SP

induction in BKD, I tested my last major hypothesis: **SP can be induced as a result of phagocyte function and oxidant production.**

Previously, no one has investigated SP expression in fish phagocytes or whether the oxidants which fish phagocytes are known to produce cause a SP response in the phagocytes. Since they are the source of the oxidants generated, stimulated phagocytes are exposed to the highest concentrations. For these reasons it seemed reasonable to test the possibility that the fish phagocytes themselves have a SP response as a result of the oxidants that they produce. I used cultures containing rainbow trout phagocytes and mixed cultures of phagocytes together with hepatocytes in order to model, in a simplified way, some of the interactions that might occur in the tissue of fish with BKD. In particular I wanted to examine whether the action of phagocyte oxidants either on the phagocytes themselves or on adjacent hepatocytes could explain the induction of SP observed in BKD.

4.1.1 Benefits of an *in vitro* model.

Experiments with isolated cells in culture allow a greater control over the conditions than is possible in a living animal. The fact that SP is induced in fish with BKD provides a relevant basis for proposing that SP could play a role in the disease. Without corroboration of this sort the relevance of any amount of evidence of SP induction *in vitro* would be suspect. However, there are many factors which are unknowns in a whole animal and which make it difficult to interpret *in vivo* data. It is difficult to control or even monitor the components at a site of infection or inflammation *in vivo* because the site is connected to rest of the animal. Cells, plasma components, and mediators can move into and out of the site. Furthermore, sampling disturbs the animal and usually involves killing it. This makes

replicating conditions difficult since the levels of hormones, proteins, and cells which could affect the course of the disease pathology may differ between individuals and can change over time.

I chose to use an *in vitro* model system in order to efficiently study the relationship between a limited number of components which I could control. I expected that using an *in vitro* system would reduce variability between treatment groups, enable me to control what components were present, and thereby allow identification of some processes of SP induction which could explain the patterns I saw *in vivo*.

4.1.2 Phagocytes and phagocyte oxidants.

Phagocytes are an essential component of both specific and non specific defenses. Since the discovery of phagocytosis by Eli Metchnicov in the late 1800's (Karnovsky & Bolis, 1982), phagocytes have been a subject of intermittent interest. Over time Metchnicov's belief in their central importance has been vindicated. Innate cellular immunity and the role of phagocytic cells are indispensable for normal life and even small defects in phagocyte function can cause susceptibility to chronic and repeating infections (Foster *et al.*, 1998). Defensive phagocytic cells are present in all known vertebrates and a wide range of invertebrate animals (Dikkeboom *et al.*, 1988). In fact the smallest of animal life are themselves free living phagocytic cells.

4.1.3 Oxidant production and phagocyte function in host defense.

Phagocytes defend multicellular animals by binding to and engulfing foreign substances and organisms. In order to kill potential pathogens and degrade the engulfed material for

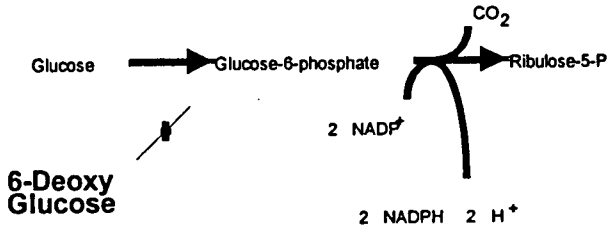
presentation to specific immune cells the phagocytes employ various lytic enzymes and toxic chemicals. One of the killing mechanisms is generation of oxidants such as superoxide, peroxide and other oxidants derived from them. To protect themselves against these toxic oxidants animals also have anti-oxidant defenses. However, oxidants can cause damage to the host tissues and this localized toxicity to host cells can induce apoptosis (Lennon *et al.*, 1991). Killing of host cells may be an important part of the defensive functions of phagocytes since this might limit the availability of suitable host cells for intracellular pathogens and allow engulfment of pathogens along with cellular debris.

In fish and other vertebrates superoxide, which give rises to other oxidants, is produced by the enzymatic single electron reduction of molecular oxygen as illustrated below (Figure 4-1). There are also some other mechanisms by which superoxide can be generated which do not require phagocytes and there are means of interrupting some of the pathways involved. These inhibitors and scavengers could be potentially used to study what oxidants and mechanisms are involved in a particular phenomenon.

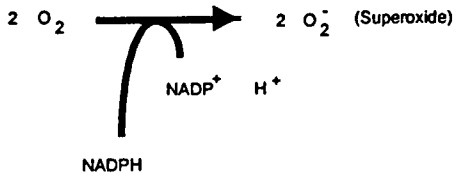
In fish, phagocytic cells have been shown to produce reactive oxidants in basically the same manner as mammalian phagocytes. Superoxide is generated from NADPH by a specific cytochrome enzyme (Secombes *et al.*, 1992). Hydrogen peroxide is derived from the superoxide generated by stimulated fish phagocytes. Some fish phagocytes also have an inducible nitric oxide synthase (iNOS) for which a gene has been cloned from rainbow trout (Secombe *et al.*, 1998). This enzyme produces nitric oxide (NO) which can combine with superoxide to produce peroxynitrite which is unstable and generates hydroxyl radicals. Hydroxyl radicals are extremely reactive and have been suggested to be particularly potent at damaging cells and inducing SP (Barazzone *et al.*, 1996; Polla *et al.*, 1998b).

Figure 4-1. Oxidant generation by phagocytic cells and reactions involving oxidants. The solid bar crossing an arrow indicates inhibition. The thin line with a cross through it indicates that the reaction cannot proceed from that substrate.

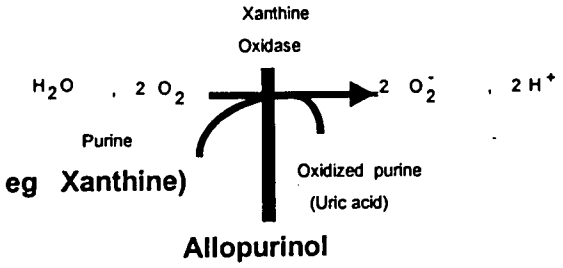
Production of NADPH by Hexose Mono Phosphate Shunt



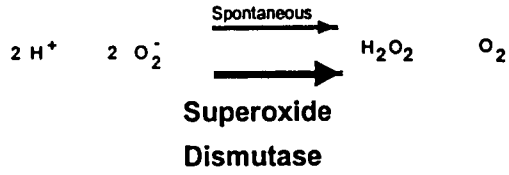
Production of Superoxide by NADPH Oxidase



Production of Superoxide by Purine Oxidation

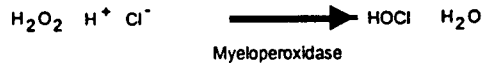


Dismutation of Superoxide

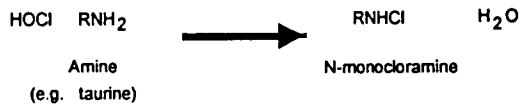


Destruction of Hydrogen Peroxide

Production of Hypohalous acids from Peroxide



Production of Chloramines from HOCl



4.2 Materials and Methods

Fish used for isolation of cells — Sexually immature rainbow trout (*Oncorhynchus mykiss*) weighing between 0.5 and 1.5 kg were obtained from local suppliers and maintained in fresh water in a 1000 L tank in the facilities described in the Materials and Methods section of Chapter II. I used rainbow trout as the source for isolation of phagocytes and hepatocytes because there are well established methods for isolation of these cells and because large rainbow trout are readily available from commercial suppliers. Rainbow trout are closely related to coho salmon and their cells can be expected to respond in a fashion similar to coho cells. Fish that were producing gametes or showed signs of final sexual maturation were not used.

Isolation procedures — The media and conditions used to isolate phagocyte and hepatocyte suspensions are described in detail in Appendices II and III. Fish were killed humanely in buffered TMS (200 mg/L). To prevent heat shock, the anaesthetic solution was chilled by addition of a small amount of ice; During dissection the fish were held on a bed of crushed ice. The appropriate tissues (liver or head kidney) were removed aseptically in the field of HEPA filtered air from a laminar flow hood. All subsequent steps in the isolation process were performed on ice or in a refrigerated centrifuge at 0°C. Leukocyte suspensions containing phagocytes, which are here after referred to as phagocytes, were isolated from head kidney tissue using discontinuous 34%/54% Percoll™ density gradients essentially as described by (Graham & Secombes, 1988). For subsequent assays and distribution into cultures the phagocytes were routinely resuspended to 2 X 10^7 cells/mL with 0.1% porcine gelatin (Sigma G-2500) in Hanks balanced salt solution without phenol red (HBSS-Gel) or with 0.1% porcine gelatin in Hanks that was calcium and magnesium

free (HBSS-CMF-Gel). Hepatocytes were isolated by *in situ* collagenase perfusion essentially as described by Mommsen et al. (1994). For distribution into cultures the hepatocytes were routinely resuspended to 1 X 10^6 cells/mL in Leibovitz L-15 medium modified (L-15M see Appendix III).

Stimulants — *Renibacterium salmoninarum* was prepared as described in Chapter II; a suspension with an optical density (540 nm) of 2.0 was pelleted and resuspended in HBSS-Gel to yield a suspension with approximately 5 X 10^8 cfu/mL. Stocks of phorbol 12-myristate 13-acetate (PMA; Sigma P-8139) were stored frozen in individual portions at 200 μg/mL in dimethylsulphoxide (DMSO) and were diluted in HBSS-Gel to 500 ng/mL immediately before use. These stimulants were added to cultures at 1/10 of the final volume.

Superoxide assay — Superoxide generated by phagocytes was measured by the ferricytochrome c reduction method in a spectrophotometric assay which is described in more detail in Appendix II. Briefly, phagocytes were added in triplicate to the assay mixture to a total volume of 0.25 mL and a final concentration of 2 X 10^6 cells/mL, 1.24 mg/mL ferricytochrome C (Sigma C2506), 500 units/mL catalase (Sigma C-10) and 50ng/mL (except where stated otherwise) PMA in HBSS-Gel. Assay blanks were created by including superoxide dismutase (SOD Sigma S2515). The assays were incubated for 30 min at the ambient temperature from which the fish were taken, chilled on ice, centrifuged at 13000 rpm for 30 sec in a microcentrifuge. The assay supernatants were diluted 1/5 and the difference (A_{MAX}-A_{MIN}) between the maximum absorbance at approximately 550 nm

and the minimum absorbance at approximately 530 nm was measured in a scanning spectrophotometer. The amount of superoxide generated over the course of the assay period was calculated as nmoles superoxide per 10^6 cells = the dilution factor X (A_{MAX}-A_{MIN}) X 0.021. The blank value (SOD treated) was subtracted and was always near zero.

Phagocyte cultures — The cultures were incubated for an initial 30 min period with the same conditions as the superoxide assays except that ferricytochrome c was not added to any cultures and other components were omitted from some cultures. Components omitted from individual cultures were replaced by an equal volume of an alternate component or the medium it was dissolved in. The catalase stock was treated to inactivate more than 99 % of the contaminating LDH activity by titration with 1.0 N HCl to pH 4.5, incubating on ice for 20 min, and then neutralizing with 1.0 N NaOH. All components for the incubation media, except the cells, were mixed together in 1.5 mL polypropylene tubes and held on ice. The phagocytes were added to a final volume of 0.5 mL and a final concentration of 1 X 10^7 cells/mL. The tubes were closed and mixed by gently inverting the tubes and placed in a flowing water bath at the ambient temperature which varied by less than +/- 0.5°C during each experiment and was between 5°C and 7.5°C for all of the phagocyte culture experiments. Some cultures were harvested (see below) immediately after set up (Time zero controls). Samples of the unused media without cells were saved as blanks for the LDH assay. At the end of the 30 min incubation the tubes were chilled on ice and centrifuged for 10 min at 300g. The supernatants were removed and saved in labeled tubes for later measurement of LDH and 0.5 mL of culture medium (L-15 with 10 % FCS) was added. The cultures were returned to the water bath and incubated until 18 h after the

beginning of the experiment. At the end of the experiment the tubes were harvested as follows: the tubes were chilled on ice and centrifuged again; the supernatants were saved; and the pellets were immediately frozen on dry ice in the original tube. All samples were stored frozen at or below -70°C.

Hepatocyte cultures — Rainbow trout hepatocytes were isolated and cultured as described in Appendix III at 4°C in 24 well plates with 5 X 10^5 cells/well in 0.5 mL of L-15M. For all handling steps, the plates were placed on a bed of wet crushed ice. Samples of the initial isolated hepatocyte suspension were saved and some cultures were harvested at various times after plating. Some cultures from each batch of hepatocytes were subjected to a heat shock to verify that they could mount a SP response. For co-culture experiments 0.2 mL of media was removed from each well (except some controls) and replaced with HBSS and/or 50 μL of stimulant (PMA or Rs) and/or 50 μL of phagocytes to a total of 0.2 mL. Components omitted from individual cultures were replaced by an equal volume of an alternate component or the medium it was dissolved in. The resulting cultures either contained no stimulant or contained 50 ng/mL PMA or 5 X 10^7 cfu/mL of Rs. The cultures also contained either no phagocytes or phagocytes added to a final concentration of 2 X 10^6 or 5 X 10^5 cells/mL. Each combination of components was repeated in triplicate. Three wells were harvested as a presample before the media was removed. Three wells on each plate were left untouched. These are the "nothing done" controls and were harvested at the same time as the other wells. Other control cultures had the medium replaced with no stimulant and no phagocytes. The plates were incubated for 24 h at 4 °C and then harvested as follows: the plates were chilled on ice and a micropipetter set to 0.4 mL was

used to flush the cells off the plastic. Medium was drawn into the tip and expelled with the end of the pipette tip 2-3 mm away from the bottom of the well. In normal cultures, a single flush dislodged the cells from an area approximately 8 mm in diameter. In some wells, the cells were non adherent and a single flush dislodged the cells from the entire well. The flushing was repeated seven times in each well (once in the middle and at six points in a regular hexagon around the well). The suspension was then collected into prelabeled tubes and centrifuged for 30 sec at 6500 rpm in a microcentrifuge, the supernatants were saved, and the pellets were immediately frozen on dry ice. Samples of the unused media without cells were saved as blanks for the LDH assay. All samples were stored frozen at or below -70°C.

Processing of cell pellets — Frozen cell pellets were resuspended in 50 mM HEPES (pH 7.5) containing 1 mM PMSF and were sonicated for 2 min on ice in a sonicator bath (Fisher FS-30). An aliquot was removed for measurement of protein and LDH and the remaining sample was immediately refrozen on dry ice. Protein was measured using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). Bovine serum albumin (BSA) was used as a standard and all reported protein values are in BSA equivalents. Lactate dehydrogenase (LDH) activity was measured in a kinetic assay using a microplate reader (Molecular Devices THERMO$_{max}$™) as described by Vijayan et al. (1996).

Measurement of SP — The ELISA for HSP-70, SDS PAGE and Western blotting were performed as described in the Materials and Methods section of Chapter II. However, for quantitation of HSP-70 by Western blots the sections of the gels containing HSP-70 were cut out prior to equilibration and all of the samples to be compared were blotted onto a

single nitrocellulose membrane. The flood fill mode of Sigma Scan Image™ was used to automatically select and measure the average intensity of bands on Western blots. Liver lysates containing various amounts of HSP-70 were loaded on each gel to provide a positive control and a standard for comparison of band intensity. For proteins radioactively labeled with ^{35}S, equal counts were loaded in each lane for SDS page and the separated proteins were detected by autoradiography with Kodak XAR X-ray film which was pre-flashed to enhance the sensitivity and linearity of detection (Laskey & Mills, 1975).

4.3 Results

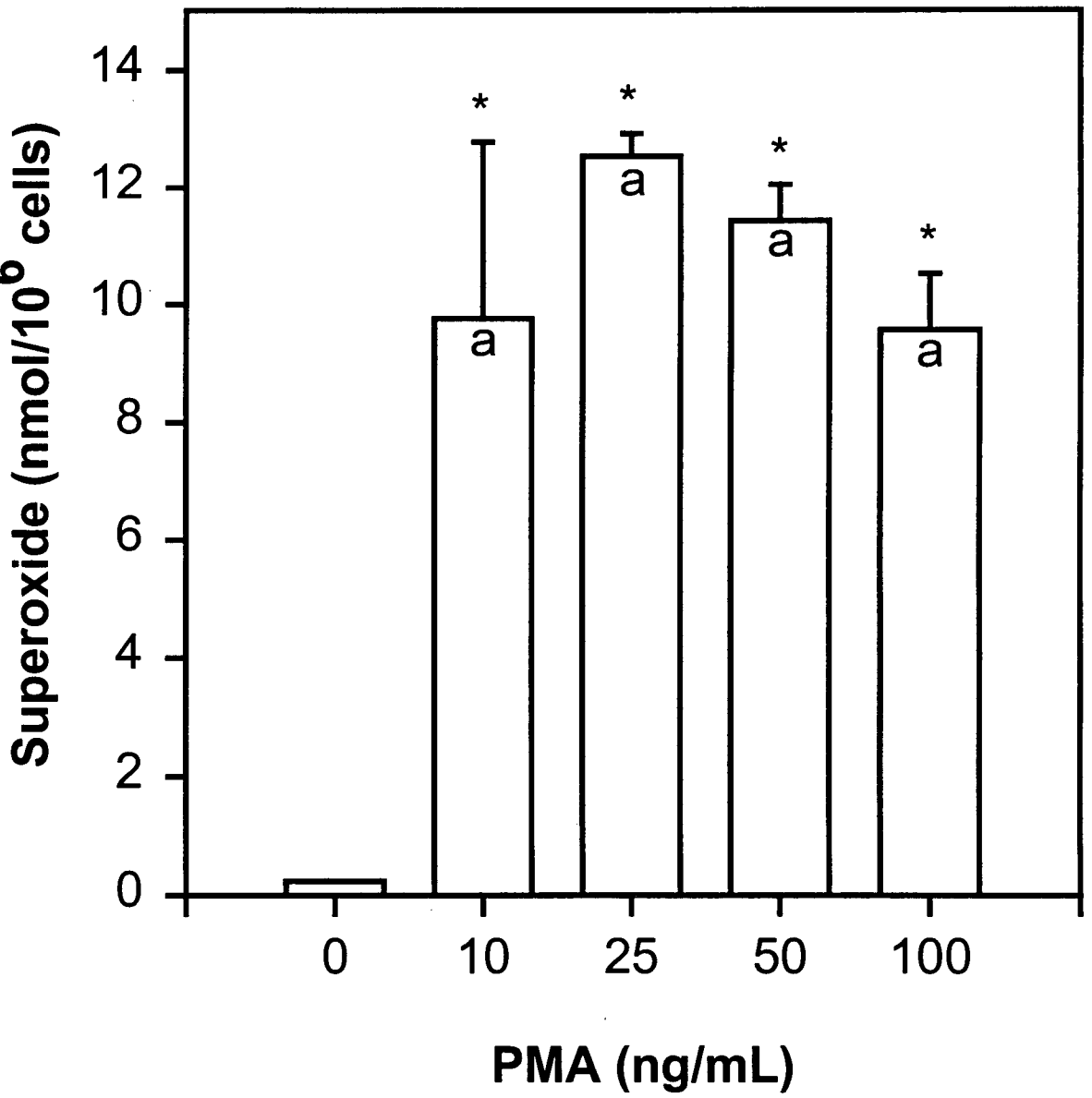
4.3.1 Phagocyte oxidant production.

Oxidant production by fish phagocytes has not been studied as extensively as oxidant production in mammals. Before proceeding with the studies of SP induction I wanted to make sure that the isolated phagocyte population behaved as would be expected based on the literature. As expected, isolated suspensions containing rainbow trout phagocytes generated superoxide when stimulated with PMA (Figure 4-2). The lowest dose of PMA (10 ng/mL) was nearly as effective at stimulating superoxide production as the highest dose (Figure 4-2). When maximally stimulated these phagocyte suspensions produced between 12 and 18 nmoles of superoxide per million cells in 30 min at ambient water temperatures of 5.8 to 9.0 °C .

4.3.2 SP in fish cell line exposed to hydrogen peroxide.

As a preliminary experiment to determine if oxidants induce SP in fish cells, I exposed cultures of a chinook salmon cell line (CHSE-214) to different concentrations of hydrogen peroxide and labeled the newly synthesized proteins with ^{35}S-methionine. Autoradiographic detection of these proteins by SDS PAGE showed increased synthesis of bands including those with molecular weights of approximately 70, 60, 45 and 30 kD (Figure 4-3). This SP response appears to be somewhat dose dependent and at the highest dose (1 mM H_2O_2) the synthesis of proteins in general was inhibited.

Figure 4-2. Stimulation of superoxide production in phagocytes treated with PMA. The assay was incubated at 5.8°C for 30 min (N=3 replicate cultures per group). Groups marked with an asterisk are significantly different from the unstimulated group (0 ng/mL) ANOVA on Ranks ($P_{\alpha} < 0.05$). Groups with the same letter are not significantly different from each other. The error bar is one SD.


Superoxide (nmol/10^6 cells)
14
12
10
8
6
4
2
0
*
*
*
*
a
a
a
a
0
10
25
50
100
PMA (ng/mL)

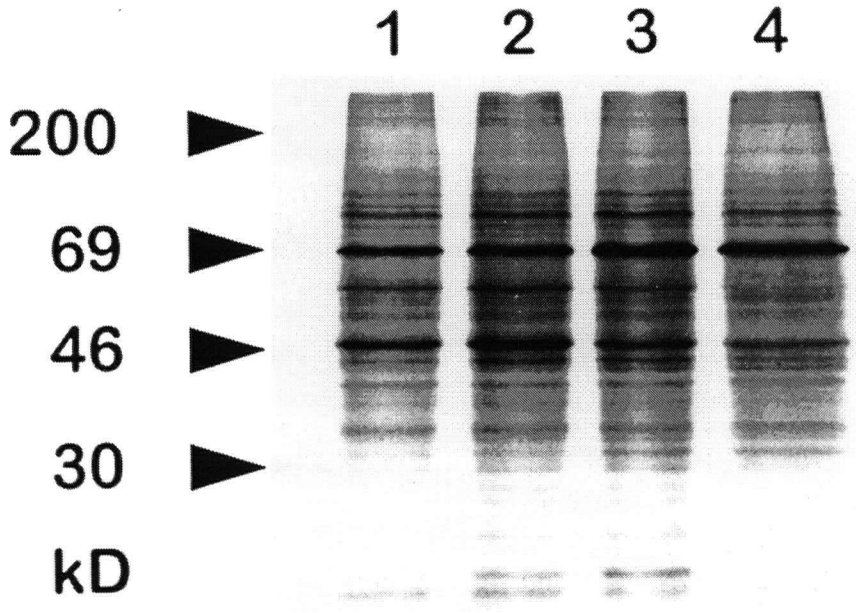


Figure 4-3. Newly synthesized stress proteins induced in CHSE-214 cell line by H_2O_2. Proteins labeled with ^{35}S-methionine were separated by SDS PAGE (12.5% acrylamide) and detected by autoradiography. The cells cultured at 18°C in Eagle's MEM with 10% heat inactivated Fetal Bovine Serum, were rinsed once in phosphate buffered saline (PBS), and exposed for 30 min to hydrogen peroxide at 0, 10, 100 or 1000 μM in PBS (lanes 1 – 4 respectively), and rinsed two times with PBS prior to addition of label (^{35}S methionine in methionine free Eagle's MEM) for 2 h. Equal counts were loaded in each lane.

4.3.3 HSP-70 in isolated rainbow trout phagocytes.

I investigated HSP-70 induction in isolated rainbow trout phagocytes using Western blotting. On Western blots of control cultures of phagocytes there was little or no detectable HSP-70 (Figure 4-4). There was no significant difference in the scanned intensity of the bands from the control cultures incubated for between 0 and 18 h without stimulation and there was no significant elevation of HSP-70 levels in heat shocked cultures (Figure 4-5). In one out of three experiments, significantly higher levels of HSP-70 were found in phagocyte cultures stimulated with PMA (Figure 4-5). Inclusion of superoxide dismutase (SOD) and catalase (CAT) to remove the generated oxidants had no effect on HSP-70 levels in any of the stimulated or unstimulated cultures. This suggests that either oxidants did not induce a SP response in these phagocyte cultures or that the oxidants were acting while they were still inside the cell and inaccessible to the enzymes.

The retention of lactate dehydrogenase (LDH) was used as an indicator of membrane integrity. Phagocyte cultures retained most of their LDH for the duration of the experiment (Figure 4-6) but heat shock or stimulation of phagocyte cultures with PMA caused substantial losses of the LDH. In different experiments PMA caused a loss of 50% to 90% of the LDH. This destructive effect of stimulating the phagocyte cultures was present even at a PMA concentration of 10 ng/mL and at all concentrations tested (Figure 4-7).

Figure 4-4. Western blots of HSP-70 in unstimulated phagocyte cultures. Rectangular strips from the regions containing HSP-70 were cut from four gels and transferred onto the same membrane. The cultures were incubated with (gels 1 and 2) and without (gels 3 and 4) SOD and catalase. There are no significant differences between the band intensities of the cultures. Lanes 1, 4 and 7 are from time zero controls; Lanes 2,5 and 8 are from cultures incubated for 18 h. Lanes 3, 6, 9 on each gel are standards prepared by blending induced and uninduced coho liver lysate to give 5X, 2X and 1X the HSP-70 content of uninduced coho liver lysate (15 μg/lane).

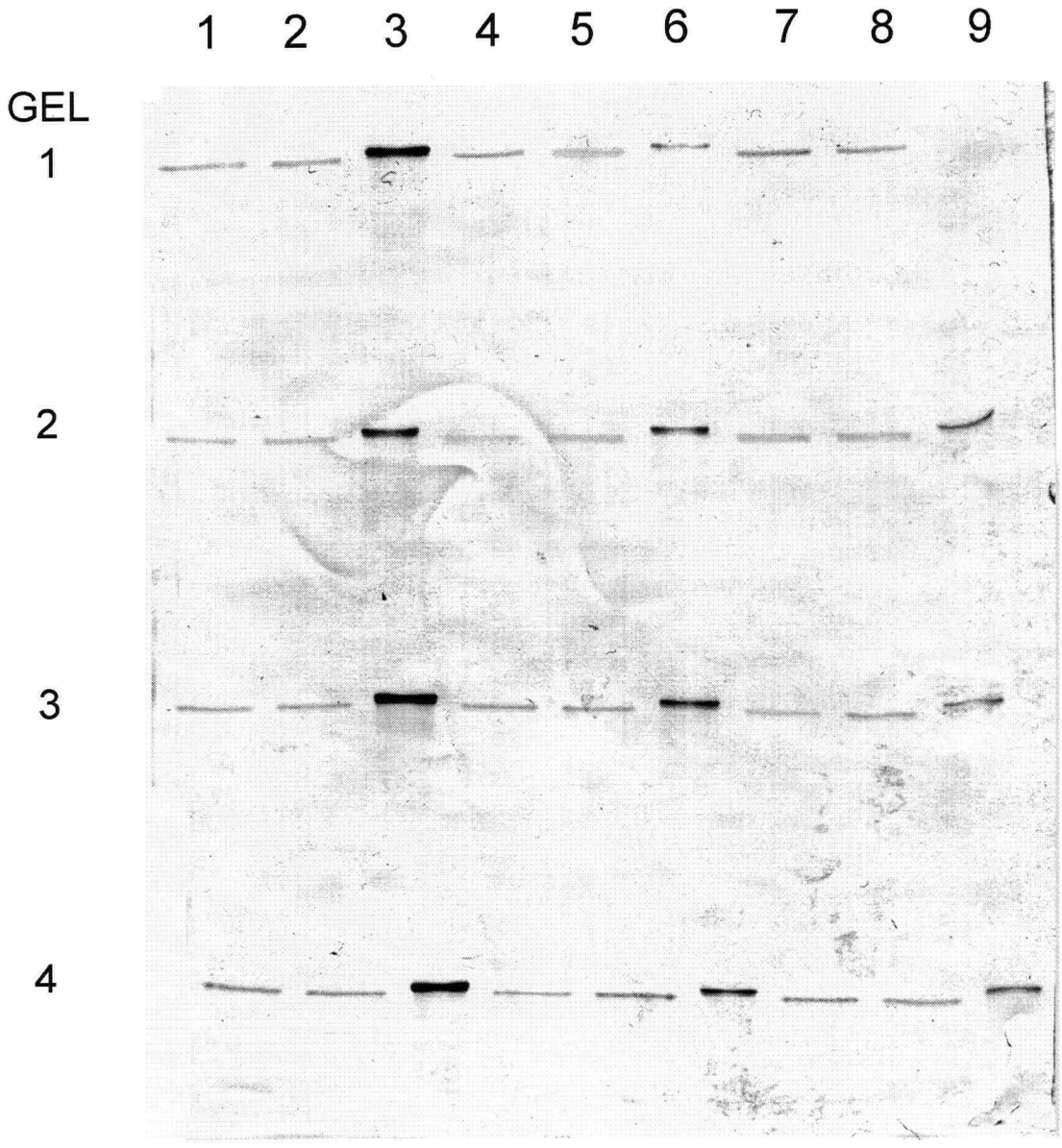

1 2 3 4 5 6 7 8 9
GEL
1
2
3
4

Figure 4-5. Band intensity of HSP-70 in stimulated and unstimulated phagocyte cultures on Western blots. Relative HSP-70 units measured as band intensity were standardized relative to the time zero control (T=0). The group marked HS was subjected to a 2 h heat shock at 15°C and then returned to the ambient water temperature (5°C). The groups marked No Stim. were unstimulated control groups (0 ng/mL). The groups marked PMA were treated with PMA at 50 ng/mL and the groups marked DMSO were the vehicle controls (treated with DMSO at 50 ppm). Groups marked with an asterisk are significantly different from the unstimulated group ($P_{\alpha} < 0.05$). Groups with the same letter are not significantly different from each other. The error bar is one SD (N=3 replicate cultures per group).

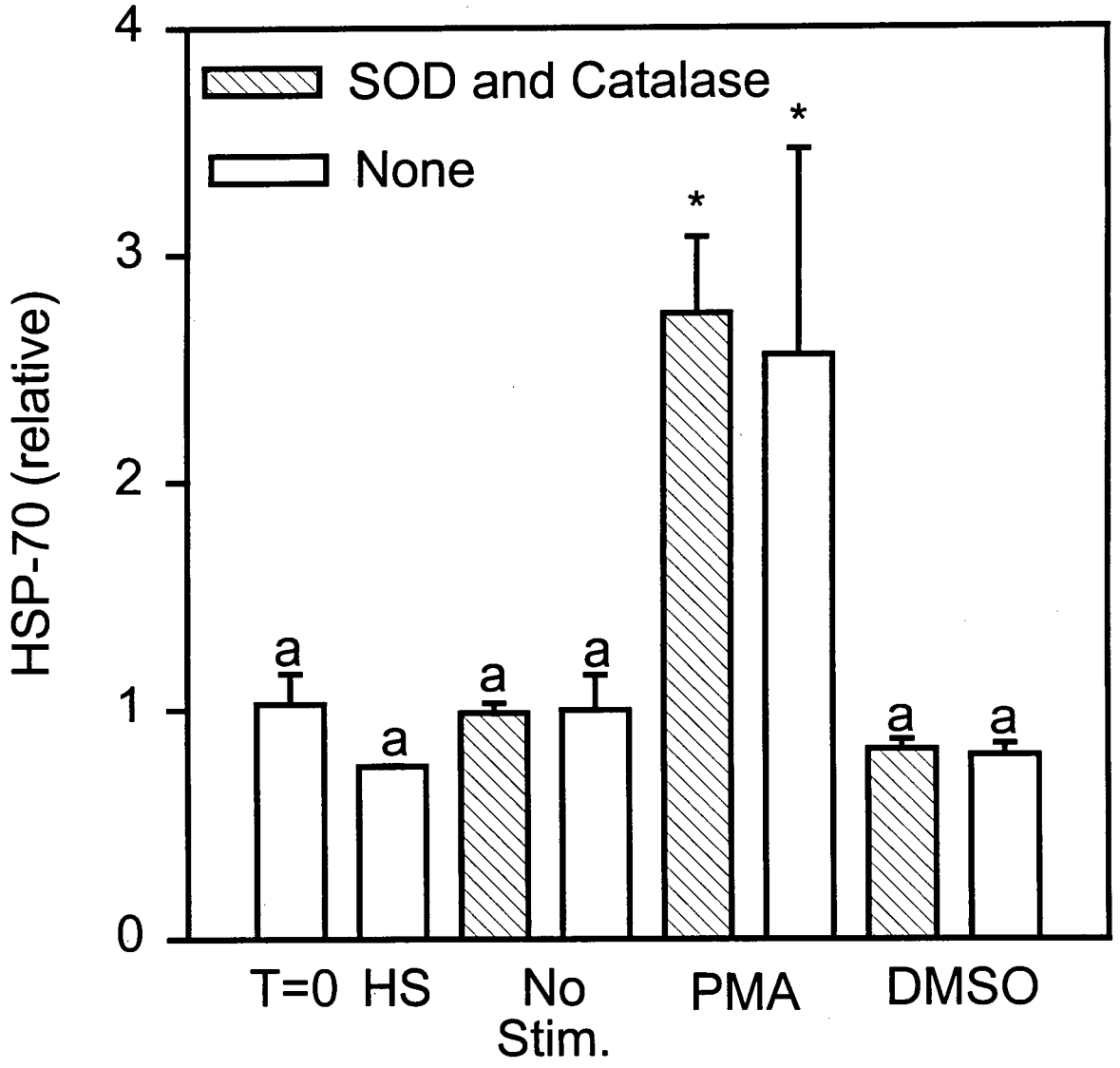

4
3
2
1
0
HSP-70 (relative)
SOD and Catalase
None
*
*
a
a
a
a
a
a
T=0
HS
No Stim.
PMA
DMSO

Figure 4-6. Retention of cytoplasmic contents by phagocytes in culture. The groups marked PMA were treated with PMA at 50 ng/mL and the groups marked DMSO were the vehicle controls (treated with DMSO at 50 ppm). The group marked HS was subjected to a 2 h heat shock at 15°C and then returned to the ambient water temperature (5°C). Groups marked with an asterisk are significantly different from the unstimulated group (None) sampled at the same time ($P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).

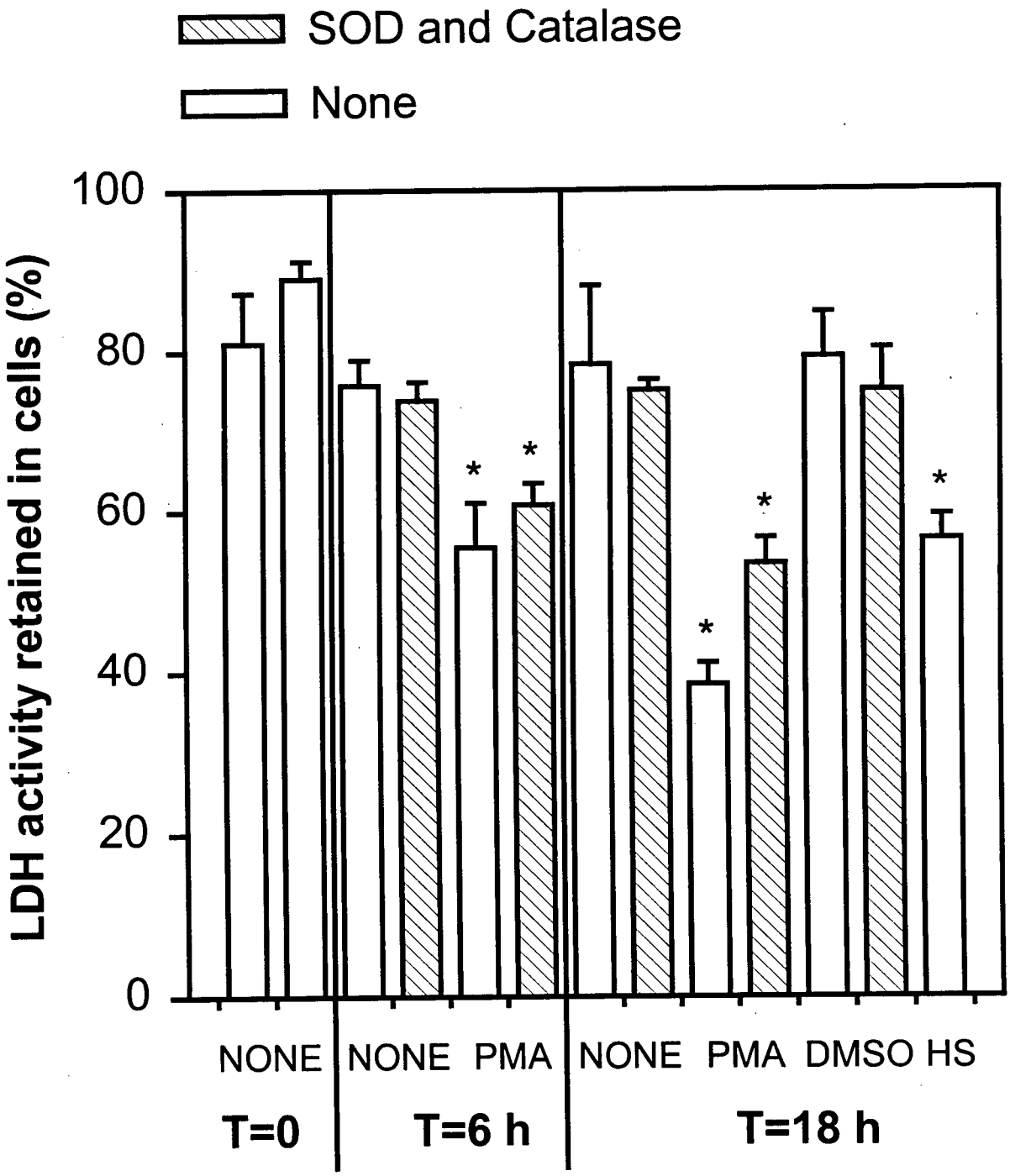
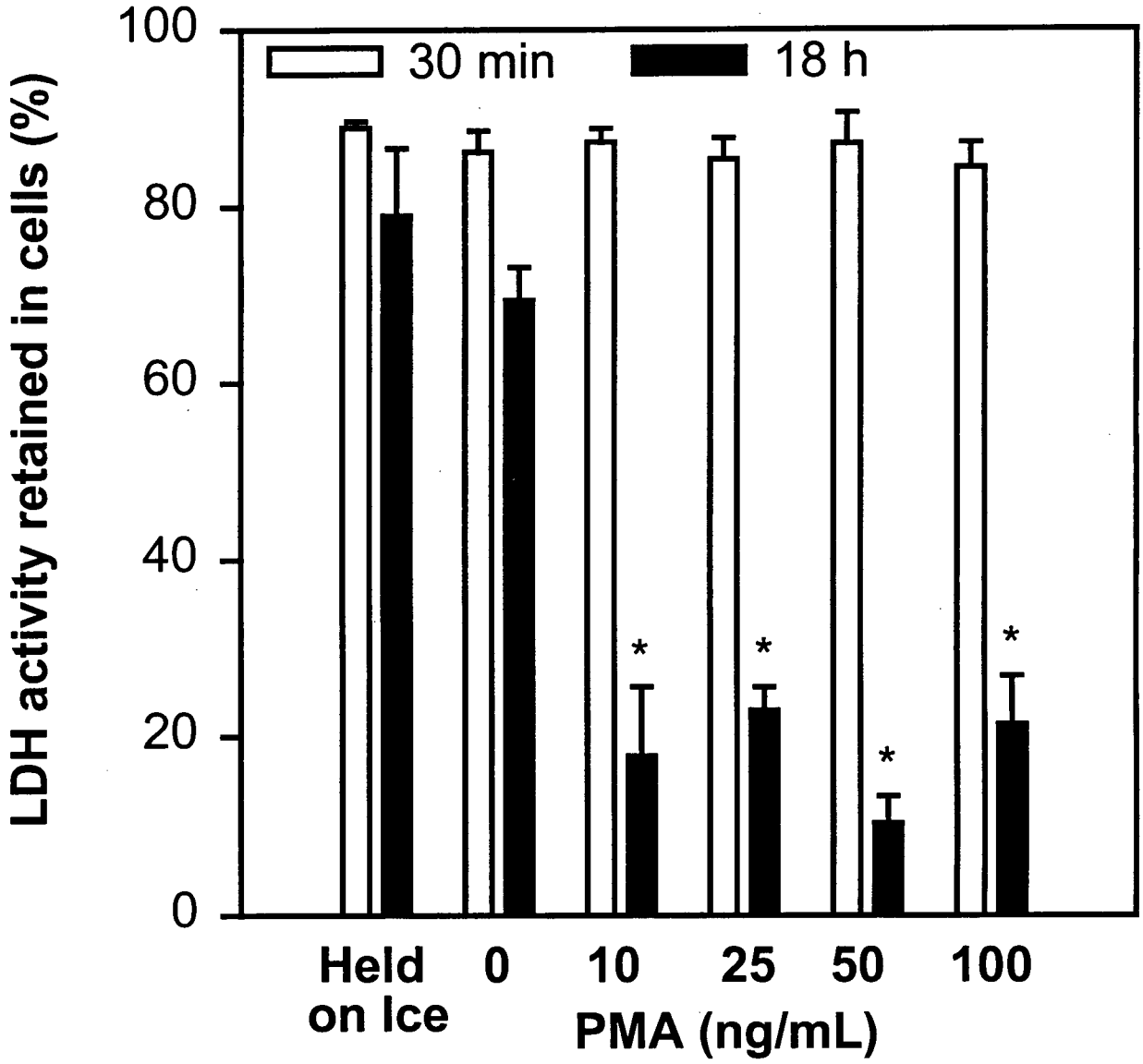

SOD and Catalase
None
LDH activity retained in cells (%)
100
80
60
40
20
0
*
NONE
NONE
PMA
NONE
PMA
DMSO
HS
T=0
T=6 h
T=18 h

Figure 4-7. Loss of cytoplasmic contents by phagocytes cultured with PMA. Groups marked with an asterisk are significantly different from the unstimulated group (0 ng/mL of PMA) held on ice and sampled at the same time($P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).


100
80
60
40
20
0
30 min
18 h
LDH activity retained in cells (%)
*
*
*
*
Held on Ice
0
10
25
50
100
PMA (ng/mL)

4.3.4 HSP-70 in mixed cultures of hepatocytes and phagocytes.

Since the SP response in the phagocytes themselves does not seem to explain the expression which I observed in the livers of fish with BKD, I conducted a series of experiments with mixed cultures of hepatocytes together with phagocytes to explore the possibility that oxidants produced by phagocytes might induce SP in adjacent cells.

HSP-70 levels in hepatocyte cultures were measured by ELISA. Cultures from each batch of hepatocytes were subjected to positive control heat shock experiments to ensure the validity of the coculture experiments and to give a basis for comparing the magnitude of induction. Shifting of hepatocytes to a higher temperature caused up to approximately a ten fold increase in HSP-70 over the levels present in the freshly isolated hepatocytes (Figure 4-8). Heat shock caused only a modest loss of LDH from the cells (Figure 4-9) indicating that the hepatocytes maintained good cellular integrity.

Figure 4-8. Heat shock induction of HSP-70 in hepatocytes shifted from 4 ° C to 15 °C. Relative HSP-70 units measured by ELISA were standardized to the HSP-70 content of heat shocked hepatocyte cultures with the maximum HS response observed in that batch of hepatocytes. Groups marked with an asterisk are significantly different from the time zero control ($P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).

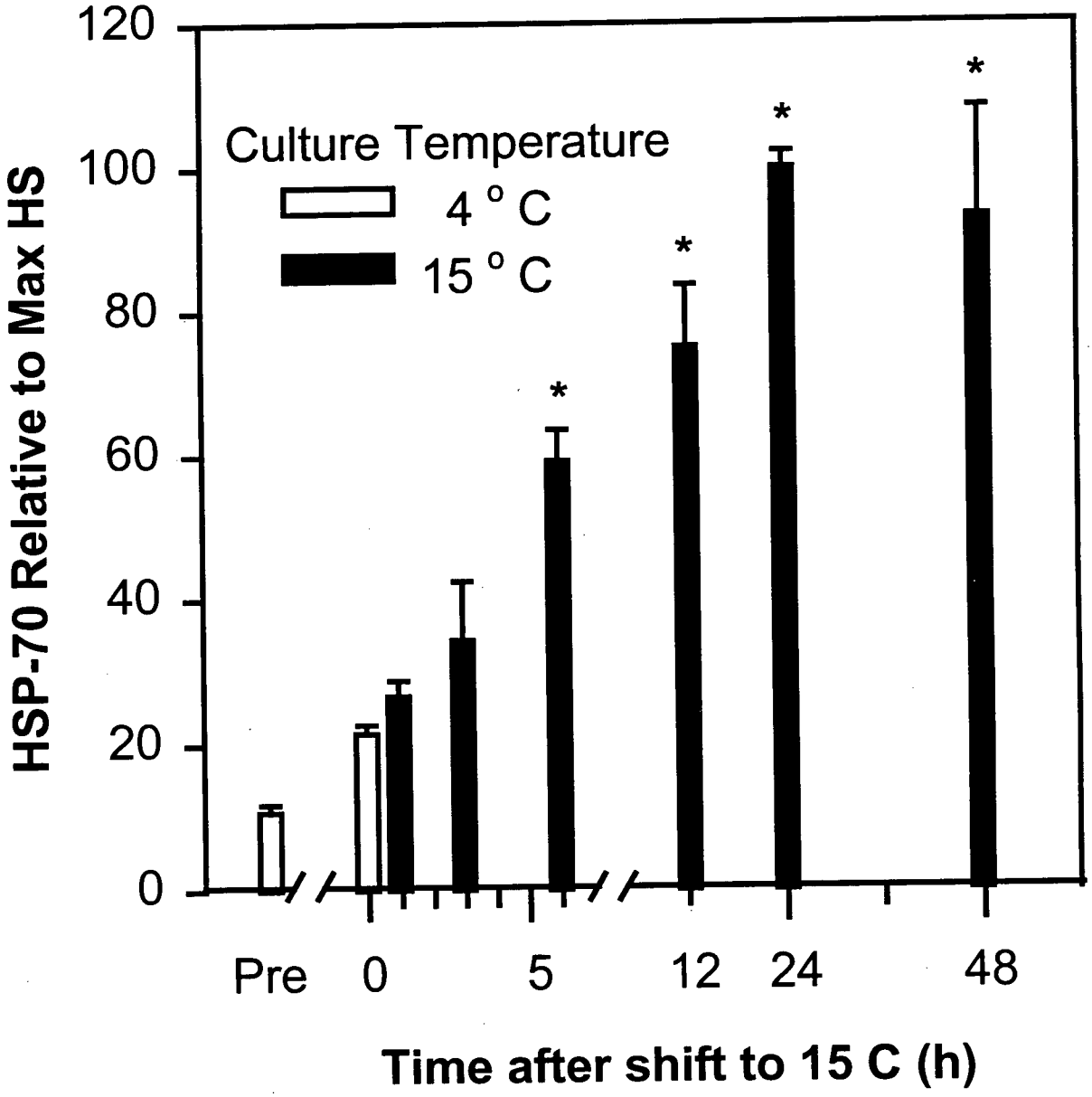
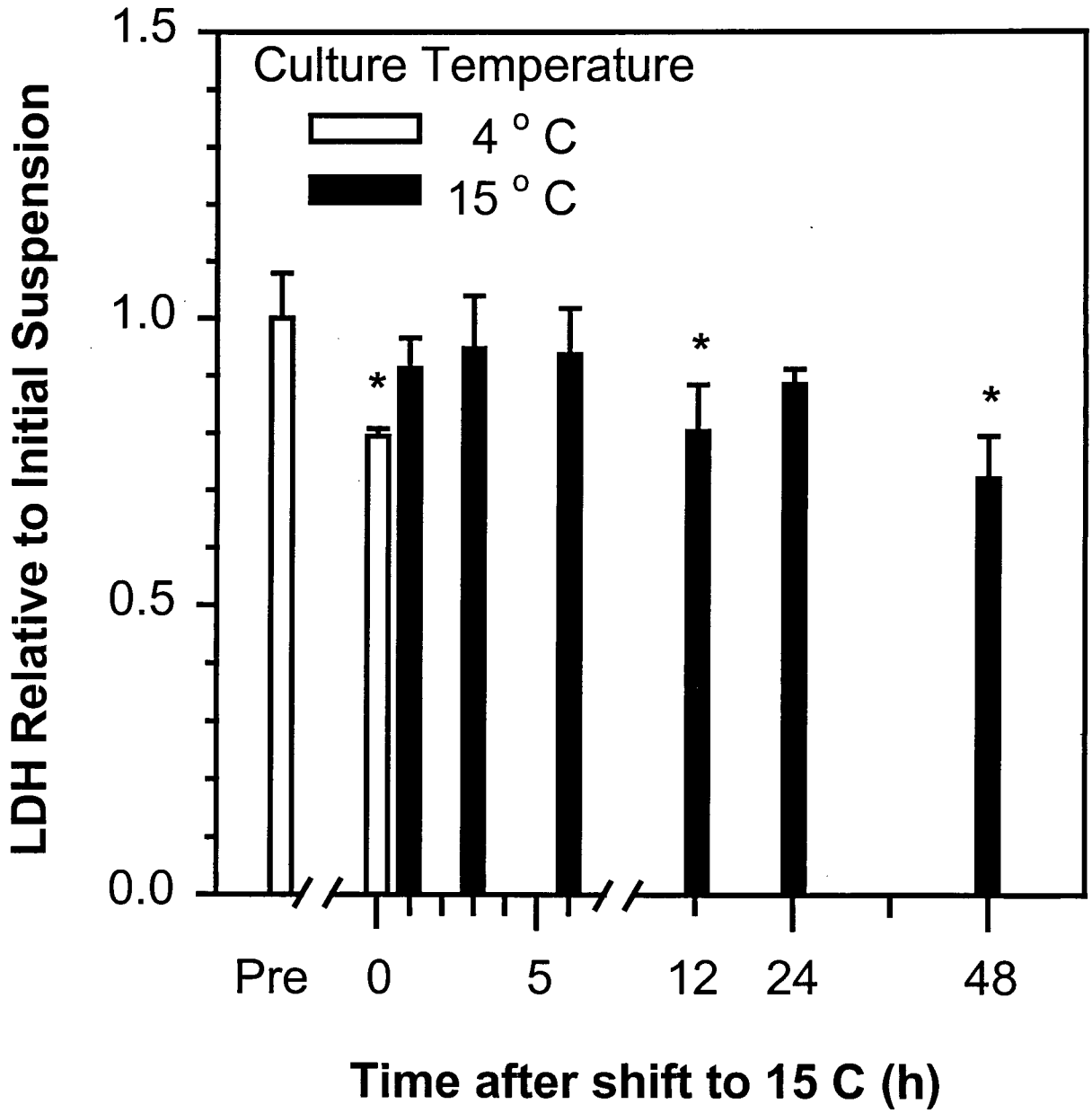

120
100
80
60
40
20
0
HSP-70 Relative to Max HS
Culture Temperature
4 ° C
15 ° C
*
*
*
*
Pre
0
5
12
24
48
Time after shift to 15 C (h)

Figure 4-9. Retention of cytoplasmic contents by heat shocked hepatocytes. Groups marked with an asterisk are significantly different from the initial isolated hepatocyte suspension (Pre) control ($P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).


1.5
1.0
0.5
0.0
LDH Relative to Initial Suspension
Culture Temperature
4 ° C
15 ° C
*
*
*
Pre
0
5
12
24
48
Time after shift to 15 C (h)

In the absence of phagocytes neither PMA nor Rs caused significant HSP-70 induction in the hepatocyte cultures. In experiments with three batches of phagocytes isolated from separate fish, incubation of hepatocytes with phagocytes did not induce HSP-70 regardless of whether or not stimulants were added (Figure 4-10). In fact, there was significantly less HSP-70 in cultures containing unstimulated phagocytes than in cultures containing only hepatocytes (Figure 4-10). The hepatocyte cultures in wells with phagocytes were also dramatically less adherent. When harvested, the cultures containing phagocytes were dislodged by a single flushing with medium rather than the usual seven point flushing that was required to dislodge hepatocyte cultures in the absence of phagocytes. The failure of the phagocytes to cause induction in short term cultures can not be explained by lysis of the hepatocytes because the retention of LDH in those cultures was not significantly different from the control cultures (Figure 4-11).

Figure 4-10. HSP-70 content of hepatocytes cultured with phagocytes and stimulants. Relative HSP-70 units measured by ELISA were standardized to the HSP-70 content of heat shocked hepatocyte cultures with the maximum HS response observed in that batch of hepatocytes. Groups marked with an asterisk are significantly different from the control cultures to which nothing was done ($P_{\alpha} < 0.05$). The precontrol cultures were sampled before setting up the experiment (one day earlier than the other cultures). The error bar is one SD (N=3 replicate cultures per group).

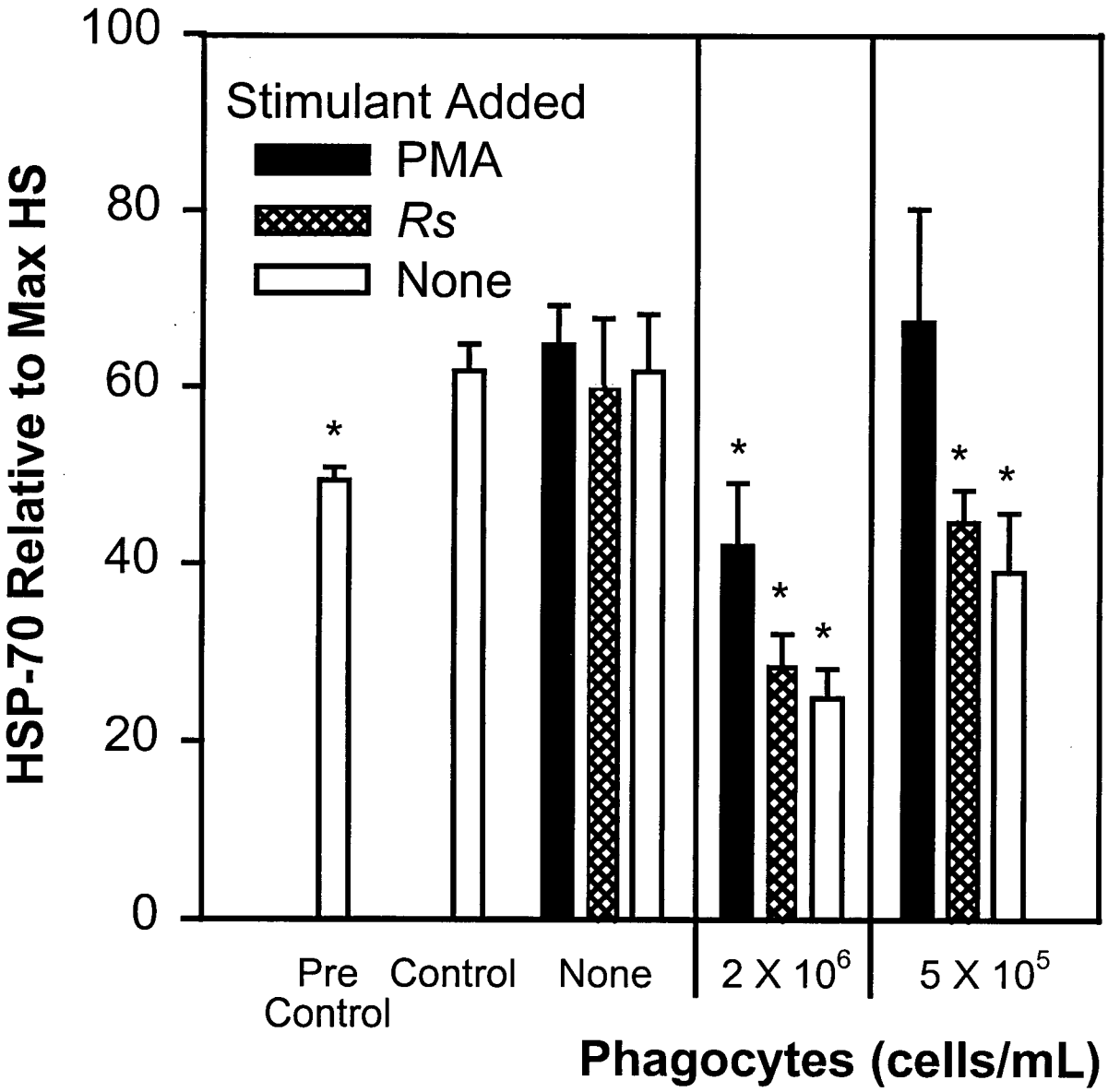
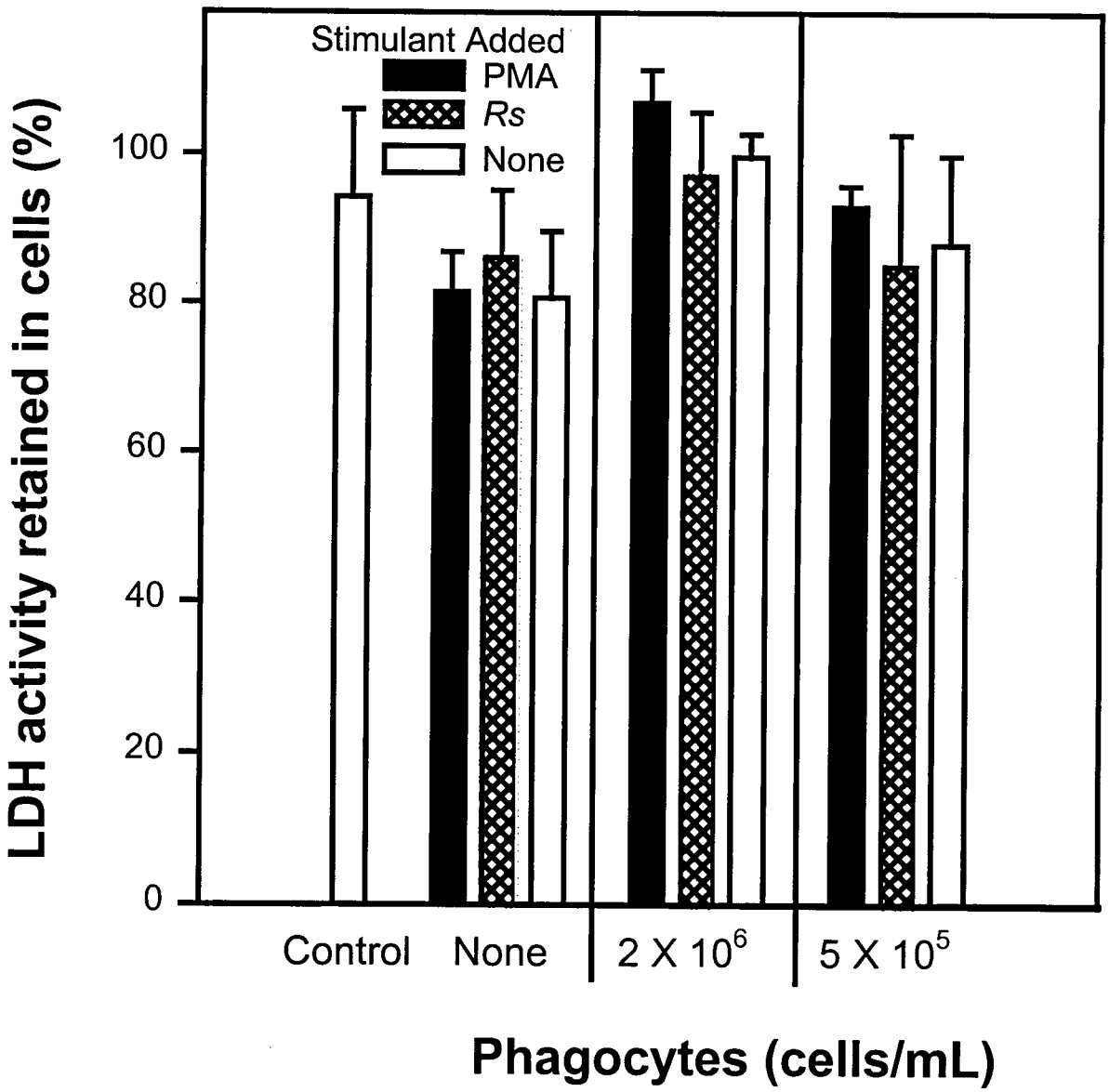

100
80
60
40
20
0
HSP-70 Relative to Max HS
Stimulant Added
PMA
Rs
None
*
Pre Control
Control
None
2 X 10^6
5 X 10^5
Phagocytes (cells/mL)

Figure 4-11. Retention of LDH by hepatocytes cultured with phagocytes and stimulants. None of the groups are significantly different from the control cultures to which nothing was done. The error bar is one SD (N=3 replicate cultures per group).


Stimulant Added
PMA
Rs
None
LDH activity retained in cells (%)
100
80
60
40
20
0
Control
None
2 X 10^6
5 X 10^5
Phagocytes (cells/mL)

4.4 Discussion

The results from the *in vitro* experiments in this chapter do not provide an explanation for what causes SP induction in BKD. In discussing the results of the experiments in this chapter I concentrate on exploring some possible explanations for the results of the *in vitro* experiments and how they relate to the expression of SP in tissue from fish with BKD.. Since culturing hepatocytes with stimulated phagocytes did not seem to cause a stress response and hepatocytes responded to heat shock in the expected way, the experiments with hepatocytes require little discussion.

The apparent SP response of the phagocytes to PMA can not be taken as reliable evidence for induction of SP by some phagocyte functions other than oxidant production. I discuss the limitations of the results and some possible mechanisms that may explain the response of phagocytes to PMA and the failure to respond to heat shock. Although I deal with the implications of these observations mainly in the General Discussion chapter, I also briefly discuss in this chapter the possible value of a weak SP response to the role of phagocytes in defense. Finally, I discuss how the results from experiments with cultured cells contribute to understanding SP induction in fish with BKD and point out some tools needed for future studies.

4.4.1 SP response in phagocytes stimulated with PMA.

The possibility that PMA induces HSP-70 in fish phagocyte suspensions must be interpreted cautiously. This data could be interpreted as evidence for induction of HSP-70 by some phagocyte function that does not involve superoxide or peroxide. However, such a speculation is doubtful because only a fraction of the initial cells survived in these PMA

stimulated cultures and elevated HSP-70 was not seen in all experiments. It is possible, for instance, that the change in HSP-70 content is the result of selective destruction of cells which happened to contain lower HSP-70 levels rather than a genuine induction of SP. It is also important to point out that all of the experiments on phagocyte cultures should be viewed with caution since heat shock treatment did not cause an increase in HSP-70 in phagocyte cultures.

The apparent induction of HSP-70 in phagocyte suspensions stimulated with PMA may have been a direct result of exposure to PMA. Many phorbol esters including PMA activate protein kinase-C and cause a wide range of changes in cell physiology including cell proliferation and differentiation (Buchner, 1995). Exposure to PMA did not cause HSP-70 induction in hepatocytes, and, to my knowledge, PMA itself does not induce a SP response in any cell type. However, in some cell types, PMA has been reported to increase synthesis of HSP-70 without new synthesis of HSP-70 RNA (Jacquier-Sarlin *et al.*, 1995) and protein kinase-C inhibitors (staurosporine and H-7) can inhibit induction of HSP-70 in oxidant stressed phagocytes (Kantengwa & Polla, 1991). Furthermore, PMA can increase the response of cells to stressors such as sodium arsenite and heat (Ito *et al.*, 1995; Holmberg *et al.*, 1997). This enhancement of stress responses by PMA is consistent with the fact that protein kinase C causes phosphorylation and transcriptional activation of HSF-1 which has already been trimerized by a previous stressor (Ding *et al.*, 1997).

4.4.2 SP response in stressed phagocytes.

The failure of heat shock to cause elevation of HSP-70 levels in isolated phagocytes may be an artifact of isolation but may also be a characteristic of these cells. Isolated hepatocytes

had a strong SP response when heat shocked and even when cultured at 6.5°C, the ambient temperature of the fish from which they were isolated. In contrast, Western blots of the cultured phagocyte suspensions did not show any significant induction of HSP-70 in cultures held at ambient temperatures or in heat shocked cultures. Clearly, the absence of a heat shock response in isolated phagocytes could be evidence that they were damaged in the isolation process. However, the condition of the isolated phagocytes seemed generally good since the phagocyte suspensions were capable of producing superoxide, had high retention of the cytoplasmic marker enzyme LDH when held in culture, and over 95% of the isolated cells excluded trypan blue (see Appendix I). The phagocytes may have had subtle impairments in their function, but it is just as reasonable to speculate that a weak SP response to some stressors may be one of the characteristics of salmonid phagocytes.

A weak SP response in isolated rainbow trout phagocytes might also relate to the role of phagocytes in general. Published studies of SP induction in mammalian phagocytic cells support the general conclusion that phagocytes themselves may not have particularly strong stress responses to oxidant production (Kantengwa & Polla, 1991). A strong SP response is induced in human monocyte-macrophages by oxidant production in the presence of additional factors such as phagocytosis of red blood cells (Kantengwa & Polla, 1991) or eosinophils (Polla *et al.*, 1993).

Since erythrocytes were almost completely removed in the process of preparing the phagocyte suspensions, they could not have acted as cofactors in the experiments with cultured phagocytes. Such cofactors may be important *in vivo* and might play a role in the induction of SP which has, for example, been reported in arterial plaques (Kleindienst *et al.*, 1993) and in bronchoalveolar lavage cells from inflamed lungs (Polla *et al.*, 1993). The

phagocytosis of other cell types might also play a role in SP expression in BKD. The major surface protein (p57) of Rs can bind to host cells including erythrocytes and make them targets of phagocytosis *in vitro* (Gutenberger *et al.*, 1997). It is not known whether p57 mediates phagocytosis of erythrocytes in fish with BKD nor whether phagocytosis of erythrocytes causes SP induction in fish, but such possibilities could be tested.

4.4.3 Self destruction of stressed phagocytes.

Another possible explanation for the low levels of HSP-70 in the phagocyte cultures is that the production of oxidants or the release of lytic enzymes may destroy the phagocytes before they can mount a stress response. Lytic enzymes could also degrade any SP which was induced before the cells died. Indeed, there was a definite destruction of phagocytes when they were stimulated with PMA. These phagocytic cells may not need to survive after they have successfully performed their functions of producing oxidants and releasing lytic enzymes. A large fraction of the phagocytes present in the isolated phagocyte suspensions were neutrophils and these are responsible for most of the oxidant production in phagocyte suspensions (Plytycz *et al.*, 1989; Hamdani *et al.*, 1998). In mammals, neutrophils are short lived and undergo apoptosis after participating in an inflammatory response (Hart *et al.*, 1996). A SP response to oxidants might not be needed or useful if these fish neutrophils are terminal and short lived or if their role in phagocytosis and killing is inherently self destructive. To my knowledge, there have been no studies of the life span of phagocytic cells in salmonids or whether the cells survive after engulfing and destroying microorganisms. However, as I discuss in the General Discussion chapter, it may be an advantage for the phagocytes to die and thereby present a less suitable environment for the

growth of any intracellular pathogens which survive phagocytosis and the first line of killing mechanisms.

4.4.4 Implications for understanding SP in coho with BKD.

The negative results from the experiments with cultured cells are of limited value in understanding how SP might be induced in BKD. If SP had been induced in the coculture experiments such results would have supported the idea that phagocytes could be causing SP induction in fish with BKD. Unfortunately, the fact that phagocytes did not induce HSP-70 in the *in vitro* experiments does not rule out the possibility that phagocyte derived oxidants cause SP induction in living fish with BKD. There are a number of factors in the conditions of the coculture experiments which differ from the situation *in vivo* and may not have been optimal for induction or detection of SP. For instance, the phagocyte concentration was chosen in order that the phagocytes would produce measurable amounts of oxidants. As such, the ratio of phagocytes to hepatocytes may not have been optimal. Furthermore, the phagocytes added were obtained from healthy rainbow trout and as such the leukocyte suspensions probably did not contain Rs specific immune cells. In mice, T-cells have been shown to be required for induction of HSP-60 in macrophages of *Toxoplasma gondii* infected mice (Barazzone *et al.*, 1996). Such lymphocytes might produce proinflammatory cytokines and might even cause the HSP-70 induction in the fish with BKD by directly damaging tissue.

In a similar way, the fact that Rs didn't induce SP when added to these short term *in vitro* cultures does not rule out the possibility that it might induce SP in a living animal or in a longer term culture experiment. Rs was grown on culture media and added as a stimulant

rather than being chronically present as an intracellular parasite within the cells as it would be in an infected fish. The short culture period might not have allowed the time necessary for Rs to affect the hepatocytes directly by toxin production or cellular invasion for instance.

However, even in the negative results, there is some value for understanding SP induction BKD. The low levels of HSP-70 in stressed phagocyte cultures is consistent with the fact that in the tissue of fish with BKD there was little HSP-70 staining in the cells which contained Rs and which may have been phagocytes. In this light, the fact that the hepatocytes had a strong SP response is consistent with fact that there was intense HSP-70 staining in the liver tissue surrounding the colonies of Rs. Finally, the fact that there was no evidence for SP induction by phagocytes oxidants or direct induction by Rs *in vitro* suggests that other less obvious mechanism should be considered. These results also suggest that the process of induction in BKD may be more complicated than can easily be modeled in a simple culture system.

4.4.5 Tools needed to study SP in BKD.

Given that there was no direct evidence that phagocytes are involved in SP induction it did not seem reasonable to conduct an exhaustive series of experiments with many permutations of phagocyte, hepatocyte, and stimulant concentrations.

For future experiments to elucidate the cause of SP induction in fish with BKD an improved model is needed to study infection by Rs and microcolony development. This could be accomplished either with a more complicated *in-vitro* system with components which closely mimic those present in the tissue of diseased fish or with a more simple *in*

vivo system which would allow convenient monitoring of the progress of pathology over time. The pursuit of an improved system in either of these directions is beyond the practical scope of this thesis, but I discuss some possible approaches in the General Discussion below.

4.4.6 Conclusion.

In conclusion, the experiments with cultures of isolated rainbow trout cells showed that hepatocytes were able to respond to heat shock with elevated HSP-70 levels, but there was little or no induction of HSP-70 in phagocyte cultures and no evidence that oxidant production induced HSP-70 in the phagocytes themselves nor in adjacent hepatocytes. The apparent induction of SP by stimulation of phagocyte cultures with PMA was only seen in one out of three experiments and was not prevented by consuming the superoxide and H_2O_2 so it is unreasonable to attribute the induction to the effects of phagocyte generated oxidants. My results do not support my third major hypothesis: **SP can be induced as a result of phagocyte function and oxidant production.** The null hypothesis appears to be supported: HSP-70 is not induced by isolated rainbow trout phagocytes *in vitro.*

Chapter V General Discussion

In the work for this thesis I sought evidence for induction of stress proteins by physiological mechanisms which operate in a living animal (fish). I looked for SP in fish with bacterial kidney disease (BKD) and sought to determine whether phagocytes might cause SP induction in these fish. My results confirm the major hypothesis of this thesis: **SP are induced in fish with BKD. (This appears to be an example of SP induction by a physiological response to a stressor.)**

My results also support the secondary hypothesis: **SP induction is associated with the sites of infection and phagocyte infiltration in tissue from fish with BKD.**

The central observation of this thesis is that in coho salmon with BKD, there were localized areas with elevated SP levels in the host cells surrounding bacterial microcolonies in diseased tissue. This is the first example of SP induction in diseased fish and is one of a very few examples of SP induction in animals with bacterial diseases. Beside being new, this observation may be important if such localized SP plays a role in the pathology of BKD or of other bacterial diseases.

Although these results do not support the hypothesis that SP induction was caused by phagocyte function, it is likely that SP was induced in the fish with BKD by some localized physiological response to the presence of the stressor (bacteria). Such a physiological response seems unlikely to be peculiar to BKD. The presence of SP at the sites of infection in BKD raises the possibility that this pattern of SP distribution may occur in other bacterial infections. To put it another way, the confirmation of my major hypothesis supports an

inductive argument that local physiological responses to infections are important potential causes of SP induction *in vivo.*

I am confident that this thesis points toward valuable new avenues of research. The impact of host SP on interactions between the host and bacterial pathogens has not been studied. My results lead me to conclude that it will be valuable to explore the role of host SP in the pathophysiology of BKD and of other bacterial diseases.

The work for this thesis has also yielded new insights and ideas with practical and conceptual value. First of all, this work has lead to some new ideas which may be of practical value for studying and treating BKD. The conceptual impact of the observations, however, goes beyond their value in BKD. It requires no additional postulates to speculate that areas with elevated levels of host SP are likely to be present in some bacterial diseases other than BKD and especially in some diseases caused by other Gram positive bacteria and intracellular pathogens. These two classes of pathogens cause some of the most important bacterial diseases and if this speculation is true it could provide valuable leads for human medicine as well as for the study of animal diseases.

It is also reasonable to speculate that elevated levels of host SP in cells near bacterial microcolonies could have an effect on whether the bacteria survive or not. For instance, SP are known to protect cells against self destruction (apoptosis) which some bacteria inhibit and others induce. The death of host cells and the mechanism of their death could affect the ability of the pathogen to survive in the tissue. Some other known functions of host SP that could potentially influence the interaction between the host and bacteria include: acting as adjuvants to stimulate immunity, preventing apoptosis of neutrophils,

and acting as targets for immune cross reactivity by T-Cells specific for SP of the pathogen. Whether host SP at sites of infection benefits the host or the pathogen and whether it is important to the outcome of the infection might vary between diseases. However, this possibility is worth studying if host SP have an impact in diseases caused by even a subset of bacteria (intracellular pathogens for instance).

We do not know to what extent host SP are induced in the tissue of animals with bacterial diseases because this has either not been studied or has not been reported in most cases. There is still very little knowledge about induction of host SP in bacterial diseases even though the need for study in this area was pointed out almost a decade ago (Young & Mehlert, 1990). I have been able to find fewer than 10 papers that report host SP induction in tissue infected by bacteria. (Emoto et al., 1992; Emoto et al., 1993; Khanolkar-Young et al., 1994; Kol et al., 1998) Even recently, some researchers have reported SP of a pathogen (*Helicobacter pylori*) in infected tissue but failed to look for expression of host SP (Ko *et al.*, 1998). The importance of host SP in bacterial diseases will remain hypothetical until more researchers look for and report whether host SP is elevated in diseased tissue.

At the start of the research for this thesis nothing was known about SP expression in BKD or any other fish disease and little was known about SP expression by host tissue in bacterial diseases of any species. My results open up new approaches to the study of BKD and the eventual control of this important fish disease. Although I was pursuing the relatively limited questions of whether SP are induced in fish with BKD and whether phagocytes might play a role in SP induction, the observations also have implications and relevance which may reach beyond the practical value in BKD and other fish diseases.

If localized SP induction occurs with a similar pattern in diseases other than BKD and in vertebrates (such as humans) other than salmon, such host SP near colonies of bacteria might affect the outcome of infection. Elevated levels of host SP near colonies of a pathogen might also be involved in stimulating or maintaining T-cells which react with SP from both the host and the pathogen and which may play an important role in autoimmune inflammatory diseases such as reactive arthritis. Thus the localized elevation of host SP levels observed in BKD might occur in other bacterial infections and influence the course of human and animal diseases caused by bacterial infections.

5.1 New approaches and directions for research on BKD

The most immediate practical value of this thesis work is that it has lead to ideas for some new approaches to studying BKD and to realize that a quorum sensing system may be present in Rs. Although I did not pursue these possibilities as part of this thesis, I think that these new ideas would be fruitful directions for future research. Control of BKD will require sufficient understanding of the disease to permit the design of preventive and therapeutic approaches. Unfortunately, BKD has been difficult to study because of the slow development of disease in infected fish. Any preventative or therapeutic approach developed using model systems will ultimately need to be tested for its ability to prevent infection or mortality in fish with BKD, but there is a need for more rapid experimental approaches. Simplified model systems should help us to rapidly explore the virulence strategies of Rs and to determine the cause and significance of SP expression in BKD.

5.1.1 Improved *in vitro* culture system.

The first new approach is a potential improvement to the *in vitro* culture system which stems from the possibility of growing Rs in tissue culture with a fish cell line (McIntosh *et al.*, 1997). *Renibacterium* will multiply intracellularly in these cultures for several weeks. It would be worthwhile as a future project to determine what are the longer term effects of infecting hepatocyte cultures with Rs and then adding immune and non-immune leukocytes to infected cultures. In the experiments with phagocyte suspensions and Rs added to cultures of hepatocytes, the conclusions were limited by the fact that there may not have been sufficient time for Rs and the cells to interact in the way that they do in an infected fish. Allowing more time for interaction may permit the pathogen to become intracellular and perhaps express virulence factors. In addition to being a more realistic *in vitro* model in which to explore the cause of SP elevation in BKD, this culture system might also be useful for studying intracellular persistence by Rs. It might allow rapid screening of drugs and immunization approaches for their ability to eliminate intracellular Rs.

5.1.2 Simplified *in vivo* model system.

The second approach is the use of a septic inflamed peritoneum model for BKD that might be a good simplified *in vivo* system for investigating intracellular persistence and the effects of Rs on its host cells. In the course of the experiments with Rs, pus like fluid collected in the peritoneal cavities of fish injected with large numbers of bacteria. At necropsy, this material contained many leukocytes and large numbers of Rs associated with cells or cellular debris. Others have made similar observations and peritoneal fluid from fish infected with Rs can be used as a source of virulent inocula for disease challenges (McIntosh *et al.*, 1997). Fluid can easily be collected from the peritoneum without killing

the fish. Repeated sampling from a single fish would allow the progress of an infection to be monitored over time. A reasonable control peritoneal fluid could be produced by injecting fish with killed Rs or with other materials to stimulate an inflammatory response and cause infiltration of cells into the peritoneum. The ability to collect sequential samples would allow examination of the time course of SP induction and the effects of Rs on the infiltrating cells. Studying Rs infection in the peritoneum may also allow screening of the effects of therapeutic agents in living fish more rapidly than would be possible using mortality as an end point.

5.1.3 Method for following the growth of colonies in BKD.

The third new approach to studying BKD is a way of observing the effects of small colonies of Rs in the tissue of infected fish before obvious disease develops. Host SP was present in cells surrounding relatively small colonies of Rs in the livers of coho with BKD. If SP levels are elevated near microcolonies of Rs early in the infectious process it might influence whether the infection progresses to disease. However, studying these early colonies may be difficult. There was no detectable Rs on frozen sections of liver from fish without obvious disease. Presumably, there were too few colonies to be found or any colonies that may have been present were too small to be easily found. It is likely that small or sparsely distributed colonies could be located and studied early in the infectious process using confocal microscopy of relatively thick sections of tissue. This approach has been used to study early stages of infection by *Salmonella* which is another intracellular pathogen (Richter-Dahlfors *et al.*, 1997). Confocal microscopy would reveal the patterns of SP distribution and tissue pathology at progressive stages of disease and microcolony development in BKD.

5.1.4 Identifying the quorum-signal and sensor in Rs.

Finally, the possibility that Rs may have a quorum sensing system presents an exciting direction for future research on BKD. Although I had no intention of investigating quorum sensing in BKD, the results of this thesis and results of other groups make it seem plausible that Rs has a quorum sensing system to regulate growth rate in culture and perhaps to regulate virulence. Research on quorum regulation in Rs may provide additional targets for therapeutic approaches to BKD. It could be very fruitful to identify the molecule or molecules involved and explore how the signaling system affects the pathogenesis of BKD. If the growth rate stimulating properties of culture supernatants from Rs are due to the presence of a quorum-signal, it should be relatively easy to purify and identify the quorum-signal molecule from Rs using a simple growth promotion assay.

The quorum-signal molecules in most Gram positive bacteria are peptides. It is likely that any quorum-signal in Rs will also be a peptide. Sequencing the purified peptide would allow rapid identification of its gene and any precursor proteins. A complimentary approach to identify and clone the genes regulated by the system might be to use subtractive hybridization to select for genes which are expressed at high culture density (or in the presence of the purified quorum-signal) but not at low culture density.

There are several potential ways that the isolated quorum-signal could be used in studying and treating BKD. Since spent culture media promotes rapid growth of Rs in culture, one potential application would be to use the purified quorum-signal molecule or a recombinant peptide as a reliable and reproducible component for culture media. Such a reliable media could be used to isolate and enumerate Rs and could also be used in bulk production of Rs for vaccines under conditions which would ensure that a consistent product was produced.

The purified quorum-signal molecule itself or an understanding of its regulation might provide the basis for new tools to prevent or treat BKD.

Identifying the quorum-signal and the genes involved in quorum regulation would provide a new target for therapeutic approaches to BKD. Quorum sensing systems have been shown to be involved in the induction of virulence factors such as exotoxins in plant pathogens (Liu *et al.*, 1998) and in the important human pathogens *Pseudomonas aeruginosa* (Sawa *et al.*, 1998) and *S. aureus* (Kleerebezem *et al.*, 1997). Peptide signal molecules involved in quorum sensing have been suggested as suitable targets for vaccine production (Balaban *et al.*, 1998). The use of inhibitors or other means to interfere with quorum sensing might also help to prevent disease caused by bacteria that use such a system (Barrett *et al.*, 1998). Any of these potential applications would make it worthwhile to isolate the quorum-signal from Rs.

5.2 Conceptual implications of my results for BKD and other diseases

5.2.1 Quorum sensing systems and their role in disease.

The observation that the timing but not the severity of disease and mortalities in BKD was highly dependent on the dose of Rs points not only toward the presence of a quorum sensing system but also toward an important role of quorum regulation in the biology of the interactions between the host and pathogen in BKD. I didn't set out to study quorum sensing and it would be inappropriate to stray too far from the focus of this thesis. Nevertheless, the observation may be important for the control of BKD and has some

important conceptual implications which bear mentioning before returning to the discussion of possible implications of elevated host SP levels in diseases caused by bacterial infections.

Quorum sensing has been shown to be involved in controlling pathogenesis in both plant and animal pathogens. In the Gram negative plant pathogen *Erwinia carotovora*, enzymes involved in damaging host tissue are expressed when the bacteria has reached a sufficient density (Costa & Loper, 1997). A similar regulation of virulence factors occurs in the Gram positive animal pathogen *S. aureus* (Ji *et al.*, 1997). The selection and retention of such a control systems indicates that there must be a selective pressure on these pathogens not to cause damage to the host tissue all of the time. One explanation is that damage to the host tissue triggers host defenses and that the bacteria may need to reach a critical density before it is able to avoid being eliminated by these host responses (Swift *et al.*, 1996).

Since BKD is caused by a slowly growing pathogen which does not grow in the environment it may be advantageous for the pathogen to regulate its virulence so as to cause pathology in two phases in a quorum-dependent way. One of the important modes of transmission of BKD is via infection of eggs while they are still in the mother. The ability of Rs to grow slowly without killing the host is biologically important because it allows the host an opportunity to reach sexual maturity and transmit the pathogen before dying. At the end, however, production and release of large numbers of Rs favours transmission since the incidence of infection and disease in the progeny increases with high concentrations of bacteria in the mother (Elliott et al., 1995).

The role of quorum sensing in regulating the virulence of several pathogens has lead others to suggest that suppression of the quorum-sensing mechanisms and other regulators of virulence by vaccination or drugs might prevent disease by inhibiting expression of virulence factors. Suppression of virulence by vaccination against a regulator peptide has been shown to protect mice infected with *S. aureus* (Balaban *et al.*, 1998). Vaccination against such a regulator peptide might also protect fish from BKD.

As an alternative approach, induction of bacterial virulence factors at a time during the infection which does not favour the pathogen might be a simple and direct way to treat infections by exploiting quorum sensing systems. Pathogens would not maintain such a system of regulation if there were not some selective pressure favouring control over virulence. The presence of regulation implies that expression of these virulence factors may be detrimental to the pathogen at some stage of the infectious cycle. Mutants which continuously express the virulence factors normally regulated by the quorum-system would probably be less successful as pathogens and therefore good candidates to test as vaccine strains.

For similar reasons, I suspect that stimulating the quorum sensing pathways of such pathogens early in the infectious process might prevent disease. The quorum-signal molecules themselves or drugs designed to stimulate the quorum-sensor could be used to induce premature expression of host virulence factors. This might lead to elimination of the pathogen by host defenses before the infection could cause overt disease. The quorum-signal or an inexpensive analogue could be used as a therapeutic agent. This approach might be feasible especially for slowly developing diseases like BKD and tuberculosis. Now that the entire genome of *Mycobacterium tuberculosis* is known, elucidation and

exploitation of quorum sensing and other autoinducing regulatory systems may be a worthwhile avenue for research on Mycobacteria. Since it would operate by a completely independent pathway, treatment to induce premature expression of virulence might also act synergistically with other therapeutic approaches such as vaccination or antibiotic treatment.

5.2.2 The importance of locally increased levels of host SP in BKD.

In the course of the disease the levels of SP were elevated in host cells adjacent to bacterial microcolonies in the tissue. Although I did not identify any specific mechanism which explains the elevated SP levels, the fact that SP was localized rules out induction by any of the systemic changes in physiology and points toward some local mechanism of induction. The close association of elevated SP to colonies of bacteria also raises the possibility that SP or the localized mechanism that induced it could have an impact on the bacteria and on the pathology of BKD.

From a practical point of view, it is also important to point out that these results suggest the potential for an artifact when using SP levels as an indicator of environmental stress. Since it is clear that HSP-70 was elevated in fish with BKD, researchers who measure SP expression in fish sampled from natural populations should keep in mind the possible effects of infectious disease. It is likely that in regions where BKD is enzootic, surveys of SP expression in wild fish would include some fish with BKD. It is also possible that SP may be induced by other pathogens. Induction of SP in a diseased subset of fish might seriously interfere with the interpretation of such surveys. When differences in disease prevalence could exist between populations being studied, investigators studying SP

expression should be careful to examine the sampled animals for signs of infectious disease as a potential confounding factor.

It is of course possible that the pattern of SP distribution in BKD is merely a consequence of disease pathology in general and of no particular importance. With hindsight some may raise the criticism that host SP would obviously be induced in diseased tissue by inflammation and by the processes leading to tissue necrosis. Clearly, I would not have set out to look for elevated SP in BKD without expecting to find something. However, the highly localized pattern of SP distribution is not an obvious observation. Although I hypothesized that host SP would be associated with sites of infection and inflammation, it was surprising that the association was so strong. This restricted pattern of SP distribution is evidence that a restricted and perhaps even controlled process induced SP at those sites.

The most likely explanation for localized induction of SP is that the local presence of the bacteria triggered SP induction either directly or by causing some local pathological reaction such as inflammation, clotting, tissue necrosis or apoptosis. Although there was no evidence *in vitro* of direct induction by Rs, it is possible that Rs *in vivo* might be able to induce SP directly. This pathogen has been reported to produce a variety of mildly toxic activities (Shieh, 1988; Daly & Stevenson, 1990; Griffiths & Lynch, 1991; Bandin *et al.*, 1992; Grayson *et al.*, 1995a; Grayson *et al.*, 1995b; McIntosh *et al.*, 1997). It may also produce additional toxins *in vivo* since it has been reported to become more virulent when it grows as an intracellular pathogen (McIntosh *et al.*, 1997). Any of these toxins could be potential causes of SP induction.

Whatever its cause, areas with elevated SP levels were clearly associated with the bacteria and may play an important roll in the interactions of the host and pathogen at the site of infection. This elevation of SP appeared to occur in association with relatively small microcolonies and may occur early in the development of such colonies. It might therefore have a greater potential to influence whether the colonies persist and form an established infection. Analogous SP expression might occur in association with bacterial microcolonies in the early stages of other bacterial disease or even in healthy tissue persistently colonized by potential pathogens or commensal bacteria. Such expression might be difficult to observe because most bacterial diseases do not involve such a large build up of bacteria within the tissue of a living animal. However, as I discuss below such local elevation of SP levels could quite reasonably be expected to influence whether intracellular bacterial microcolonies evade eradication or control by the host defenses and thus influence the outcome of bacterial infections.

5.2.3 BKD as a model for diseases of Gram positive and intracellular pathogens. Any of the implications of these findings with BKD may also be of value in understanding other important diseases. There are many important diseases of both humans and farmed animals caused by Gram positive pathogens and some of these (staphylococcal infections in particular) have been difficult to prevent by vaccination or hygiene. In addition to intellectual value, a better understanding of the interactions which allow Gram positive pathogens to live inside host cells would produce new approaches to preventing or treating disease.

BKD is somewhat unusual in that it is caused by Gram positive bacterium which is not a mycobacterium and appears to be a primarily intracellular pathogen. As I mentioned in the Introduction, Rs might be a useful model for the pathogenesis of intracellular infection by Gram positive bacteria in general. Rs causes a slowly progressing disease in which large amounts of bacteria and bacterial protein can build up in the tissue before the fish dies. A better understanding of BKD is likely to uncover mechanisms that might play a part in infections by other Gram positive pathogens which are intracellular at significant stages of the infectious process. Studying BKD is also likely to cast light upon the host pathology associated with this kind of intracellular pathogenesis. It is surprising that there is not more interest in studying BKD given the relative scarcity of good models for Gram positive intracellular bacterial infections and the fact that the horrible disease that Rs causes cannot affect humans.

In recent years there has been an increasing awareness of the importance of intracellular persistence as part of pathogenesis of many important bacterial pathogens. The study of invasion and the intracellular pathogenesis of Gram negative bacteria has revealed elegant mechanisms which are increasing our understanding not only of the pathogens but also of the host cell's pathways which they subvert (Finlay & Cossart, 1997). In general much less is known about the mechanisms important to the intracellular persistence of Gram positive pathogens (Finlay & Falkow, 1997). The Gram positive mycobacteria, and several genera of Gram negative bacteria have been long known to have members which are intracellular pathogens but this persistence within host cells was once thought to be a relatively rare strategy. Intracellular persistence is now known to be an important feature of many kinds of bacterial infections even by bacteria such as Staphylococci which were once

thought to be exclusively extracellular or only accidentally intracellular. My results and future studies on how host cell SP and other factors impact the intracellular survival of Rs may shed light on the interactions which allow other Gram positive pathogens to live inside host cells.

5.2.4 How common is localized SP expression in bacterial infections?

Although it is reasonable to speculate that focal SP expression will occur in some bacterial diseases other than BKD, the proportion of bacterial diseases that are likely to cause focal SP expression will depend on whether or not the mechanism of induction is highly specific to BKD. If a relatively nonspecific mechanism causes SP induction in BKD it is likely that a similar pattern of expression might be very common in other bacterial diseases. For example, SP may have been induced in BKD as a result of the bacteria triggering a host response such as oxidant production from phagocytes or clotting in the local microvasculature. If such a mechanism was the cause then it is very reasonable to expect that microcolonies of most if not all other bacteria would be just as effective as Rs at inducing localized SP expression. Furthermore, even if SP induction in BKD was caused by a toxin released from Rs or from host cells infected with Rs, other Gram positive bacteria and intracellular pathogens might produce similar toxic effects. Given the wide range of stressors which induce SP responses such a toxin would probably not need to be very specific in its effects in order to cause SP induction. Rs might of course have a highly specific toxin or virulence mechanism which it has evolved to induce SP in its host cells. Such a specific toxin would probably not occur in most other bacteria but would be an even more interesting discovery.

It is not clear why there have been few reported studies of host SP expression in bacterial infections. The unusually slow progress of BKD may account for why I observed elevated SP levels while there are few published reports of host SP in the tissue of animals with bacterial infections. There may simply have been a greater opportunity to observe elevated SP levels in fish with BKD. A much longer time can pass and an unusually high level of sepsis can be reached in BKD before the fish dies. This creates a higher percentage of tissue which could be stressed and a longer window of opportunity to observe SP. For this reason induction of detectable levels of SP in whole tissue may be unusual among bacterial diseases other than BKD. Furthermore, apparent elevation of SP levels in severely morbid tissue might be disregarded as being merely an uninformative consequence of the disease pathology. Without a compelling observation of elevated SP levels in whole tissue, researchers may not have had the motivation to look for more subtle patterns of induction such as the focal distribution observed in BKD. If it turns out that localized SP induction occurs in bacterial diseases other than BKD, then useful information might come from looking for localized SP expression at infected sites in the tissue of hosts with other bacterial diseases. Such localized SP expression might have an impact on a variety of diseases caused by bacterial infections.

5.2.5 Potential impacts of host SP on survival of bacteria and host cells.

The most obvious way that locally elevated SP levels could influence bacterial infection is by influencing the survival of the bacteria or of the host cells. However, it is not clear from published information whether host SP would help or harm the bacteria. Some researchers have speculated that SP might protect host cells from damage caused by factors such as oxidant production triggered by the pathogen (Kantengwa *et al.*, 1991). An

elevated level of HSP-70 has also been shown to protect cells from apoptosis (Buzzard *et al.*, 1998). However, protection of host cells from killing might not serve to protect the host against infection. Delayed killing and induction of apoptosis rather than necrosis during staphylococcal infections (Wesson *et al.*, 1998) may be a specific virulence mechanism to allow survival of a larger numbers of bacteria from each infected host cell (Bayles *et al.*, 1998). SP production in such infected cells might only serve to let the cells live longer and permit even more pathogen growth. Conversely, if SP prevented apoptosis in the cells infected with *Staphylococci* the cells might die later by necrosis, a mechanism which may be less favourable to the bacteria since necrosis generally causes more stimulation of the inflammatory response (Hart *et al.*, 1996).

Host SP might also influence the survival of bacteria in less direct ways. For instance host SP might inhibit the infectious process if it serves to present bacterial antigens and stimulate immunity (Suzue & Young, 1996) or if it helps to direct the host's defensive cells to kill or compartmentalize infected cells. More research will be needed to determine whether SP in the host enhances or reduces the survival of intracellular bacteria nearby.

5.2.6 Potential impacts of localized SP on Inflammatory and autoimmune diseases. Another way that elevated host SP levels might influence the outcome of bacterial infections is by stimulating immune responses that could be beneficial or pathological. If SP is induced in host cells associated with microcolonies of bacteria other than Rs it may help explain the mechanisms of induction of diseases such as reactive arthritis (ReA). Various bacterial infections act as triggers to induce the autoimmune cycle which is at the basis of the joint inflammation in reactive arthritis (Gaston, 1997). Tissue damage by the

inflammatory process induces host SP which is thought to stimulate further autoimmune reactivity. It is not yet clear how this process occurs and what roles SP from the host and the pathogen play.

In forms of ReA induced by chlamydial infections there is evidence of persistent chlamydial antigens and DNA in the synovial membranes of the affected joints (Schumacher, 1998). Some researchers believe that a persistent bacterial infection is generally involved in ReA because long term antibiotic therapy has been found to be helpful in treating the disease (Cimmino, 1997). Whatever the ultimate cause, there is considerable evidence of an autoimmune component involving tissue damage caused by T-cells which react with epitopes from HSP-70 and HSP-60.

It is not clear whether the T-cells which cause these sort of inflammatory diseases are specific to host SP, specific to SP from the pathogen or crossreactive to SP from both the host and the pathogen (Schultz & Arnold, 1993; Gaston, 1998). In animal models (e.g. adjuvant arthritis) some T-cell lines which crossreact with both host and pathogen have actually been shown to be protective. Whether particular sets of T-cells cause or prevent disease may be determined by factors in addition to their epitope specificity (Gaston, 1998).

It is reasonable to speculate that the induction of host SP in close proximity to bacterial microcolonies might provide simultaneous stimulation to T-cells by both host and bacterial SP and this could favour the selection of crossreactive T-cells. The combination of antigen stimulation by elevated host SP together with stimulation of the inflammatory system by other bacterial components from the nearby microcolonies creates a special microenvironment for the selection of T-cells which might somehow favour pathological

reactivity. For example, the cytokine profiles of the T-cells at these sites might be skewed toward stimulating inflammation and tissue destruction. In rats with adjuvant-induced arthritis, disease-causing T-cells which react to mycobacterial HSP-60 are stimulated by whole mycobacteria and the other components of Freund's adjuvant. In contrast, immunization with purified recombinant mycobacterial HSP-60 stimulates T-cells which actually prevent disease (see review Gayton 1998). In a similar way, T-cells stimulated by host SP at the sites of bacterial infections might cause disease.

The possibility that immune responses by anti-SP T-cells in autoimmune diseases are provoked or perpetuated by host SP induction at sites of bacterial infection presents a rationale for using treatments which alter host SP expression. If a means could be found to prevent host SP induction at the inflamed sites, this might break the autoimmune cycle. Alternatively, induction of generalized host SP expression (by heat shock) might draw the T-cells away from the affected sites and thereby reduce both the symptoms and stimulation of the T-cells. This reasoning might provide part of the explanation for why hot tub therapy is beneficial in relieving the pain of arthritis. Perhaps earlier intervention in the disease process would allow simple approaches such as a warm bath to prevent the combination of host SP at the site of a persistent bacterial infection from leading to the development of reactive arthritis and similar diseases.

Concluding remarks

In discussing the implications of my observations I have speculated about the possible impacts of localized expression of host SP in bacterial disease. This might be criticized as being overly bold in light of the fact that host SP expression has been reported in very few bacterial diseases. My hope is that by pointing out the potential value of studying host SP expression at sites of infection I may stimulate research in this area. If only one conclusion is taken from this work, it should be that those studying other diseases caused by bacterial infections should look for host SP in association with bacterial microcolonies and consider what impact it has on the course and outcome of those diseases.

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Appendix I. ELISA for HSP-70

In this appendix I present a more complete explanation of the ELISA for HSP-70 in samples of fish tissue. I also present some of the key data from the development process which illustrates the performance and reliability of the ELISA. To ensure clarity I repeat some material presented in the main body of this Thesis. The data and discussion presented in this and the other appendices is intended to illustrate the workings of the techniques and record some conclusions from my experience with the development and use of the techniques. As such, these appendices are intended not to deal with points of scientific interest but rather to provide guidance to those who wish to use the techniques in the future.

1.1 Materials and Methods

Antibodies —— The primary antibody was a polyclonal rabbit antibody raised against rainbow trout HSP-70 as described in the Materials and Methods of Chapter II. The secondary antibody, alkaline phosphatase conjugated goat anti-rabbit IgG (GAR-AP), was from GIBC0-BRL (9815SA). The antibodies were diluted with 2% w/v skim milk powder in TBS-T (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5).

Buffers, reagents and basic method — The method is based on the buffers and reagents recommended by Kemeny (1991). After binding of proteins, ELISA plates and nitrocellulose were blocked for 1 h with 2% w/v skim milk powder in TBS-T and were washed three times with TBS-T between each subsequent assay step.

In this ELISA, the proteins in the sample bind directly to the plastic plate. The HSP-70 in the population of bound proteins is then detected using rabbit antibody against HSP-70 followed by an enzyme labeled secondary antibody against rabbit immunoglobulin. The ELISA is based on the principle of competition for a limited binding capacity. The HSP-70 in the lysates must compete with all of the other proteins present to bind to the plastic. If we assume that the proteins all have the same probability of binding then the amount of HSP-70 which binds will be related to how large a proportion of the total protein is HSP-70. Since HSP-70 is a reasonably abundant protein enough of it binds to yield a measurable signal even for non induced control samples.

For the basic ELISA, the aliquots of lysate were rendered fully soluble by adding an equal volume of 0.1 N NaOH and then diluted to the desired concentration in coating buffer (50 mM sodium carbonate-bicarbonate, pH 9.6). Wells of ELISA plates (Corning 25801 or Dynatech Immulon-4™) were coated with 50 μL of diluted lysate overnight at 4 °C. After blocking, the plates were incubated at room temperature (20 °C) for 1 h with rabbit anti HSP-70 (100 μL/well) and 1 h with GAR-AP (100 μL/well diluted to the concentration suggested by the manufacturer). Various antibody concentrations were used in different optimization experiments. The plates were incubated at room temperature with 100 μL/well of substrate (1 g/L disodium p-nitrophenol-phosphate, 10% w/v diethanolamine, pH 9.5). For most experiments the plates were incubated with substrate until the absorbance of the highest standard was between 0.5 and 1.0. Absorbance was measured at 405 nm with a microplate reader (Molecular Devices THERMO$_{max}$™).

In order to optimize the assay, the conditions were varied to obtain a strong and consistent signal. I performed checker board titration experiments in which increasing concentration of lysate were used to coat sequential wells on the ELISA plate and increasing concentrations of antibody (lower dilutions) were used to detect HSP-70 bound to the various wells. The signal increased with anti HSP-70 antibody concentration up to the highest concentration tested (1/1000 dilution). This concentration was sufficient to produce a reliable, linear response to increased antigen and was used in all subsequent assays.

1.2 Determination of optimum coating conditions

Coating buffer — I explored the optimal conditions for coating ELISA plates by diluting lysates containing HSP-70 in carbonate buffer or in a series of buffers with several components which were varied to give a consistent buffer concentration and different pH values between approximately 3 and 11. The amount of HSP-70 bound was compared by measuring the resulting absorbance in the ELISA. The optimum binding occurred at an alkaline pH around 9.5 and was only slightly greater than binding of HSP-70 of lysates dissolved at a similar pH (9.6) in a simple carbonate bicarbonate buffer. Since the commonly used carbonate-bicarbonate buffer (CB) worked nearly as well as did the more complex buffer, CB was used to dissolve samples for coating ELISA plates in all other experiments.

Coating concentration — The signal also increased rapidly with increasing lysate protein concentration until a plateau was reached as the binding capacity of the plastic was saturated at a protein concentration of between 10 and 30 mg/L in CB (Figure AI-1). This

plateau region is optimal because in this range of coating concentrations the ELISA is sensitive to the amount of HSP-70 in a sample but not to minor variations in the total protein concentration of the samples. For measuring HSP-70 in experimental samples I coated the wells at 30 mg L^{-1}. At this point in the binding curve the slope is very shallow (Figure AI-1). A three fold increase in the protein concentration used to coat the plate had little effect on the signal or the linearity of the standard curve (Figure AI-2).

Figure AI-1. Effects of antibody and antigen concentration on the ELISA for HSP-70. In a typical checker board titration experiment the plates were coated with 0-30 mg/L of protein from positive control (arsenite induced coho liver) lysates dissolved in CB. Each point is the absorbance of a single well with the blank subtracted. For clarity the lower end of the concentration ranges have been omitted.

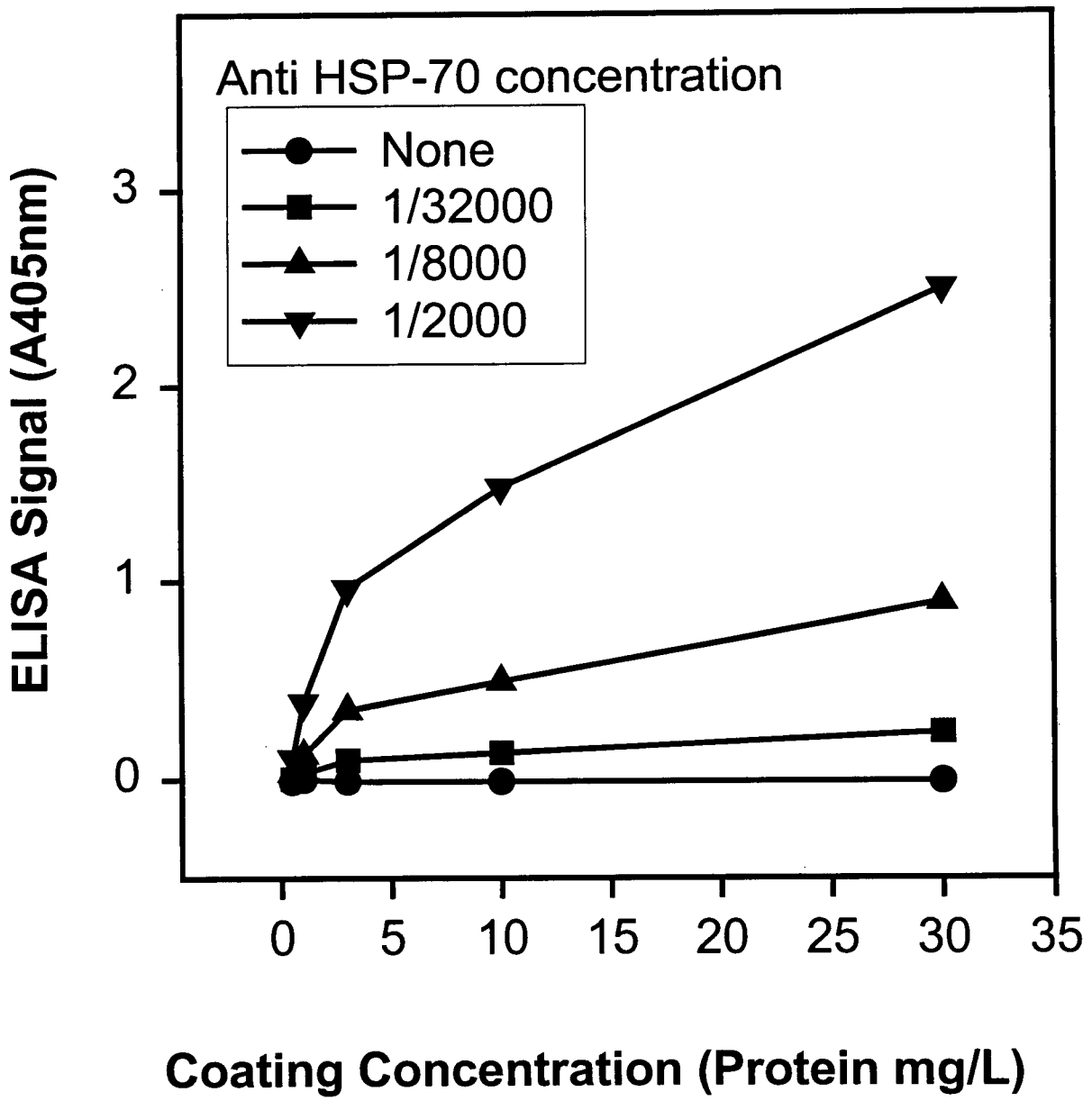
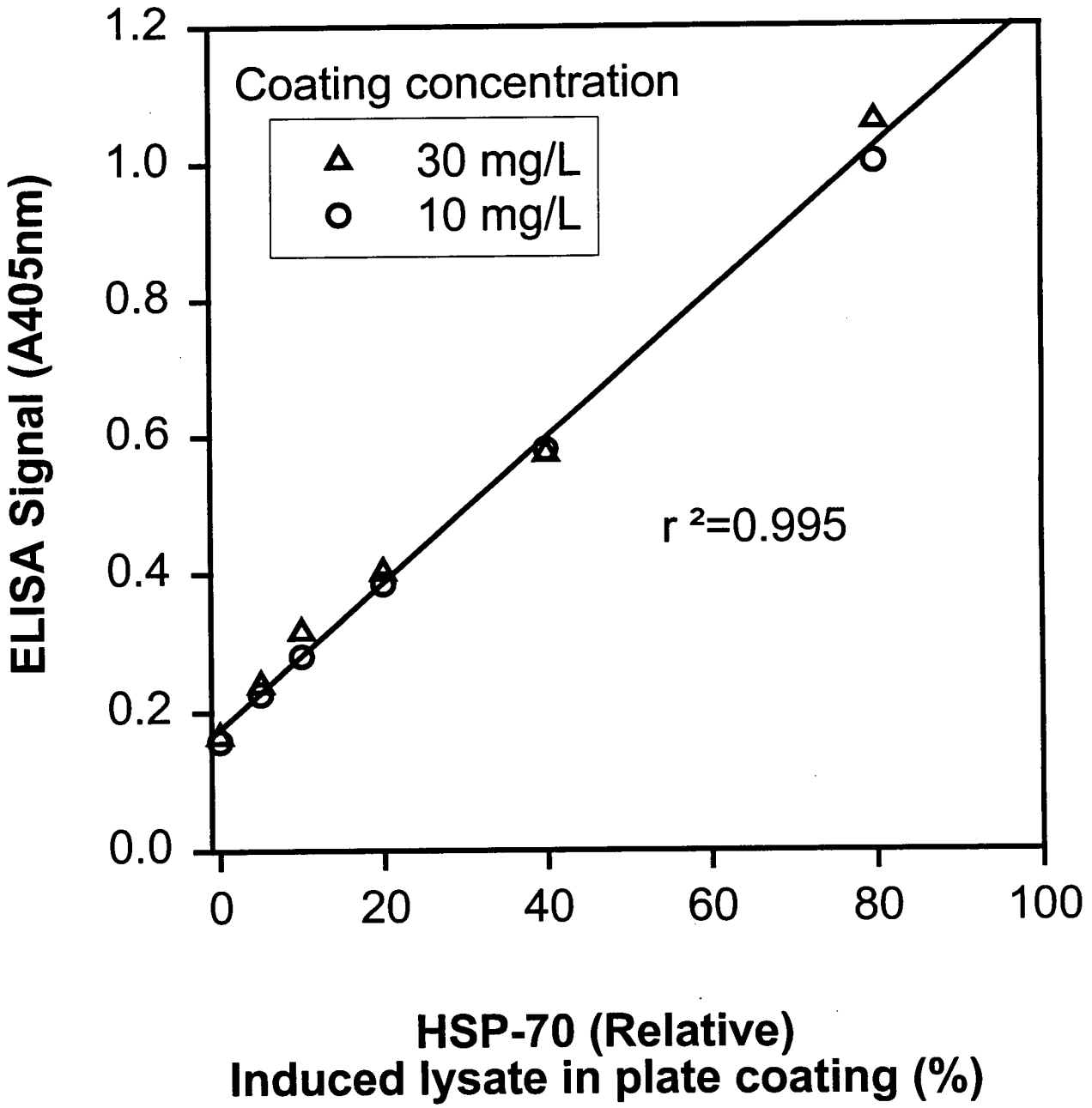

Anti HSP-70 concentration
None
1/32000
1/8000
1/2000
ELISA Signal (A405nm)
0
1
2
3
0
5
10
15
20
25
30
35
Coating Concentration (Protein mg/L)

Figure AI-2. Standard curves for the HSP-70 ELISA with saturating protein concentrations. Blended standards containing different amounts of HSP-70 were used to coat the plates. The blended mixtures contained a constant total protein (either 10 or 30 mg/L) and various proportions of positive control lysate (arsenite induced coho liver) and control lysate in CB. Anti HSP-70 antibody was used at 1/2000. Each point is the absorbance of a single well with the blank subtracted. The line is the regression for the pooled set of data and fits both the 10 mg/L and the 30 mg/L curves well. The response of the ELISA for the two coating concentrations was not significantly different ($P_{\alpha} = 0.0798$ Two Way ANOVA).


1.2
1.0
0.8
0.6
0.4
0.2
0.0
ELISA Signal (A405nm)
Coating concentration
30 mg/L
10 mg/L
r ²=0.995
0
20
40
60
80
100
HSP-70 (Relative)
Induced lysate in plate coating (%)

1.3 Set up, standards, linearity

Standards — I used a set of relative standards for the ELISA which I produced by blending lysate from induced and control (non induced) tissue. The SP content contributed to the blends by each of the starting (induced or control) lysates is a linear function of the SP content of each of the two starting lysates. The sum of two linear systems is also linear. If the assumption of equal competition holds then the signal in the ELISA should be linear with the amount of HSP-70 in the blended lysates. The signal in the ELISA is in fact highly linear (Figure AI-2). The HSP-70 content of samples can be reliably estimated relative to the standards.

The total amounts of protein bound to each well were assumed to be equal and to be representative of the proteins present in the lysate. A standard curve was constructed using a positive control liver lysate containing approximately 20 times more HSP-70 than normal liver. Positive control lysate and normal coho liver lysate were mixed to produce a series of standards containing 30 mg/L protein in coating buffer of which between 0 and 80 % was contributed by the positive control lysate and the balance contributed by the normal control lysate. The standard curve was linear over the assay range (Figure AI-2). The signal contributed by the control lysate content was subtracted from each standard and a linear regression of the standards against the signal produced in the ELISA was used to estimate the HSP-70 content of each sample in relative units as a percentage of the HSP-70 content of the positive control.

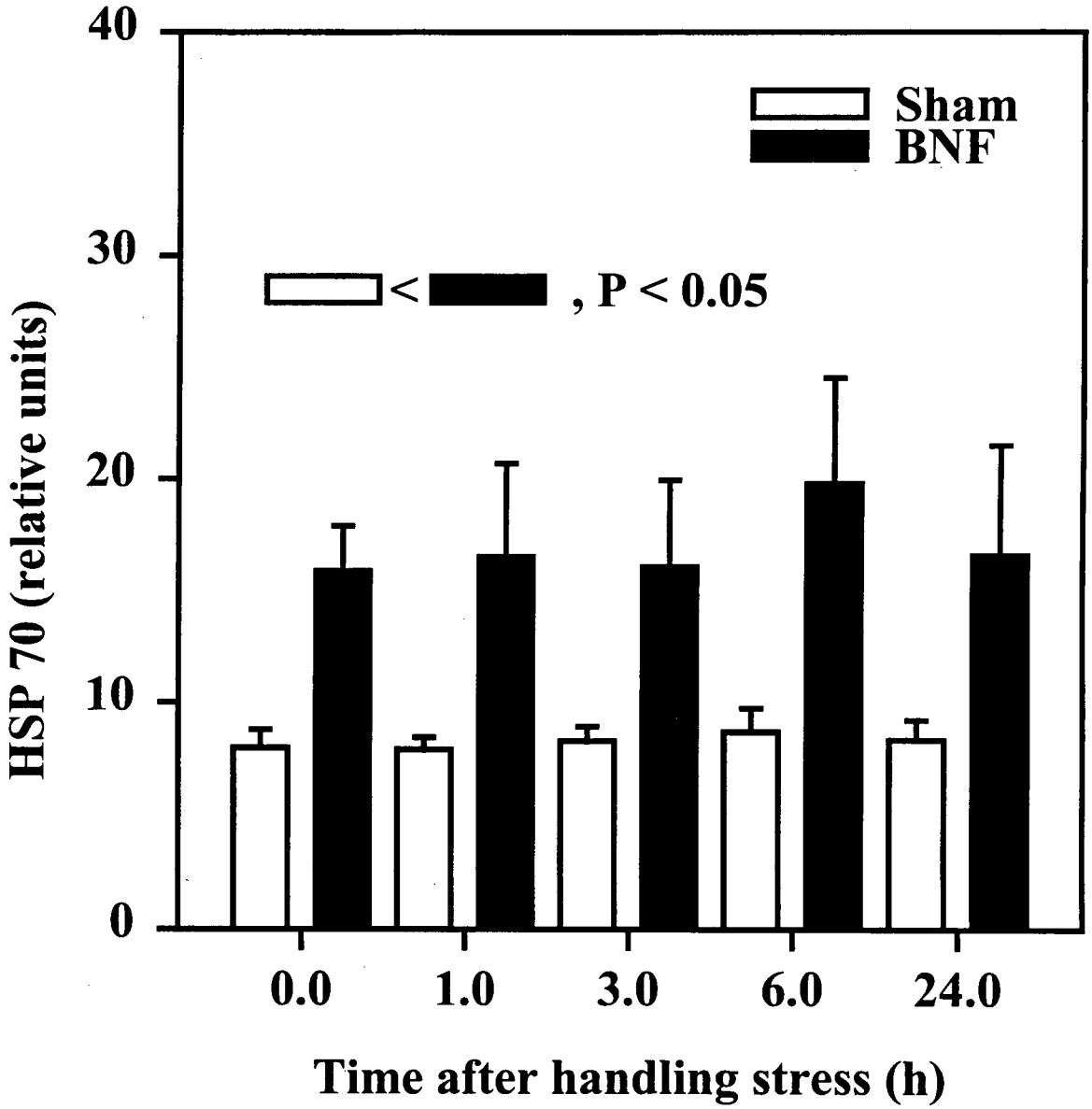
1.4 Plate to plate CV: Reliability in measuring HSP-70

In order to improve the precision of the estimates in the ELISA, I repeated each sample in triplicate with one replicate on each of three plates and then took the mean of the three estimates. This also yielded estimates of the plate to plate variability of the assay. The plate to plate coefficient of variation (CV) was less than 10% for most samples and the average plate to plate CV for a typical large data set (Figure AI-3) was 4.8 ±2.0 % (Mean ±SD).

1.5 Example data: Reliability of ELISA

One of the problems with measurement of proteins by Western blotting is that the measurement method adds a considerable amount of variability to the data. The ELISA on the other hand gives highly repeatable measures of the HSP-70 content in samples. The advantage of this precision is illustrated nicely by some data (Figure AI-3) from another project on which I collaborated (Vijayan *et al.*, 1997). The bars shown are the standard error of the mean for groups of eight to twelve fish. Clearly the HSP-70 levels are quite consistent over time even though the groups are composed of different fish at each time point. The difference between the treatments is clear even though the change is modest. Lack of repeatability (precision) in the measurement of the levels in each fish would have introduced an artificially high variability which could have clouded the interpretation of this data set.

Figure AI-3. Stress protein (HSP-70) in rainbow trout injected with β-Naphthoflavone. ELISA of HSP-70 in the livers from fish injected ip with β-Naphthoflavone (BNF) at 50mg/kg or from sham (vehicle) injected controls. The first sample (time 0) is 3 d after injections. The fish were subjected to a 3 min handling stress immediately after Time 0 to determine if stress would affect the HSP-70 levels. Bars are the standard error of the mean (N=8-12 fish per group). The β-Naphthoflavone treated groups (dark bar) were significantly different from the control group ($P_\alpha < 0.05$) but time after handling had no effect (2 way ANOVA).


40
30
20
10
0
HSP 70 (relative units)
Sham
BNF
< , P < 0.05
0.0
1.0
3.0
6.0
24.0
Time after handling stress (h)

Appendix II. Isolation and purification of fish phagocytes.

2.1 Materials and Methods

Methods for isolating phagocytes — I used rainbow trout head kidney as a source of phagocytes. The initial cell suspension for all of the methods was prepared in essentially the same manner except for the composition of the medium used. At all steps the cell suspensions were held on ice and all centrifugation steps were performed in a refrigerated centrifuge at between 0 and 5 °C. To prepare a cell suspension free of large clumps, the anterior portion of the kidney (head kidney) was removed aseptically, placed in ice cold medium (approximately 5-10 mL per 100 g of fish) over a 40 μM nylon mesh sieve and gently squashed apart with the plastic end of a sterile plunger from a 1 cc tuberculin syringe. The cell suspension passed though the screen and was collected in a sterile plastic Petri plate held on a frozen gel filled ice pack. The suspension was removed from the plate and from the bottom of the sieve. An additional 5 mL of media was then placed in the sieve and collected from the plate to rinse the remaining cell suspension through the mesh. The resulting suspensions contained monocytes (macrophages), granulocytes with polymorph nuclei (PMN), lymphocytes, thrombocytes, immature blast cells and varying numbers of contaminating erythrocytes.

In determining what method to use for isolating the fish phagocytes, I tried three basic methods to remove contaminating erythrocytes and enrich the head kidney cell suspension for phagocytes. All of the methods use Percoll™ (colloidal silica coated with polyvinylpyrrolidone) as a density gradient media. I initially assessed the performance of two continuous density gradient methods. In both methods centrifugation at high speed

causes the Percoll to sediment and form a continuous density gradient. When cells are centrifuged through the density gradient they move to their buoyant densities and collect there. In the method of Garduno and Kay (1994), the density gradient is formed by high speed centrifugation (20,000 g for 15 min) of a mixture containing the cells to be separated along with the density gradient medium. In the method described by Plytycz *et al.* (1989) a continuous density gradient is formed by high speed centrifugation (27,000g for 30 min) and then the cell suspension is layered over this preformed gradient and centrifuged at a lower speed (300g for 30 min). In some replicate tubes, density marker beads (Sigma DMB-10) were included to indicate the shape of the density gradient.

The method that I decided to use for isolation of phagocyte suspensions for the *in vitro* culture experiments was the discontinuous Percoll™ gradient method described by Graham and Secombes (1988). This method is quick and requires minimal equipment. Briefly, Percoll™ (P-1644) was made isotonic and diluted with 10X HBSS and sterile distilled water (Table AII-1 at the end of this Appendix). The pH adjusted to between 7.4 and 7.6 with sterile 1N HCl. Two layers were placed into each tube so as to produce a single step discontinuous gradient with 51% Percoll™ on the bottom and 34% Percoll™ on the top. Head kidney cell suspensions in L-15 containing 2% FBS and 10 IU of heparin (Sigma H-7005) were layered over discontinuous gradients of 34% and 51% Percoll™. The gradients were centrifuged at low speed (400g for 25 min). Most of the overlying medium and debris was aspirated from the top of the tubes and the bands were collected from the 34%/51% interface of each tube. The collected band contained leukocytes (PMN, Macrophage/monocytes, lymphocytes), blast like cells, and some erythrocyte contamination due to streaking of cells on the wall of the tubes. Most of the red cells formed a pellet at

the bottom of the tube. The collected cells were washed twice by diluting the leukocyte suspension with at least five volumes of HBSS and centrifuging 10 min at 400g.

Staining and Morphology — I assessed the cell types present in cell suspensions by examining the sizes and shapes of viable cells using Trypan blue in a haemocytometer chamber and by examining the overall morphology on hand smeared slides stained with Diff-Quick and with Sudan black (Plytycz *et al.* 1989). The lymphocytes and thrombocytes are about 1/2 to 2/3 the size of the PMN and monocytes. The PMN have lobed nuclei and granules which stain with Sudan black. The monocytes have round or kidney shaped nuclei. The blast like cells have dense staining in the cytoplasm and large, round, but often indistinct nuclei. It is often hard to tell the difference between a blast and a poorly stained monocyte. To prepare slides, the cells were pelleted in 0.5 mL microcentrifuge tubes for 1 min at 6500 rpm and resuspended in a 10-20 μL of FBS. One 5 μL drop was placed onto a microscope slide and smeared using a second slide as a spreader in the same way as for preparing blood smears. The slide was air dried and either stained immediately or wrapped in aluminum foil and stored frozen for subsequent staining.

Superoxide assay — Superoxide generated by phagocytes was measured by reduction of ferricytochrome C. In this assay superoxide acts as a reducing agent and converts the oxidized form of cytochrome C to the reduced form. Reduced cytochrome C has an absorbance peak at around 550 nm and a local absorbance minimum at approximately 530 nm. Subtracting the absorbance of the sample at the wavelength of this local minimum provides an internal blank to remove interference caused by turbidity or other sources of variation in the absorbance of the assay mixture. I used a spectrophotometer with a

scanning mode (Shimadzu UV160U) to locate the actual absorbance maximum and minimum of the completed assays. I then measured the absorbance at the same wave lengths in the blank assay which included superoxide dismutase.

For the routine assay, phagocytes were added in triplicate to the assay mixture at a final concentration of 2 X 10^6 cells/mL, 1.24 mg/mL ferricytochrome C (Sigma C2506), 500 units/mL catalase (Sigma C-10) and 50 ng/mL (except where stated otherwise) phorbol 12-myristate 13-acetate (PMA Sigma P-8139) in HBSS-Gel. Assay blanks were created by including superoxide dismutase (SOD Sigma S2515). For some assays the incubation time and temperatures were varied so as to determine the effects of these parameters. In all other cases, the assays were incubated for 30 min at the ambient temperature from which the fish were taken. After incubation the assays were chilled on ice and centrifuged at 13000 rpm for 30 sec in a microcentrifuge. The assay supernatents were diluted 1/5 and the absorbance was measured in a scanning spectrophotometer. The amount of superoxide (nmoles per 10^6 cells) generated in the assay was calculated as the dilution factor X 0.021 X the difference between the maximum absorbance at approximately 550 nm and the minimum absorbance at approximately 530 nm. The blank value was subtracted and was always near zero. The following protocol sheet (Table AII-2) is a step by step guide to setting up the assay conveniently.

Table AII-1. Protocol for discontinuous Percoll™ gradients (Graham & Secombes, 1988).

1. To prepare the isotonic density gradient media mix the components shown in the table below in sterile 50 mL tissue culture tubes or small glass bottles.

   HINT: Do not insert pipettes the into main Percoll stock bottle. Decant the amount needed into sterile container and pipette from that container.

   To minimize waste of pipettes add Percoll to both batches with a single sterile pipette, rinse out the pipette with sterile distilled water and use the same pipette to add water to both batches and to distribute the final mix into tubes. (Make sure that no residual water clings to the inside of the pipette.)

2. To prevent a cloudy precipitate from forming on contact with the acid, add the required volume of HCl in a drop on the inside of the tube cap and mix quickly by inverting the sealed tube. If phenol red is present in the HBSS the color will shift from a purple/red to an orange/red
3. Add 3.3 ml of 51 % into the bottoms of 15 mL round bottomed polypropylene tubes.
4. Mark the position of the interface with a fine tipped permanent felt marker.
5. Gently layer 4.8 mL of 34 % over the 51 % (allow some mixing at interface).
6. Store at 4 C until ready to use.
7. Check the pH of leftover gradient media (should be between 7.4 and 7.5)
8. For use, layer a cell suspension (4 to 5 mL) over the 34 % allowing some mixing to occur and centrifuge (400g for 25 min at 0 to 4 °C).

	34 % (10 mL)	34 % (30 mL)	51% (20 mL)	51% (30 mL)
Percoll (stock)	3.4 mL	10.2 mL	10.2 mL	15.3 mL
Hanks BSS (10x)	1.0 mL	3.0 mL	2.0 mL	3.0 mL
STERILE dH_20	5.6 mL	16.8 mL	7.8 mL	11.7 mL
1N HCl add to cap	0.010 mL	0.030 mL	0.020 mL	0.030 mL

Table AII-2. Superoxide assay protocol

(WEAR GLOVES: PMA IS A TUMOUR PROMOTER SEE MSDS)

Thaw and hold all reagents on ice during preparation.

1. Prepare reaction **Mix** as follows (enough for six assay sets of four tubes)

   3.43 mL medium (HBSS 0.1% Gelatin w/o phenol red)

   70 µL catalase stock

   0.70 mL ferricytochrome C and mix

   Just before adding to tubes with cells add

   0.70 mL PMA at 1ug/mL in HBSS-G and mix by inverting

2. Set up 0.5 mL tubes with cell suspension 50 µL at 5X 10^6 cells/mL as follows using a small blue pipette tip rack to hold the tubes (in an ice water bath).

Tube	Cells	Media	SOD	Mix
1	50 µL (source#1)	25 µL	-	175 µL
2	"	"	-	"
3	"	"	-	"
4	"	-	25 µL	"
5	50 µL (source#2)	25 µL	-	175 µL
6	"	"	-	"
7	"	"	-	"
8	"	-	25 µL	"
9	50 µL (source#3)	Et cetera		

3. Incubate at appropriate temperature i.e. 15 C or 20 C for 20 min or ambient temp for 30 min with gentle agitation (i.e. rocking on Nutator platform with rack placed on its side)

4. Remove from incubator and chill rack of tubes in ice /water slush bath.

5. Spin down each tube in sequence 30 sec at high speed in microcentrifuge.

6. Immediately remove 200 μL sample from each, dilute (5 fold) with 800 μL media or dH_2O and read scan from 560nm (559.8) to 530 nm. Use Shimadzu in spectrum mode. For positive samples use peak pick. For weak or negative samples print at 5 nm intervals and determine the absorbance at the peak and the trough.

7. Calculate the ferricytochrome C conversion in nmoles mL^{-1} which with 10^6 cells mL^{-1} in the final reaction mixture is also nmoles produced per 10^6 cells. ((Peak -Trough) / 0.021) X 5. Remember to use correct factor for dilution of sample if a dilution other than five fold is used in step 6. A conversion rate can also be calculated but is dependent on the assumption that the rate is constant over the incubation period.

Note: i) Red cells are a source of SOD and so red cell contamination could lead to abnormally low values due to dismutation.

ii) Background color change (in the presence of SOD) can be subtracted if there is a difference between the "peak" around 550 nm and the "trough". The traces with SOD are typically flat.

2.2 Comparison of three techniques: Clumping, separation, purity, speed.

Since there are several methods reported in the literature for isolation of fish phagocytes. I compared three of these methods in order to find the most suitable method. I initially tried the continuous density gradient method of Garduno and Kay (1994) because it was purported to produce highly purified preparations of monocytes. This method lead to extensive clumping of cells and poor separation. In addition I was concerned that this method subjected the cells to the extreme acceleration forces involved in generating the density gradient.

The continuous density gradient method described by Plytycz *et al.* (1989) was much superior to the method of Garduno and Kay (1994). The Plytycz *et al.* (1989) method does not subject the cells to extreme forces and effectively separated the leukocytes into a phagocyte rich band and a band rich in small (lymphocyte like) leukocytes.

The discontinuous gradient method of Graham and Secombes (1988) efficiently separated the leukocytes (a mixture of phagocytes, lymphocyte like small leukocyte and blast like cells) from the contaminating red cells. Although it did not separate the lymphocytes from the phagocyte suspension, the discontinuous gradient method recovered a high proportion of the leukocytes from the initial head kidney suspension and was faster than the two continuous gradient methods. Speed was the deciding factor for me since I was concerned about the possibility of inadvertently inducing a SP response during the isolation procedure.

The discontinuous gradient method generates a single sharp leukocyte band at the interface between the two Percoll™ concentrations. The banding patterns are more complex in the continuous gradient methods. Figure AII-1 below illustrates the banding pattern obtained

with the continuous Percoll™ gradient methods. The cell types indicated fall mostly in the major visible bands but seem to occur as contaminants over the larger area shown by the vertical bars.

Except when clumping occurred, the continuous gradient methods yielded relatively pure phagocyte suspensions which contained neutrophils (PMN), mononuclear phagocytes and blast like cells. In suspensions produced by the Plytycz *et al.* (1989) continuous gradient method, there was usually no detectable contamination with red cells (less than 0.5%) and typically less than 5 % lymphocyte like cells.

The suspensions yielded by the discontinuous gradient method were similar to the suspensions from the continuous gradients except that lymphocytes were not separated from the other leukocytes. Approximately 1/3 of the cells in these suspension were small leukocytes which appeared to be lymphocytes.

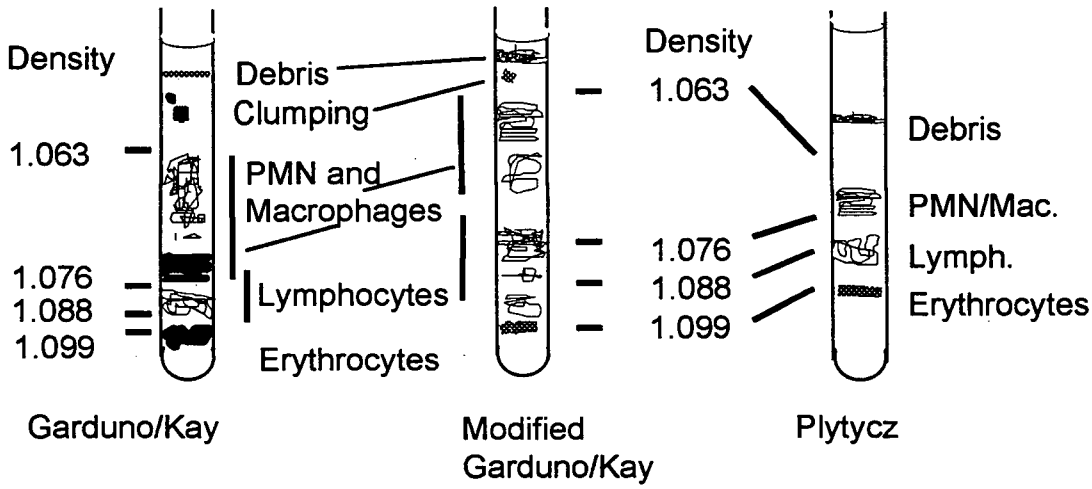


Figure AII-1. Separation of rainbow trout leukocytes on continuous Percoll™ gradients. The drawing indicates typical banding patterns in centrifuge tubes containing head kidney leukocytes separated using three types of continuous gradients. The tube on the right illustrates the pattern for gradients prepared according to the method of Plytycz et al. (1989). The other two tubes illustrate the patterns yielded by gradients prepared according to the method of Garduno and Kay (1994; left) or a slight modification of that method (centre) using an initial Percoll™ concentration of 53%(v/v). The density values given refer to the final positions of density marker beads in replicate tubes as indicated by the tube ward ends of the associated horizontal or angled lines. The labeled vertical bars delineate the areas in which the indicated cell types were typically found.

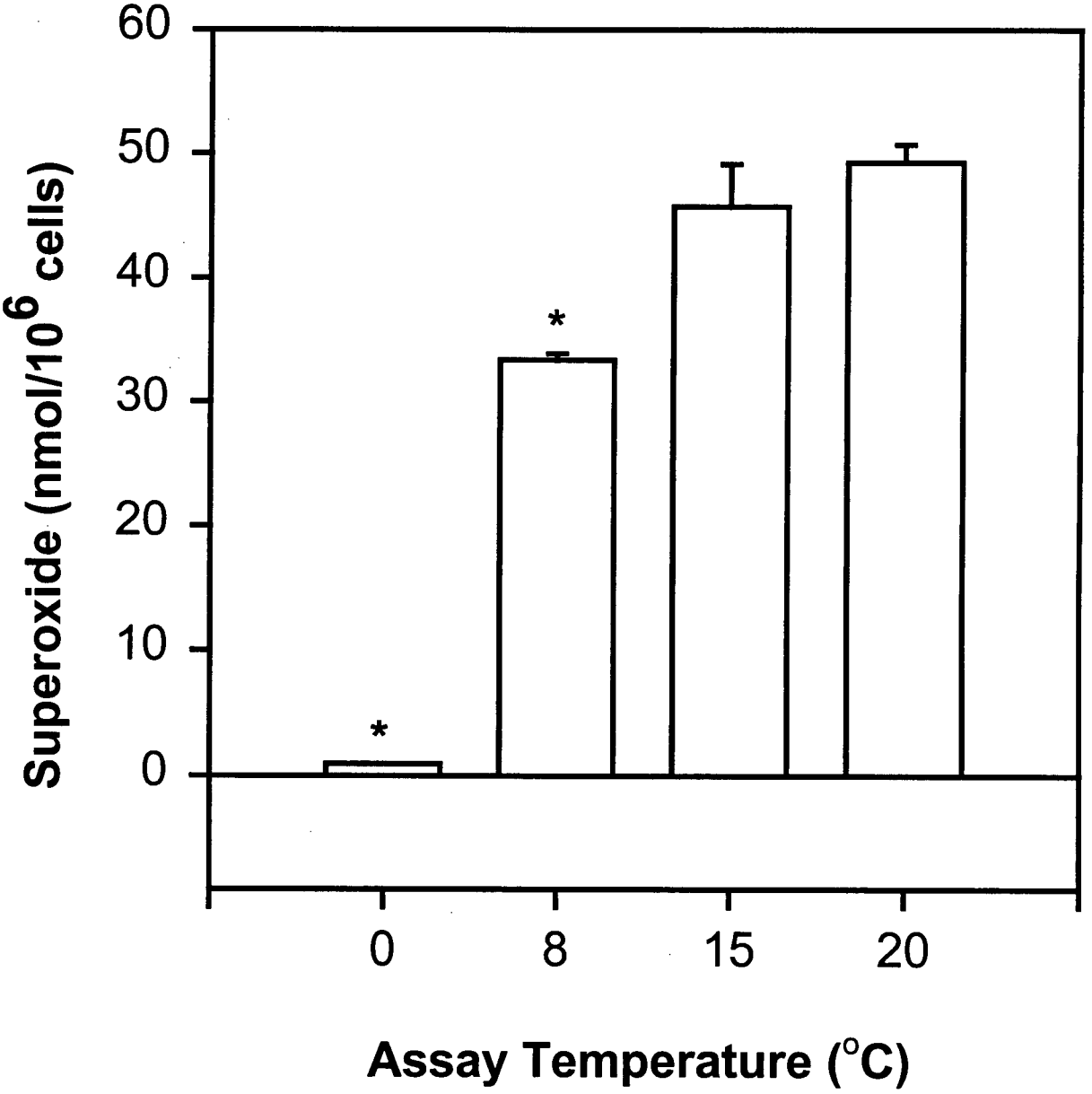
2.3 Oxidant production by fish phagocytes: temperature and stimulation.

The amount of superoxide generated by fish phagocytes was dependent on the temperature at which the cultures were held. When stimulated with PMA, phagocyte suspensions generated the most superoxide at 20 °C (the highest temperature tested) and essentially the same amount at 15 °C. Superoxide production was significantly lower at 8 °C and almost absent at 0 °C (Figure AII-2). This temperature dependence is important because the *in vitro* phagocyte culture experiments were conducted at ambient temperature so as to avoid a heat stress to the isolated cells. In a typical experiment (see Figure AII-3) with isolated trout phagocytes at the lowest ambient water temperature used for the phagocyte culture experiments (5.8 °C), superoxide was generated at a rate of 1.2 nmols superoxide $min^{-1}/10^6$ PMN which is about 1/5 of the production observed at the optimal temperature (see below).

The phorbol ester PMA is a convenient and effective stimulant to trigger oxidant production by fish phagocytes. The dose of PMA used to stimulate the phagocytes did not strongly influence the amount of superoxide produced. All of the doses of PMA that I tested stimulated vigorous superoxide production. The lowest dose of PMA (10 ng/mL) was nearly as effective at stimulating superoxide production as the highest dose (Figure AII-3). When maximally stimulated, rainbow trout phagocyte suspensions isolated by the method of Plytycz et al. (1989) produced approximately 50 nmoles of superoxide per million cells in 20 min at 15 or 20 °C. Since these suspensions contained approximately 40 % PMN, this level of production corresponds to a rate of 6 nmols superoxide $min^{-1}/10^6$ PMN in the suspension. This is similar to the production rate reported for trout neutrophils

purified using a monoclonal antibody (4.85 nmols superoxide $min^{-1}/10^6$ cells ; Hamdani et al. 1998).

Figure AII-2. Temperature dependence of superoxide production by trout phagocytes. A head kidney phagocyte suspension was isolated by the method of Plytycz et al. (1989) from a fish maintained in 8 °C water. Replicate assays were incubated at various temperatures for 20 min. Groups marked with an asterisk are significantly different from the 20 °C group ($P_{\alpha} < 0.05$; ANOVA on Ranks). The error bar is one SD (N=3 replicate cultures per group).


60
50
40
30
20
10
0
Superoxide (nmol/10^6 cells)
*
*
0
8
15
20
Assay Temperature (°C)

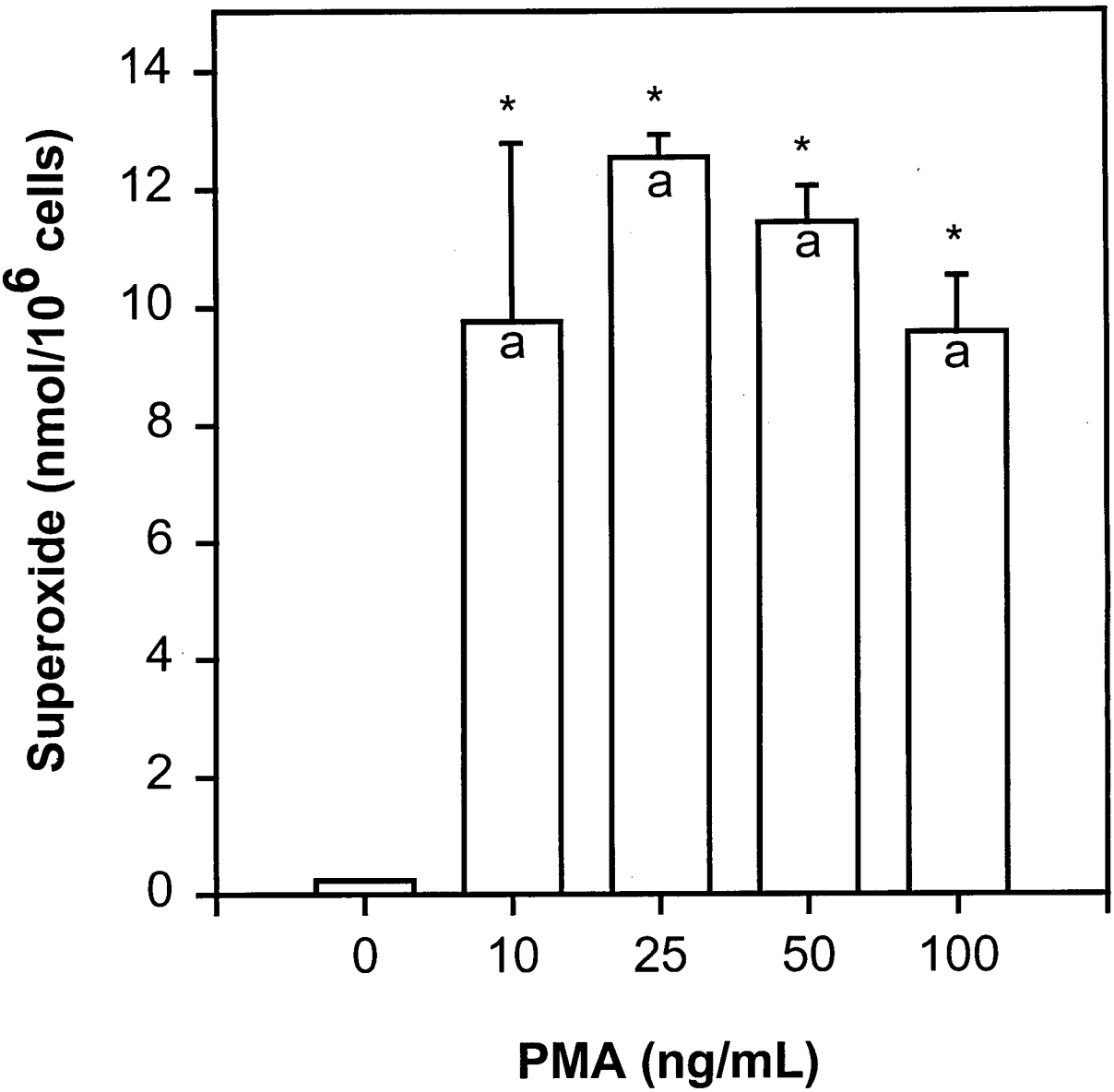


Figure AII-3. Stimulation of superoxide production in phagocytes treated with PMA. Assay was incubated at 5.8°C for 30 min. Groups marked with an asterisk are significantly different from the unstimulated group (0 ng/mL) ANOVA on Ranks ($P_\alpha < 0.05$). Groups with the same letter are not significantly different from each other (ANOVA). The error bar is one SD (N=3 replicate cultures per group).

Appendix III. Hepatocyte culture

3.1 Materials and Methods

Hepatocyte isolation and culture — The hepatocytes were isolated by *in situ* perfusion with collagenase (Mommsen *et al.* , 1994). The solvent in all steps of the perfusion procedure was sterile modified Hanks saline with HEPES (HS-H: 136.9 mM NaCl; 5.4 mM KCl; 0.8 mM $MgSO_4$; 0.33 mM Na_2HPO_4; 5 mM $NaHCO_3$; 5 mM HEPES; 5 mM HEPES monosodium salt; 3 mM glucose; pH 7.63 at 22 °C). All steps were conducted on ice and all solutions were held on ice during the procedure. Rainbow trout (400-1000 g) were rapidly killed using an overdose of buffered TMS (200 mg/L TMS and 400 mg/L $NaHCO_3$) in aerated water which was chilled by adding a small amount of ice so that the temperature remained below the ambient water temperature. The outside of the fish was flushed with cold running tap water at the ambient temperature and then dried with clean paper towels, swabbed with 70 % v/v ethanol and placed right side down on a bed of crushed ice. The abdominal cavity was opened by aseptically cutting a flap out of the left hand abdominal wall of the fish without nicking the liver. The hepatic portal vein was exposed by moving the liver slightly to one side and pulling the intestine gently away from the liver to expose the mesenteric vessels. With the peristaltic pump set to the lowest setting (less than 0.5 mL of HS-H /min), the hepatic portal was nicked using ophthalmologic scissors and the cannula was immediately inserted into the vein at a distance of 2-3 cm from the liver and advanced until the tip passed the last junction of veins near the liver. The vessels at the junction were compressed around the cannula with a stainless steel bulldog clamp. Any other veins allowing back flow of saline were also clamped (back flow occurs if one of the

veins joins the main portal vein inside the liver or too close to the liver to allow successfully clamping). The heart was then excised to prevent pressure buildup and the flow rate of HS-H was increased to the maximum setting (2.5 mL/min). The liver immediately went pale as the blood was flushed from the liver. This flushing was continued for 5 min. The outside of the liver was kept moist throughout the perfusion procedure by irrigating it every few minutes with 1 to 2 mL of ice cold, sterile HS-H. Forceps were used to clear blood clots out of the pericardium so that the waste saline could flow away freely. Excess saline and ice melt water was periodically removed from the surgical tray with a suction connected to a water driven aspirator. After flushing the blood from the liver the pump was stopped briefly and the perfusate was changed to collagenase (Sigma type IV: 175 to 300 mg/L in HS-H) at 1.5 mL/min. The liver was gently massaged for about 5 min with a finger tip (in sterile latex gloves) at least two times in the first 20 min of collagenase perfusion. The liver was perfused for 25 to 30 min until the tissue began to separate inside the capsule. The liver was then carefully dissected away from the other viscera leaving the intact gallbladder behind. The liver was aseptically chopped with razor blade on a watch glass and the hepatocyte suspension was rinsed with HS-H through a nylon mesh filter. The resulting hepatocyte suspension was washed by centrifuging and resuspending twice with HS-H; once with HS-H containing 1.5 mM $CaCl_2$ and 1% Bovine Serum Albumin; and twice with L-15 essentially as described by Mommsen *et al.* (1994).

For long term culture, hepatocytes were maintained in L-15-HB (Leibovitz's L-15 (GibcoBRL 41300-021) with antibiotic/antimycotic supplement (Sigma A-7292 100X stock penicillin 10,000 IU/mL, streptomycin 10 mg/mL and fungizone 25 μg/ml); 2 mM HEPES; 5 mM $NaHCO_3$; ; pH 7.6). In some experiments at 15° C, 10% heat inactivated FBS

(GibcoBRL 16140-071) was also added to the medium. Hepatocytes were resuspended to 10^6 cells/mL in L-15-HB and 0.5 mL was added to the wells of 24 well Primaria™ cluster well tissue culture plates (Falcon 3847, Becton and Dickinson). The plates were chilled on ice while dispensing the ice cold cell suspension and incubated at 4° C in sealed chambers with 0.25% to 0.5% CO_2 in air.

The culture medium contained a small amount of $NaHCO_3$ (5 mM) which is appropriate for trout hepatocytes (Mommsen and Hochachka, 1994). If the cultures were incubated in air the pH of the media would rise due to loss of bicarbonate as CO_2 gas. After 12 h of equilibration the pH of media held in unsealed culture dishes in the incubator rose to pH 8.1 (measured at 20° C). To prevent the loss of CO_2 from the medium, I held the cultures in sealed boxes with atmospheres of between 0.25% and 0.5% v/v CO_2 in air. I generated the CO_2 by placing a loosely capped tube containing 0.66 mL of 7.5% w/v $NaHCO_3$ and 6 mL of 0.1 N HCl into each 2.5 L sealed plastic culture chamber. Low levels of CO_2 in this concentration range are physiologically appropriate for trout cells (Mommsen and Hochachka, 1994). Medium removed from hepatocytes held in these chambers for various lengths of time at 4°C, ranged in pH between pH 7.48 and 7.65 (measured at 20° C).

3.2 Primaria™ culture wells and adherence

The strength of adherence of hepatocytes in culture varied with the incubation conditions and was best when using tissue culture cluster wells with specially treated plastic surfaces to enhance binding (Falcon Primaria™, Becton Dickinson). I assessed adherence when I changed the media and when I harvested the hepatocytes. Hepatocytes that were adherent remained on the bottom of the wells when fresh medium was gently added to the wells.

Cells that were not adherent lifted off of the bottom of the plate as a result of the gentle swirling that occurred when the medium was replaced or when the plates were moved. While changing the medium strong sheer forces or ice cold media might dislodge adherent cells from the plastic. For this reason I warmed fresh medium to the culture temperature and directed the stream against the sides of the wells to reduce the shear forces on the cells.

Although at higher temperatures hepatocytes will adhere to the plastic in regular cluster wells (Falcon 3047, Becton Dickinson), they did not adhere to this plastic at 4°C. The hepatocytes cultured at 4°C did adhere to Primaria™ cluster wells. Hepatocyte cultures maintained at 4° C in Primaria™ cluster wells were attached strongly enough that the medium could be changed without dislodging them. However, they could still be removed without a cell scraper even after 10 d in culture. Hepatocyte cultures could be conveniently harvested by gently flushing them off of the surface with a direct stream of medium from a 1 mL automatic pipetter.

3.3 Prevention of heat shock during isolation and culture.

Cultures of isolated hepatocytes can be used to study biochemical and physiological responses under controlled conditions (Mommsen *et al.* , 1994). Their use for studying SP expression however requires some caution to maintain suitable conditions so that a SP response is not induced by the basic culture conditions. Obviously, the culture temperature is one of the conditions which must be carefully maintained. However, it must be recognized that a heat shock should also be avoided during the isolation procedure.

Although I used them without testing their importance, I suggest the following precautions to avoid heat shock during the isolation procedure. The fish should be anaesthetized in water at or below the rearing temperature. A practical way of accomplishing this is by preparing the anaesthetic bath from the rearing water and placing a small amount of ice into the bath so that it will not warm up to room temperature as it sits in air. The running water which is used to rinse the fish should be also be the same temperature as the rearing water. The fish should be placed on top of a sealed plastic bag filled with water and crushed ice. The perfusion media and the hepatocyte suspension should be held on ice at all stages and the saline which is used to irrigate the outside of the liver capsule should also be held on ice.

3.4 Culture temperature

Previously, it has been shown that trout hepatocytes can be cultured at 15°C and that this leads to strongly adherent healthy hepatocyte cultures. These cultures initially under go a HS response as a result of the stress of being isolated and incubated at a temperature higher than that of the water in which the fish were reared. After recovering in culture for 3 d these cells have a higher baseline level of HSP-70 but are capable of a further induction of SP expression in response to chemical stressors or additional heat stress (Vijayan, M.M. personal communications; Iwama *et al.*, 1998).

I found that a HS response occurred in the isolated hepatocytes not only if they were cultured at 15°C but even if they were cultured at the ambient temperature (7.5 °C) of the water in the tanks from which the donor fish were obtained (Figure AIII-1). In order to

avoid interference from this increased baseline of expression and to avoid exposing the phagocytes to a heat shock I chose to culture the cells at 4°C. It is not clear why there should be an induction at the temperature (7.5 ° C) from which the fish were obtained but not at a temperature a few degrees lower.

Whatever the reason for the beneficial effect of culture at 4 °C, hepatocytes cultured at 4°C for 3 d are adherent if the appropriate plastic substrate is used and had no significant increase in HSP-70 nor loss of LDH relative to the original suspension (Figures AIII-1 and AIII-2). Hepatocytes cultured at 4° C seem to have remained healthy for as long as 10 d after isolation since they maintained good cellular integrity (retention of LdH; Figure AIII-2) as well as a relatively low baseline of HSP-70 expression and the ability to respond to a even a modest heat shock at 7.5 °C (Figure AIII-1). When hepatocytes held at 4°C were shifted to 15°C the HSP-70 levels increased up to 10 fold (Figure 4-8). This dramatic increase in SP reflects the low baseline SP levels which existed in the hepatocyte cultures relative to cultures held at higher temperatures.

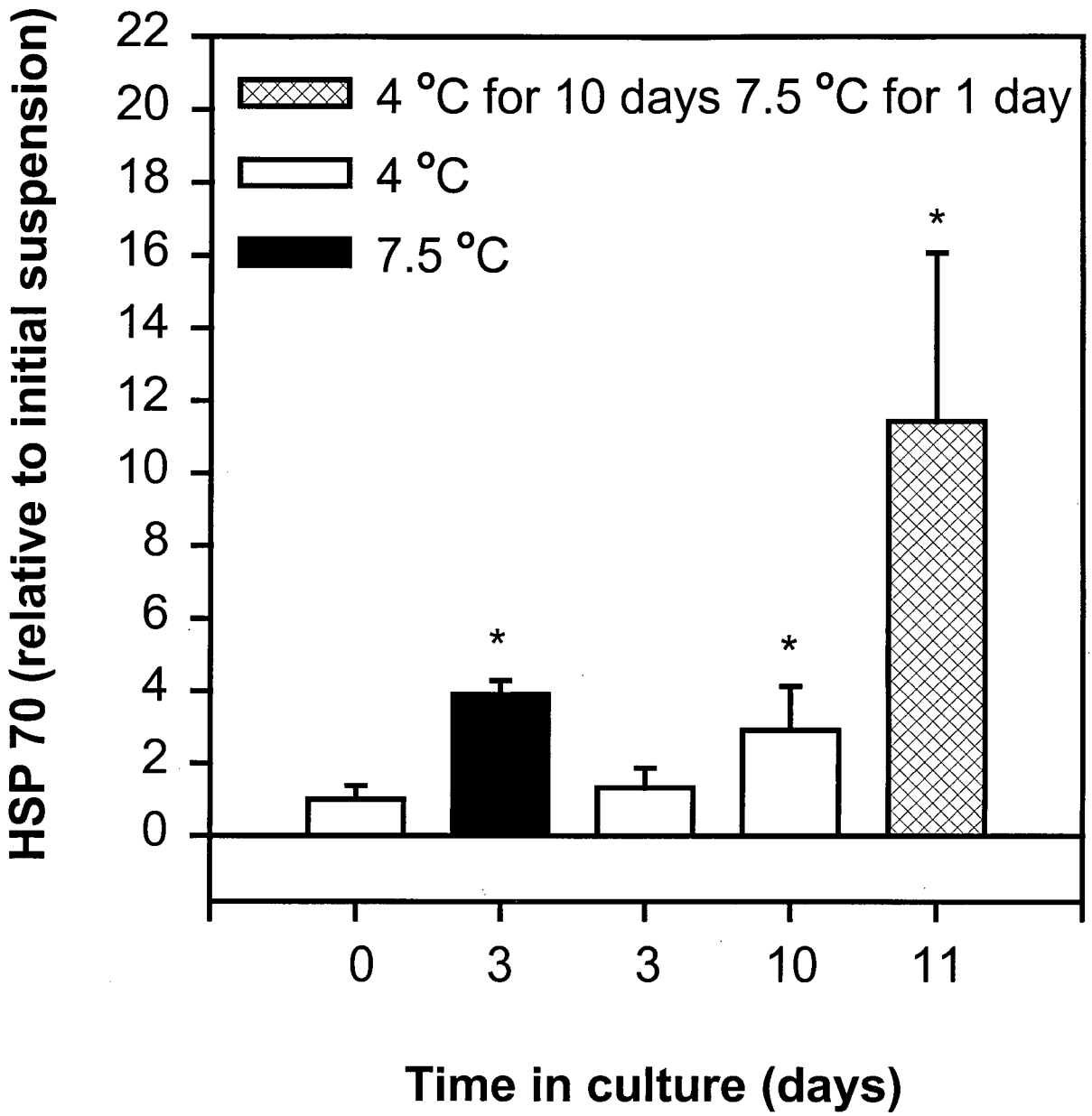


Figure AIII-1. Heat shock response of hepatocytes cultured at ambient temperature. Rainbow trout hepatocytes were held in culture at 4 °C (light bar) or at 7.5 °C (dark bar) or held at 4 °C for 10 d and then shifted to 7.5 ° C for 1 d (hatched bar). The day 0 group is the original suspension of hepatocytes. Groups marked with an asterisk are significantly different from day 0 (ANOVA; The hatched bar group is different by ANOVA on Ranks; $P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).

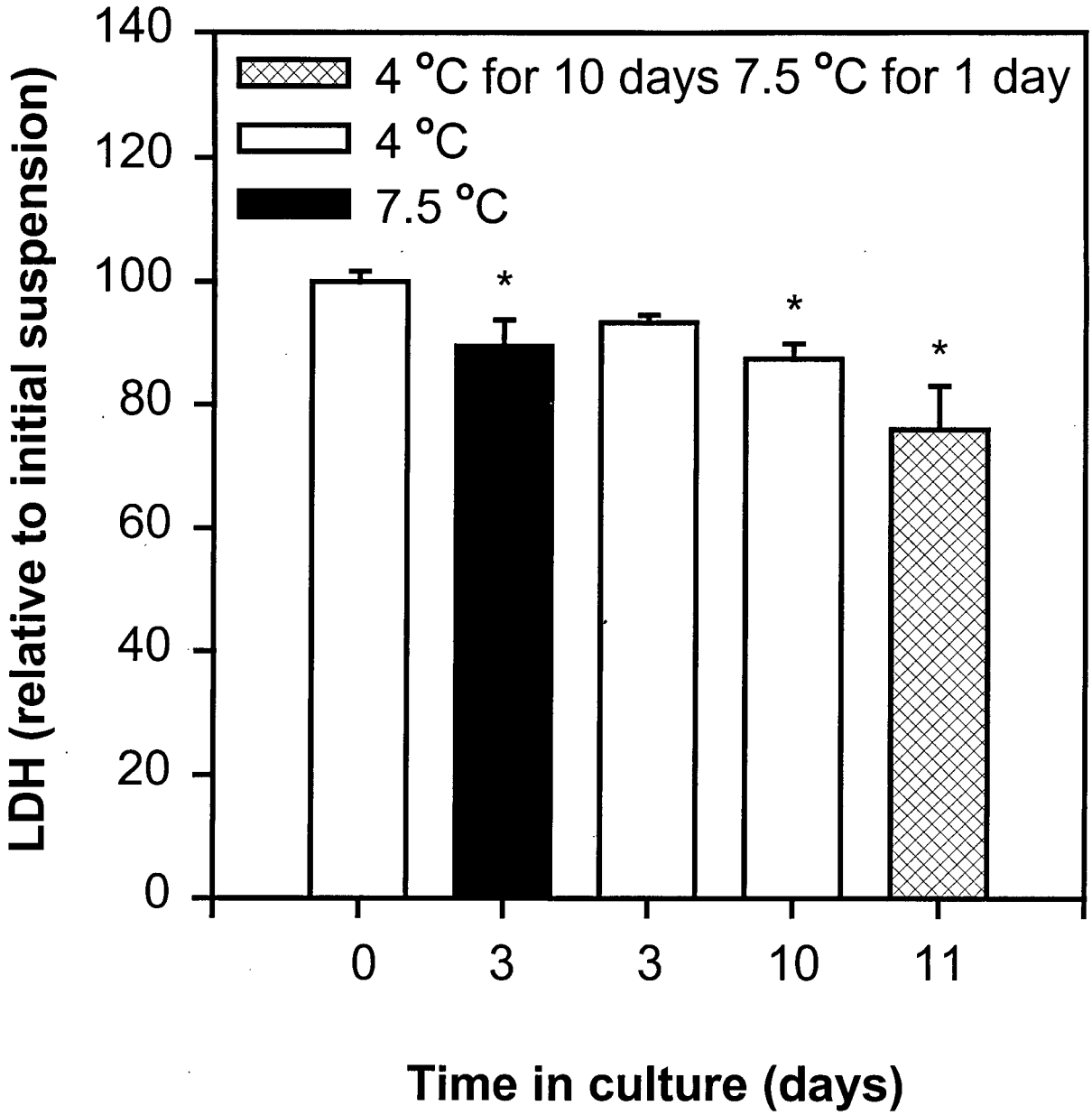


Figure AIII-2. Retention of cytoplasmic contents by hepatocytes in culture. Rainbow trout hepatocytes were held in culture at 4 °C (light bars) or at 7.5 °C (dark bar) or held at 4 °C for 10 d and then shifted to 7.5 ° C for 1 d (hatched bar). The data is retention of LDH in the hepatocyte cultures for which HSP-70 is shown in Figure AIII-1. The day 0 group is the original suspension of hepatocytes. Groups marked with an asterisk are significantly different from day 0 (ANOVA; $P_{\alpha} < 0.05$). The error bar is one SD (N=3 replicate cultures per group).