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The Dynamics and Effects of Bacterial Kidney Disease in Snake River Spring Chinook Salmon (*Oncorhynchus tshawytscha*)

by

Owen Sprague Hamel

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2001

Program Authorized to Offer Degree: Quantitative Ecology and Resource Management

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Graduate School

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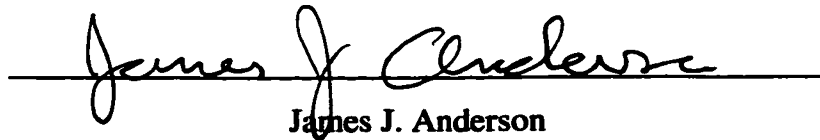

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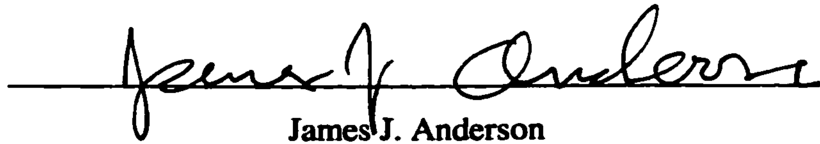

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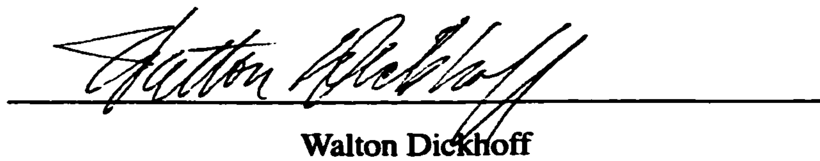

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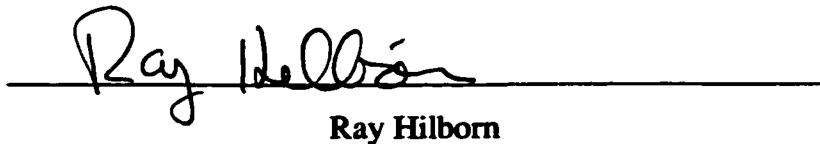

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Abstract

The Dynamics and Effects of Bacterial Kidney Disease in Snake River Spring Chinook Salmon (*Oncorhynchus tshawytscha*)

by Owen Sprague Hamel

Chairperson of the Supervisory Committee:
Professor James J. Anderson
Quantitative Ecology and Resource Management

Bacterial kidney disease (BKD) is endemic in Snake River spring chinook salmon (*Oncorhynchus tshawytscha*) stocks of both wild and hatchery origin, and may be partially responsible for their poor returns in recent years. BKD is particularly difficult to control due to the chronic nature of the disease. *Renibacterium salmoninarum*, the causative agent of BKD, can survive and reproduce intracellularly within the host, thus avoiding the humoral immune system. Numerous factors and processes are important in determining the survivorship or mortality of individual salmon infected with *R. salmoninarum*. Here, a number of these are explored using mathematical models and statistical techniques applied to data from the published literature. Antigen density, an important factor as the p57 antigen of *R. salmoninarum* has immunosuppressive properties, is related to bacterial load, and possible explanations for the observed relationship are suggested. The likelihood of vertical transmission of the disease (from spawner to offspring) or of in-ovum antigen inclusion induced immunotolerance is related to ovarian fluid infection levels. The effects of temperature on the ability of the immune system to fight off the disease and on the growth rate of the bacteria are explored. These factors are then brought to bear on the results of a broodstock segregation study, suggesting that immunotolerance and temperature effects likely account for many of the observed results.

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Dedication

This dissertation is dedicated to my parents, Ray and Judy Hamel,

and to the students, faculty and staff of Verde Valley School.

Introduction

Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD), one of the most problematic diseases of farmed, hatchery-reared and wild salmonids worldwide (Fryer and Sanders 1981). *R. salmoninarum* is an obligate salmonid pathogen (Evenden et al. 1993) which reproduces very slowly with a generation time approximating one day. Mortality due to BKD often occurs months or even years after initial infection, making it difficult to quantify its effect on salmonid populations. The disease affects anadromous salmon throughout their lifecycle, including while in ocean residence (Banner et al. 1986) and upon returning upriver to spawn. BKD is entrenched in Snake River spring chinook salmon (*Oncorhynchus tshawytscha*) stocks (Elliott et al. 1997), and is likely a significant factor in the poor survival of those stocks (Raymond 1988).

Many factors affect the progression and outcome of BKD in individual salmon including effects of temperature, stress and life-stage on the immunological response to the disease. Management of BKD aims to reduce disease-induced mortality and end the cycle of infection. The goal of this dissertation is to create a framework for understanding what cofactors are responsible for BKD-induced mortality in spring chinook, and to explore which management actions appear most promising and what research would be most useful. Models of the dynamics of the bacteria within the host and the immunological response to the disease can be used to predict mortality throughout the lifecycle and to suggest management actions that may reduce the effects of the disease.

The ecological situation presents a somewhat unique modeling system. Ectothermic animals in general present an extra complication in immunological modeling as temperature affects not only the rate at which processes occur but may affect the ability of immune cells to function. The dynamics of disease transmission are unique due to the aqueous medium and directional flow in the riverine environment and also due to the existence of vertical transmission from spawner to offspring which is rare among bacterial

diseases. Another complication is that the entry of a particular antigen of *R. salmoninarum* into the egg before spawning may result in life-long immunotolerance of the disease, far decreasing the probability of survival to spawning.

A number of factors and processes relevant to the success of *R. salmoninarum* infection are considered here. The relationship of antigen density to bacterial load is examined using data collected with two methods of quantifying infection levels, one which detects antigen and another which detects the whole bacteria. The prevalences of vertical transmission versus antigen-inclusion induced immunotolerance are examined using direct tests for in-ovum infection and indirect evidence of immunotolerance, namely infection prevalences and mortality rates, in offspring of highly infected female spawners. Temperature effects on the success of infections are examined using laboratory experiments of artificial infection followed by holding at various temperatures, with further evidence provided by hatchery rearing data.

Both mathematical and conceptual models are developed to explain the observed data. Suggestions are made for future experiments to test and further develop the proposed models and hypotheses and to provide guidance for future management of salmon stocks to minimize the negative effects of BKD on their survival.

1. Background

1. 1. Bacterial kidney disease in Snake River spring chinook salmon

Naturally spawned Snake River spring chinook salmon (*Oncorhynchus tshawytscha*) emerge in the winter and spend more than a full year in stream residence before migrating to the ocean in the spring and early summer. Hatchery spawned and reared spring chinook hatch as early as late fall, and are released in the early spring after nearly a year and a half in hatchery residence. The majority of both wild and hatchery spring chinook spend two to four years in the Pacific ocean before returning upstream to spawn. Return migration occurs in the spring to early summer, and spawning in the late summer to early fall, such that spring chinook often spend months on their spawning grounds before actually spawning.

Snake River spring chinook salmon returns have been consistently low in recent years. This is especially true for hatchery spring chinook. During the 10 year period from 1975-1984, the average smolt to adult return rate (SAR) of hatchery spring chinook smolts was only 0.4%, while wild spring chinook (far fewer in number) returned at a rate of 1.6%. Snake river steelhead (*O. mykiss*), in comparison, had SARs of 2.4% for hatchery origin and 2.8% for wild origin (Raymond, 1988). An SAR of 2% is typically considered necessary for sustaining a population.

The difference in return rates of these two species may be in part due to the particular effect of bacterial kidney disease (BKD) on survivorship of spring chinook. BKD is caused by *Renibacterium salmoninarum*, a fastidious, slow growing bacterium (generation time > 18 hours), which is an obligate salmonid pathogen (Evenden et al., 1993). BKD is prevalent in salmonids throughout the world, and in Pacific salmon is found throughout the lifecycle, including while in ocean residence (Banner et al. 1986) and upon returning upriver to spawn (e.g. Pascho et al. 1991).

BKD was first discovered in Scotland in the 1930's, then known as Dee disease,

but was soon found in North America as well. The now discontinued practice of feeding hatchery salmon unpasteurized fish meal undoubtedly contributed greatly to the spread of the disease.

While BKD can occur in epidemics, it is generally a chronic disease, and is completely entrenched in Snake River spring chinook salmon populations. Almost all fish in these populations are infected with *R. salmoninarum* at some point in their lifecycle (Elliott et al. 1997), or throughout it. Low-level infections may exist for long periods of time with no apparent adverse effects, followed by an eruption of the disease during or after stressful events. Salmon with low levels of the bacteria in their system may act as carriers of the disease. Thus the primary question is not whether a salmon is infected with the disease, but what factors affect the course and outcome of the infection given the genotype, condition, and immune functioning of the salmon and the life stages, environmental conditions and stressors experienced by the salmon while infected.

1. 2. Advances in detection

Traditional methods of detecting and quantifying bacterial infections are problematic for *R. salmoninarum*. Culture of the slow growing *R. salmoninarum* is both time consuming and difficult due to a number or complicating factors including the possibility of contamination with heterotrophic bacteria and the growth inhibitory effects of kidney tissue in vitro (Weins and Kaattari 1999). Gram stain is relatively insensitive, requiring 10^6-10^9 cells g^{-1} for detection in tissue (Bullock et al. 1980, Pascho et al. 1987).

Advances in the sensitivity of diagnostic tests for *R. salmoninarum* over the past two decades, especially with the advent of the enzyme-linked immunosorbent assay (ELISA), have allowed researchers to detect much lower level infections. This ability has proved invaluable in researching routes of transmission and determining prevalences of BKD in salmon. ELISA detects soluble antigens of *R. salmoninarum* rather than the bacterium itself and has been shown to be very sensitive for tissues. Other techniques to

detect soluble antigens include immunodiffusion and counterimmunoelectrophoresis. The direct fluorescent antibody technique (FAT), in contrast, is used to directly count tagged bacteria. Two other techniques, the dot-blot and the polymerase chain reaction (PCR) detect, but do not quantify, *R. salmoninarum* infections.

Pascho et al. (1987) tested samples of kidney and spleen tissue, ovarian fluid and blood from 56 sexually mature female coho salmon (*O. kisutch*) for *R. salmoninarum* using five techniques: ELISA, direct or direct-filtration FAT (the latter for ovarian fluid), culture, counterimmunoelectrophoresis. and agarose gel immunodiffusion. ELISA revealed the highest prevalence, finding evidence of infection in all fish (though not in all tissue or fluid samples). The FAT found infection in 86% of fish, with culture finding bacteria in 39%, and the last two techniques finding only 11% and 5% infection respectively. These results clearly indicate the sensitivity of ELISA and the FAT in diagnosing BKD.

Elliott et al. (1997) found evidence of infection in 86-100% of downstream migrating smolts at dams on the Columbia and Snake rivers during 1988-1991 using ELISA, whereas only 4-17% of these fish tested positive via FAT. ELISA appears to be more sensitive in detecting infections in tissues (Meyers et al. 1993), with FAT being more sensitive in ovarian fluid (Pascho et al. 1998).

Rhodes et al. (1998) described a reverse transcription-polymerase chain reaction (RT-PCR) of 16S ribosomal RNA technique that can be used as a semiquantitative method of detecting R. salmoninarum for various tissues, including whole fry, kidney tissue, eggs, and blood. The semiquantitative property of this technique arises from the fact that the amount of amplified product reflects the original concentration, and thus a visual comparison can be made. Rhodes et al. showed that their RT-PCR technique can detect as few as 10 cells per gram of tissue, making it more sensitive than FAT or ELISA. The semiquantitative nature of the test coupled with the ability to test blood samples may prove useful for not only for detecting timing of first infection but also track the progression of

the disease in individual salmon.

1. 3. Prevalence of *R. salmoninarum* infection

1. 3. 1. Prevalence in hatcheries

Maule et al. (1996) and VanderKooi and Maule (1999) monitored the prevalence of BKD infection in pre-release juvenile spring chinook at eight hatcheries on the Columbia and Snake rivers above McNary Dam from 1988 to 1996. Each hatchery was monitored in at least 6 of the 9 years of the study. Prevalences, by the enzyme-linked immunosorbent assay (ELISA) ranged from less than 5% to nearly 100%, with each hatchery experiencing an infection rate above 90% in at least one year.

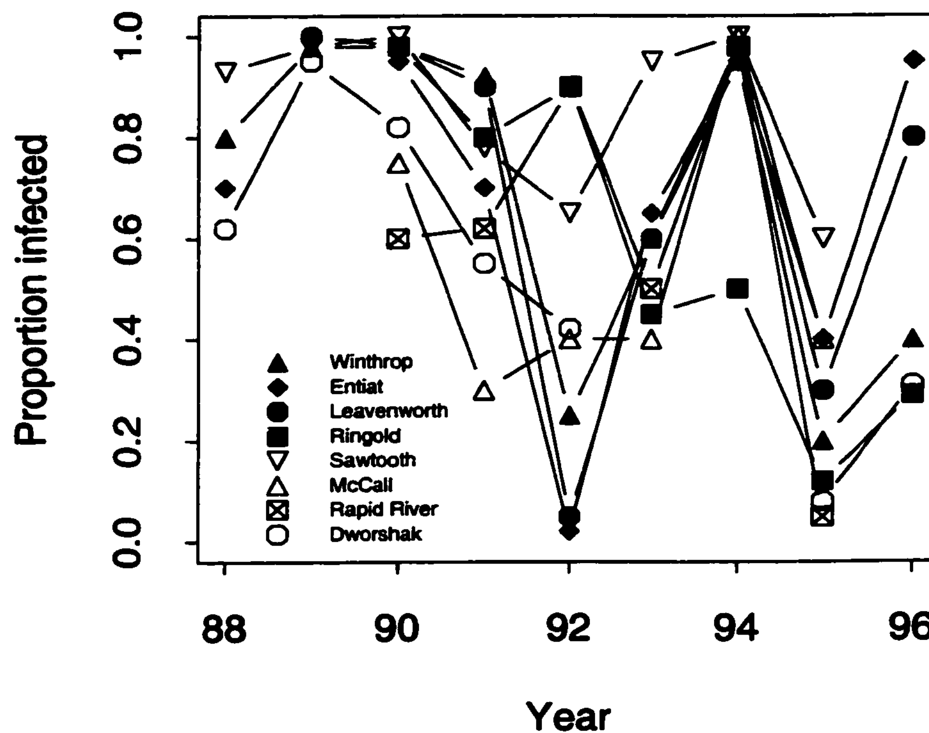

Figure 1 The prevalence of BKD at eight hatcheries on the Columbia and Snake rivers from 1988 to 1992. Data from Maule et al. (1996) and Vanderkooi and Maule (1999).The filled symbols represent data from Columbia River hatcheries, whereas the open symbols represent data from Snake River hatcheries.

There are no clear trends in BKD prevalence (Figure 1) despite improved hatchery practices to reduce prevalence and severity of BKD infection, begun at each of these hatcheries at varying times during the study period. These practices included the culling or segregation of progeny of highly infected female spawners and the use of the antibiotic erythromycin to reduce infection in holding spawners and/or directly in juveniles (Maule et al. 1996). At first, these measures appeared to be effective in reducing the prevalence of the disease. From 1988 through 1992 there was an overall decrease in prevalence of BKD, as there were steady decreases in six of the eight hatcheries (three of four in each the Snake and Columbia) from 1989 or 1990 to 1992. However, prevalences did not remain low, as the trend reversed after 1992, with prevalences peaking in 1994 in 6 of the 7 hatcheries monitored that year.

There is a distinct, if imperfect, correlation among the prevalences in the eight hatcheries in any year, however there is only weak evidence of an overall temporal trend. The high level of variability in both the prevalence and severity of disease between years and hatcheries makes it difficult to determine if any hatchery practices have decreased the effects of BKD in these hatchery populations. It is evident, nonetheless, that any such effect is relatively minor.

1. 3. 2. Prevalence at dams

Elliott et al. (1997) monitored prevalences and levels of *R. salmoninarum* infection in migrating juvenile spring-summer chinook at dams on the Columbia and Snake rivers over four years (1988-1991). Prevalences ranged from 86-100% by ELISA, with the majority of positive fish having low levels of infection, except in 1989 when over half of the spring chinook tested had medium to high levels of infection at Lower Granite and McNary dams. Marked hatchery fish had in all years at all dams lesser or equal prevalences when compared to unmarked fish of both hatchery and wild origin.

During the approximate two month out-migration period, the highest levels of

infection were observed among the last 25% of out-migrating smolts. Elliot et al. (1997) suggest rising river temperatures and different timing of migration among different wild and hatchery cohorts may be partially responsible for this observation. Other factors determining migration timing such as overall fitness and physiological preparedness for smoltification may also play a role.

No consistent relationship is seen between hatchery levels and levels at dams from 1988-1991, though the highest levels at both hatcheries and dams were observed in 1989. Prevalences at any individual dam were consistently higher than the average prevalences at hatcheries monitored above that dam.

1. 3. 3. Existence of *R. salmoninarum* infection while in ocean residence

Banner et al. (1986) found *R.salmoninarum* infections in all seven North American species of *Oncorhynchus* caught in the Pacific, and suggested that BKD might be a significant contributor to mortality during ocean residence. Testing via FAT they found evidence of infection in 11% of chinook observed, and in 1-4% of each of the other species. Given the greater sensitivity of ELISA tests in tissues and of PCR in general, higher prevalences of infection might be seen if Pacific salmon in ocean residence were tested by one or both of these techniques.

1. 4. Routes of infection

1. 4. 1. Horizontal transmission

Renibacterium salmoninarum has a uniquely powerful ability to induce uptake by tissue cells including the lining of the gastro-intestinal tract (Evelyn 1996, Bruno 1986). Thus infection is likely when the bacteria are present at sufficient levels in the surrounding water or in co-habitating fish. In seawater net pens, ingestion of contaminated feces (with up to 10^7 bacteria/g) during feeding is likely a major route of horizontal infection (Balfry et al. 1996). Waterborne bacteria may infect salmon in salt or freshwater, through the gills,

eyes, ingestion, or lesions or abrasions (Evenden et al. 1993). Murray et al. (1992) found that long term (180 day) cohabitation with highly infected and dying salmon resulted in infection and death of all fish (at an average temperature of 9.9 °C). Short term exposure (15-30 minutes) to densely infected water (10^4-10^6 bacteria/ml) and subsequent rearing for 180 days in fresh and then 180 days in salt water with an average temperature of 12.6 °C resulted in significant mortality ranging from 14% to 50%. Mitchum and Sherman (1981) confirmed that horizontal transmission occurs between wild and stocked hatchery trout in natural systems.

1. 4. 2. Environmental infection

R. salmoninarum can survive for up to 4 days in river water, four to five weeks in sterile freshwater, three weeks in fecal matter (Austin and Rayment 1985) and two weeks in sterile seawater (Balfry et al. 1996). Thus it is possible for a salmon to acquire a *R. salmoninarum* infection even without being in close quarters with other infected fish. Bacteria and bacteria laden feces from upstream may be important in transferring the disease between stocks and between species.

1. 4. 3. Vertical transmission

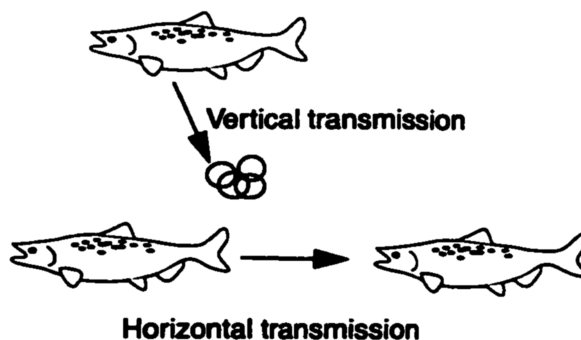

Figure 2 Illustration of vertical and horizontal transmission routes

BKD is unique in that it is the only bacterial disease of fishes that is known to be transmitted vertically (from spawner to offspring) (Elliott et al. 1989). *R. salmoninarum* has also been shown to be present in ovarian (coelomic) fluid (Evelyn et al. 1986, Elliott and Barila 1987, Lee and Evelyn 1989), which may be an important route of vertical transmission. Evelyn et al. (1984) found that the in-ovum infection rate was only 14% in eggs from a very highly infected female coho spawner ($4x10^9$ cells ml^{-1} in ovarian fluid). Challenging eggs before fertilization with highly infected ovarian fluid result in increased prevalences of BKD in 195-day old smolts (Lee and Evelyn 1989). The observed infection levels (34% in the highest challenge group) were the result of both vertical and subsequent horizontal transmission among the juvenile salmon, and thus an indication of the existence of vertical infection, but not indicative of the proportion of offspring directly infected in this manner.

1. 5. Environmental factors affecting transmission and progression of BKD

1. 5. 1. Temperature

There are many condition (internal) and environmental (external) factors that affect the transmission and pathogenesis of *R. salmoninarum*. These factors change throughout the salmon's lifecycle and the ubiquitous BKD responds accordingly. Temperature affects the health of the fish, the capabilities of the immune system and the growth of the bacteria. Sanders et al. (1978) showed that mortality due to BKD in artificially infected coho salmon (*O. kisutch*) in freshwater was highest in temperatures in the range of 6.7-12.2 °C, with decreasing mortality levels between 12.2 and 20.5 °C. Mean time to death decreased with increasing temperature. Sanders et al. and others have performed the same types of experiments with other bacterial diseases of fish, but BKD was the only disease in which higher temperatures (here 17.8 and 20.5 °C) resulted in decreased total mortality. BKD also produced the highest mortalities at low temperatures (3.9 and 6.7 °C) among all bacterial diseases tested.

Low temperatures have been shown to adversely affect the functioning of the

teleost immune system (Bly and Clem 1992, and see Chapter 1. 8. 5.). Very high temperatures or rapid changes in temperature are stressful to salmonids, but high temperatures also result in more rapid breakdown of the p57 antigen (Griffiths and Lynch 1991), perhaps reducing the virulence of the disease. *R. salmoninarum* reproduces most quickly in the temperature range of 15-18 °C (Weins and Kaattari 1999). The effects of temperature are explored more fully in Chapter 4.

1. 5. 2. Stress

Stress and environmental conditions are important proximate causes of disease outbreaks and death. One factor in stress-mediated mortality is an increase in plasma cortisol levels. Cortisol reduces immune function (see Chapter 1. 8. 4.) but provides certain temporary benefits to stressed fish such as aiding in the adaptation of gill tissue for the export of salts during the process of smoltification and adaptation to salt water (Fagerlund et al. 1995). Causes of stress include rearing density, migration, smoltification (see Mesa et al. 1999 for a discussion of the effects of smoltification on BKD), transition to salt water (Banner et al. 1983, Elliott et al. 1995), changes in travel time due to dams and impoundments (Raymond 1968, 1969, 1979, 1988), barge or truck transportation (Maule et al. 1988), dam passage (Gessel et al. 1991), and water temperature.

Banks (1994) considered the effects of rearing density and water flow on total returns of spring chinook at Carson National Fish Hatchery. He found no increase in total return per raceway above a base level of 20,000 fish per raceway, though the standard practice at the time was to stock 40,000. BKD may be a factor in affecting the severity of decreased returns in chinook under these conditions. Banks did observe increased mortality in raceways with the highest stocking densities and/or the lowest flows in the two years with highest overall in-hatchery mortality from the disease (17 and 28% total mortality). However no difference was seen in infection rates among raceways at release, and the densely stocked raceways released many more fish those that were sparsely stocked, yet in all years total returns (per raceway) were similar between raceways.

Proper conditioning to stress can reduce its effects. Artificially induced intermittent stress during rearing, especially if it is associated with feeding, can improve the ability of fish to cope with all types of stress later in life (Schreck et al., 1995). Effects of stress may be more complex than simply rapid changes in immune function followed by a slow return to normal functioning. Maule et al. (1989) studied the effects of different types of stress (30 seconds out of water in a dipnet, manipulation for 4 hours during hatchery operations, or transportation for 9 hours) on indicators of immune functioning in salmon. Cortisol levels were up at 4 hours post stress and down again at 1 day. Antibody producing cells were similarly down at 4 hours and up at 1 day but then down again at 1 week, though there was little evidence for differences in disease resistance after 1 day.

Mesa et al. (2000) found short term stress to have no discernible effect on the progression and outcome of BKD in juvenile spring chinook salmon. The short term stress applied in this case were one minute "bouts of hypoxia, struggling and mild agitation" by holding the fish in a perforated bucket out of water. The juveniles were subjected to three such bouts of short term stress separated by 2 to 3 days. This indicates that sparse intermittent stress may have little or no effect on the outcome of BKD infection. No conclusions regarding the effect of chronic stress or more frequent intermittent stress can be made from the results of this study.

1. 6. Attempts at control

Despite advances in the detection of *R. salmoninarum* (Pascho and Mulcahy 1987, Pascho et al. 1987, 1998, Jansson et al. 1996; and see Chapter 1. 2.) and in the understanding of its survival mechanisms, there is no consistent method of control for BKD (Elliott et al. 1989). Still, various methods have been shown to reduce the prevalence or effects of the disease. Use of a vaccine 2 months before a challenge with *R. salmoninarum* appears to decrease immediate mortality due to the disease (Pascho et al. 1997), injection of antibiotics into females 2-3 weeks before spawning reduces the incidence of vertical transmission (Brown et al. 1990), and the use of chlorine can greatly

diminish numbers of live *R. salmoninarum* in fresh water (Pascho et al. 1995), which should restrict horizontal transmission. Experiments with nutritional supplements have had mixed results. Some dietary modifications may, at least temporarily, improve the ability of salmon to fight off BKD (e.g. Lall et al. 1985, Thorarinsson et al. 1994).

Vaccinations have been found to temporarily, for 12 to 18 months, induce increased antibody production in Atlantic salmon (*Salmo salar*) (Paterson et al. 1985), though there was no clear evidence of decreased incidence or severity of disease in these fish, and it has been suggested that Pacific salmon (*Oncorhynchus* sp.) may be intrinsically less able than *Salmo* species to mount an immune response to BKD (Elliott et al. 1989).

More recently, nutritionally mutant attenuated strains of *R. salmoninarum* have shown promise as live vaccines. Reduced kidney infection levels were observed in Atlantic salmon vaccinated with attenuated strains of *R. salmoninarum*, when tested 14 weeks after artificial infection. The polymerase chain reaction (PCR) detected free *R. salmoninarum* in water from tanks holding unvaccinated salmon but not in water from tanks holding vaccinated salmon (Griffiths et al. 1998). Mortalities in artificially infected Atlantic salmon were reduced by up to 75%, when the intra-peritoneal injection of virulent strains took place one month after vaccination with live avirulent *R. salmoninarum* (Daly et al. 2001). Oral vaccination with whole cell $p57^-$ *R. salmoninarum* that has been heat treated at 37 °C to remove cell surface associated p57 antigen shows promise as well (Piganelli et al. 1999). The long term effects of such vaccinations have not been reported.

Hatchery fish put on diets that mimic those of wild fish during the winter (i.e. little or no feeding), followed by an excess of food in the spring, not only experience the proper growth conditions for smoltification (see Ewing et al. 1980, Dickhoff et al. 1995), but also have reduced prevalence of BKD in pre-release smolts (Dickhoff, unpublished data). Whether these observed benefits translate into decreased infection and increased survivorship later in life is yet to be proven. Still, this seems a promising tactic for improving the disease resistance of hatchery smolts.

No statistically significant effect of fasting on BKD prevalence was reported for yearling spring chinook when fasting occurred in the late spring, although a significant regression of prevalence versus ration level revealed decreasing prevalence with increasing ration (Pirhonen et al. 2000). As suggested above, feeding and growth in the spring appears to reduce the prevalence of detectable infection.

To prevent vertical transmission some hatcheries have begun culling eggs from highly infected females. Another technique to reduce the number of hatchery fish exposed to early horizontal infection due to cohabitation with vertically infected fish is brood stock segregation, in which eggs and fry from highly infected females are reared separately from the rest of the hatchery fish. The efficacy of brood stock segregation is explored in Chapter 5.

1. 7. Pathogenesis and histopathology of *R. salmoninarum* infection

1. 7. 1. Overview

R. salmoninarum is concentrated in kidney and spleen tissue in low level infections, but usually is systemic by the time BKD-induced mortality occurs. *R. salmoninarum* can avoid immune response by residing and reproducing intracellularly within phagocytic (primarily) as well as non-phagocytic cells (Gutenberger et al. 1997; Evelyn, 1996), and appears able to survive at low levels within individual fish for entire lifecycles.

Bruno (1986a,b) and Bruno and Munro (1986) examined severely artificially infected juvenile rainbow trout and Atlantic salmon held at 12 °C over 35 days post-injection to observe the progression of bacterial kidney disease in tissues. Just 45 minutes after injection, *R. salmoninarum* was detected in phagocytes in the kidney and spleen. By 6 days extracellular bacteria were observed in these organs. By 10 days bacteria were detected in blood monocytes and macrophages, apparently multiplying within these cells. By 2 to 3 weeks, the bacteria were found in the heart, and signs of the disease were

visible on numerous internal organs. Granulomas (organized collections of mononuclear phagocytes causing chronic localized inflammation) appeared, especially in the liver and kidney. Bruno concluded that death was likely attributable to destruction of normal kidney and liver tissue by bacterial lesions, possibly coupled with heart failure due to myocardial necrosis. All effects seen in artificially infected fish were confirmed in naturally infected fish, with progression of the disease taking longer in the latter, and with the additional presence of very large internal lesions perhaps due to the length of infection before death in naturally infected fish.

Many researchers have noted significant changes in blood parameters with the progression of the disease. These include decreases of up to 66% in numbers of red blood cells (erythrocytes), and more than a 10% decrease in average erythrocyte diameter (Bruno 1986a; Weins and Kaattari, 1999).

1. 7. 2. Entry of *R. salmoninarum* into host

R. salmoninarum has the ability to induce its uptake by non-phagocytic cells and can survive ingestion. This provides a means of entry into the host via the gills and the gastro-intestinal tract (Evelyn 1996; Flaño et al. 1996), though a recent study found *R. salmoninarum* not to be readily taken up by healthy rainbow trout (*O. mykiss*) gill tissue *in vitro* (McIntosh et al. 2000). Infection can also occur through external lesions or abrasions. Uptake by eggs is a possibility as well, resulting in vertical transmission in a small percentage of eggs (Evelyn et al. 1984).

1. 7. 3. Internal spread of *R. salmoninarum*

Internal spread of *R. salmoninarum* occurs via free bacteria in the blood and also through intracellular residence and reproduction in macrophages. *R. salmoninarum* is slow growing, but can reach levels of 10^3 cells g^{-1} in muscle and 10^9 cells g^{-1} in spleen and kidney tissue before induction of fish mortality (Evelyn 1996).

1. 7. 4. Survival of *R. salmoninarum* in host

R. salmoninarum can survive entry into and reproduce within phagocytic cells (immune system cells which ingest and digest bacteria, infected tissue cells and assorted debris), avoiding the humoral immune response and reproducing within the phagocyte. Opsonization (the tagging of a bacterium by immune system molecules) by antibody and/or complement has been shown to actually increase the success of the bacterium in surviving and reproducing within phagocytes rather than hastening its demise as with most pathogens. Bandin et al. (1995) found that the amount of time that *R. salmoninarum* cells can survive within phagocytic cells was related to whether they were opsonized. They found mean survival times of 4 days for bacteria not opsonized, 5 days for those opsonized by complement alone and 7 days for those opsonized by both complement and antibody.

R. salmoninarum exploits its ability to be taken up by non-phagocytic tissue cells not only as a method of infection, but as a way to hide out from the immune system (Flaño et al. 1996). *R. salmoninarum* is also resistant to lysis by lysozyme or by complement.

1. 7. 5. Growth of *R. salmoninarum*

In order to survive and reproduce, bacteria need to be able to take up nutrients from the host. Under iron depravation, *R. salmoninarum* may produce iron reductase which makes bound iron more available for bacterial uptake (Grayson et al. 1995). The bacteria reproduce in the endoplasm of phagocytic cells where nutrients are more readily available.

1. 7. 6. Effects of the p57 antigen

Particularities of *R. salmoninarum* and a 57 kilodalton surface antigen (a molecule which elicits an immune response), referred to as "p57", which it secretes in large amounts, make the bacterium highly successful in surviving from generation to generation. *R. salmoninarum* does not produce any acutely lethal toxins. *R. salmoninarum* does produce large amounts of the p57 antigen (Weins and Kaattari 1989), both in serum and while in intracellular residence, so much that it tends to neutralize the vast majority of

anti-p57 antibodies. These antibody-p57 complexes may remain in tissue and contribute to tissue destructive hypersensitivity resulting in granulomas (Bruno 1986b).

The p57 antigen is the major soluble antigen of *R. salmoninarum* (Weins and Kaattari 1999) and may be present in high densities in tissues and fluids of infected salmon (Evenden et al. 1993). Soluble antigen density (which refers to the density of the soluble antigenic fraction, which includes p57 and its breakdown products, as well as other soluble antigens) is an important virulence factor as p57 has immunosuppressive and tissue destructive properties. The p57 antigen is known to agglutinate (cause to stick together) salmon leukocytes (white blood cells including macrophages and T and B cells) (Weins et al. 1999), and to suppress antibody production against unrelated antigens *in vitro* (Turaga et al. 1987). The p57 antigen is a potent inhibitor of the phagocyte respiratory burst response at high densities and has been shown to decrease the bactericidal activity of juvenile chinook macrophages against *Aeromonas salmonicida*, an unrelated pathogen (Siegel and Congleton 1997).

It has been suggested that p57 and its breakdown products may form a protective layer (or capsule) around *R. salmoninarum* cells (Senson and Stevenson 1999). *R. salmoninarum* cells stripped of p57 have been shown to induce a much stronger immune response than those not stripped of p57 (Wood and Kaattari 1996). Cell-surface associated p57 and its breakdown products appear, then, to effectively block highly immunogenic areas of the cell surface from detection by host defences.

Studies of a virulence-attenuated strain of *R. salmoninarum* found it to produce similar amounts of free p57, but less cell-surface associated p57 than virulent strains of the bacterium (O'Farrell and Strom 1999, Senson and Stevenson 1999). The p57 from the virulence-attenuated strain was unable to restore haemagglutination activity to either the attenuated or a virulent strain of *R. salmoninarum*, whereas p57 from a virulent strain of *R. salmoninarum* was able to reassociate with and restore haemagglutination activity to both the attenuated and the virulent strain (Senson and Stevenson 1999). Chinook salmon (*O.*

tshawytscha) injected with a high dose (10^6 bacteria fish^{-1}) of attenuated *R. salmoninarum* experienced no evident kidney damage and much reduced mortality compared to chinook injected with the same dose of virulent *R. salmoninarum* (O'Farrell et al. 2000). The differences in virulence may be due in part to differences in the molecular structure of the p57 antigen produced by the attenuated strain (Senson and Stevenson 1999).

The lack of observed differences in free p57 antigen density between virulent and attenuated strains of *R. salmoninarum* does not indicate that free p57 is not a major virulence factor. Rather, the finding that the p57 from an attenuated strain of *R. salmoninarum* does not appear to attach to the surface of *R. salmoninarum* cells supports the suggestion that one of the functions of p57 is to encapsulate *R. salmoninarum* cells and shield the cell surface from the host immune system. For most naturally occurring strains of *R. salmoninarum*, high densities of free p57 should indicate high levels of surface associated p57 as well.

R. salmoninarum bacteria seem to induce their own uptake by host cells (Evelyn 1996). The p57 antigen is apparently responsible for the ease with which *R. salmoninarum* cells attach to host cells. The p57 antigen is capable of agglutinating leukocytes whether in soluble form or attached to the bacterium (Weins and Kaattari 1991). Cell-surface associated p57 appears to be an important factor in horizontal transmission as well, as attachment to host cells appears an important step before entry into host cells. Thus p57 may be the major factor inducing uptake of the bacterium by gill tissue and, as *R. salmoninarum* can survive ingestion, the lining of the gastro-intestinal tract (Evelyn 1996, Flaño et al. 1996). Ingestion of bacteria-laden feces during feeding is an important route of transmission in farmed salmon (Balfry et al. 1996). Cell surface p57 may be important in inducing the uptake of *R. salmoninarum* into eggs from the surrounding ovarian fluid before spawning, thus making BKD a rare bacterial disease that can be transmitted vertically from female spawner to offspring (Evelyn et al. 1984, 1986). It has been suggested that the p57 antigen in soluble form may also be taken up by eggs from ovarian fluid. This may result in the subsequent offspring being immunotolerant of p57 (Brown et

al. 1996) and much less capable of resisting *R. salmoninarum* infections.

After secretion, the p57 antigen may break down through autolysis (Griffiths and Lynch 1991), be taken up by antibody (Bruno 1986b) or onto or into cells (as suggested above), or be flushed out of the body. The relationship between bacterial load and antigen density *in vivo* has yet to be quantified. The long generation time (~ 1 day) of *R. salmoninarum* and slow development of the disease are reasons to believe that this relationship remains near equilibrium. This relationship is explored in Chapter 2.

Fredriksen et al. (1997) found another surface antigen (p22) to have similar immunosuppressive and agglutinating properties as p57. Siegel and Congleton (1997) studied the effects of low or high doses of whole bacteria or soluble proteins of *R. salmoninarum* (including p57 and p22) of the bactericidal activity of juvenile chinook macrophages against *Aeromonas salmonicida*. Macrophages were stimulated with low doses, but inhibited at high doses. Thus at low levels p57 may act as any other antigen, stimulating an immune response, but at higher levels may remove or inactivate immune cells.

1. 8. Teleost immunology

1. 8. 1. Overview

Immunological modeling of bacterial kidney disease in salmon poses a unique challenge for a number of reasons. First, the salmon immune system is a bit different than the human immune system, though with a similar overall structure. Second, salmon are ectothermic animals and cold water species, so that the immune system (and the pathogen) are not exposed to a fairly constant 37 °C as in humans, but rather to variable temperatures from below 4 °C to over 20 °C. Temperature can affect the functioning and effectiveness of the immune response. Third, *R. salmoninarum* has developed unique and effective ways of surviving by avoiding the immune response.

Most research and modeling in immunology has concerned the human immune system or that of related mammals. The immune system of teleosts (bony fishes) is quite similar to that of humans and other mammals, but with a few significant differences. The teleost immune system, like the human immune system, is a complex of a great many molecules and cells, comprising humoral and cellular, specific and non-specific immune defenses (Ellis, 1999). There exist, however, important differences between the human and teleost immune systems. IgM-like Ig (Immunoglobulin, or equivalently, antibody) is the only class of antibody known to be produced in teleosts and there is little evidence for the occurrence of avidity maturation (an increase in the multivalent bonding strength of the antibodies through a process of adaptation and selection which occurs in mammals). The alternative complement pathway is much more active in teleosts than in humans. The morphology (tissue structure) of the teleost immune system is very different from that of humans and other mammals (Press and Evensen 1999). An overview of the human immune system, which includes definitions of many of the components of both the human and teleost immune systems, is given in Appendix A.

1. 8. 2. Morphology of the teleost immune system

Teleosts lack bone marrow and lymph nodes, whose processes are largely delegated to specialized kidney tissues. The kidney in teleosts is divided into two distinct parts, the anterior or head kidney, which has no blood filtration function, and the middle and posterior kidney. Lymphocytes arise from stem cells within both parts, though mainly from the head kidney (Ellis and de Sousa 1974, Zapata 1979). A lymphatic system distinct from the circulatory system has been demonstrated in some teleost species. T cells develop in the thymus, as in mammals.

1. 8. 3. Components of the teleost immune system

Included in the cellular immune system of teleosts are B cells and T cells (both helper and cytotoxic), granulocytes (neutrophils, eosinophils and basophils), thrombocytes (platelets), mast cells, non-specific cytotoxic cells (NCCs) (like NK (natural killer) cells in

mammals), dendritic cells, monocytes and macrophages, including melanomacrophages which reside in the head kidney and act as antigen presenting cells (APCs), retaining antigen for long periods and thus likely active in immunological memory, though apparently not in avidity maturity, which does not appear to occur in teleosts. Humoral components include IgM-like antibody, the complement system, C-reactive protein, and cytokines including interleukins (IL) and interferons (IFN) (Ellis, 1999).

1. 8. 3. 1. Nonspecific cellular immune system

Macrophages and granulocytes (primarily neutrophils) engulf and digest bacteria. Macrophages are much more active than granulocytes in this role in fish. Phagocytes generally kill pathogens intracellularly, though extracellular killing (of larger parasites, for example) is also possible. Phagocytes kill pathogens using both oxygen dependent and oxygen independent mechanisms. Oxygen dependent killing includes the use of oxygen free radicals during respiratory burst. Opsonization by complement or antibody or particularities of the pathogen itself can increase reactive oxygen species (ROS) production. Nitric oxide (NO) can also be an important reactive factor in killing pathogens during phagocytosis. Oxygen independent killing is less well understood but probably involves lysozyme and other enzymes.

Phagocytes can also act as antigen presenting cells (APCs) in fish and, via release of cytokines, can stimulate proliferation of T cells or the production of antibodies by B cells. NCC cells are "non-specific" but do not kill indiscriminately and are not fatal to all pathogenic organisms but are particularly important in killing macroparasites and virally infected cells.

1. 8. 3. 2. Nonspecific humoral immune response:

Lysins, including lysozyme, act directly to compromise bacterial membranes. A number of different types are present in different locations in teleosts. Lysozyme is effective in splitting the cell walls of gram positive bacteria. It can also split gram negative

bacteria but only after they are damaged by other processes. Lysozyme can also act as an opsonin and may activate phagocytes. In teleosts lysozyme is found in tissues with many leukocytes, such as the head kidney, and areas likely to be primary routes of infection, such as the gills, skin and alimentary tract, and in eggs. Lysozyme is found in fairly high levels in salmonid eggs, and has been found to be bactericidal to many bacterial infectious agents, such as *Aeromonas hydrophila*, *A. salmonicida* and *Carnobacterium piscicola*, at only one third the concentration observed in salmon eggs (Grinde 1989). However even at levels comparable to those seen in salmon eggs, lysozyme was found to be ineffective at killing *R. salmoninarum*.

Complement in teleosts acts much like complement in humans, having both the classical and alternative pathways of activation, though the alternative (antibody independent) pathway is more active in fish than in humans (Ellis, 1999). Complement can be virucidal, bactericidal, parasiticidal and opsonic and can be active in chemotaxis of phagocytes (Yano, 1996). The most virulent bacteria tend not to be affected by complement (Ellis, 1999).

C-reactive protein (CRP) reacts with phosphorylcholine, a common element on the surface of many bacteria, fungi and parasites. CRP is found in many species from invertebrates to mammals and is important in activating complement and enhancing phagocytosis of some bacteria. CRP is found in low levels in mammals, with rapid increases during inflammation. In teleosts the base levels are much higher, though increases can still activate the complement system and increase the activity of phagocytes and NCCs.

Protease inhibitors may block the action of bacterially produced proteolytic toxins, whose purpose is to obtain amino acids through the digestion of host material. Transferrin may impede the uptake of iron by bacteria, thus reducing their ability to grow. Interferon simulates cells to become resistant to viral infection (Yano, 1996).

1. 8. 3. 3. Specific cellular immune system:

Fish posses both T cells and B cells, as do mammals. The exact characteristics of these cells, especially of cytotoxic T cells are still being studied. B cell activation is mediated by helper T cells, which must first be stimulated by APCs. Antigen-stimulated helper T cells also release cytokines which activate macrophages.

1. 8. 3. 4. Specific humoral immune system

Teleost Ig is a tetramer having 8 possible binding sites. Antibody can neutralize pathogens, agglutinate antigen and act as an opsonin in promoting phagocytosis. Ig can also activate the complement system. Antibodies act in many direct ways to prevent proliferation and damage by bacteria, as anti-adhesins, anti-toxins, and anti-invasins.

Teleosts do not posses germinal centers, and there is little evidence for avidity (multivalent attachment strength) maturation in teleosts. As teleost Ig is a tetramer, its avidity may be much greater than its affinity (univalent holding strength) for a particular antigen. The strength of the secondary response is due to the increase in total precursor cells rather than an increase in avidity of antibody.

1. 8. 4. Endocrine- immune system interactions and effects of stress

As in mammals there are substantial interactions between the endocrine and immune systems in teleosts. In particular, cortisol and other glucocorticosteroids suppress parts of the immune system by inhibition of lymphocyte proliferation (Weyts et al. 1999). This is possibly through suppression of cytokine production and induction of apoptosis (cell suicide). Stress causes decreased functioning of lymphocytes, including T cells, B cells and NCCs, as well as decreases in lysozyme (Mock and Peters 1990).

1. 8. 5. Temperature effects

Immune systems of fish have adapted to be optimally competent near normal summer temperatures for each species (Manning and Nakanishi 1996). Temperatures

significantly below that optimum may be immunosuppressive. In teleosts, low temperatures may seriously impair the action of helper T cells, reducing the production of cytokines and thus eliminating an important route of stimulation of phagocytes (Hardie et al. 1994). Proliferation of T cells is suppressed, though B cells are not affected (Bly and Clem, 1992). Non-specific immunity at low temperatures may help offset this setback, though low temperature affects other aspects of the immune system as well. For example, complement is most active between 15 and 25 °C, though it remains active even below 4 °C (Yano, 1996).

1. 8. 6. Developmental immunology of teleosts

There has been significant research into developmental timing of lymphoid organs in teleost fishes. Much less work has been done on the timing of immune system maturity (Zapata et al. 1996).

Immune system maturation occurs after hatch, when exposure to exogenous antigens has already begun. The timing of complete maturation varies greatly among teleost species (Tatner 1996). In rainbow trout, a complete immune response which induces subsequent immunity is not seen until several weeks after hatch (Ellis, 1988). All fish species tend to reach full immune system maturity within 2 to 8 weeks after hatch (Zapata et al. 1996). Maternal antibodies may be passed on to the eggs, providing significant temporary protection against disease in some cases (e.g. Brown et al. 1997) but not in others (e.g. Lillehaug et al. 1996).

1. 9. Immune system response against *R. salmoninarum* infection

1. 9. 1. Differences in immune response among groups of salmon

A number of factors may affect the ability of the salmonid immune system to prevent BKD-induced mortality in chinook salmon. Genotype may have some effect, but Elliott and Pascho (1995) found no evidence for differences in the genotypes of infected

and non-infected chinook spawners. Suzumoto et al. (1977) and Winter et al. (1980) found significant differences in BKD-mediated mortality among coho salmon and steelhead trout (*O. mykiss*) with different transferrin genotypes. Withler and Evelyn (1990) found a much weaker correlation between resistance to BKD and transferrin genotype in coho strains, suggesting that other factors account for most of difference observed. To this point there is no strong evidence for significant genetic effects among spring chinook. Acquired immunity after surviving BKD may reduce mortality later in life, but it appears this protection lasts for well less than a year.

Immunotolerance, due to vertical transmission and/or presence of antigens in eggs, may be the most important factor affecting BKD-induced mortality throughout the life cycle. Mortality rates of immunotolerant fish are high due to the failure of specific immunity. The non-specific immune system is still active but is often inadequate to halt the multiplication of *R. salmoninarum*, especially in the presence of other immunosuppressive factors such as stress.

1. 9. 2. Effect of macrophages

R. salmoninarum stimulates the respiratory burst mechanism of phagocytes immediately upon contact, and quickly exhausts the phagocyte bactericidal capability. Subsequently, the bacterium is taken up by the phagocyte into a phagosome, from which the bacterium escapes before multiplying within the cytoplasm of the cell. The phagocyte is apparently unable to mount a response for several days, during which time the bacteria reproduce. Secretion of p57 continues while the bacteria are in intracellular residence. Presence of p57 within the macrophage may help inhibit the bactericidal response (Seigel and Congleton 1997).

1. 9. 3. Effects of complement and antibody

Complement is not effective at directly lysing *R. salmoninarum*. Both complement and antibody act as opsonins, tagging the bacteria for uptake by phagocytic cells. The

unintended effect of this is to make it easier for the bacteria to enter, survive and reproduce intracellularly within phagocytic cells (Bandin et al. 1995).

Antibody also interacts directly with free antigen (p57), creating immune complexes which aggregate in tissue and cause hypersensitivity reactions, resulting in granulomas and tissue damage (Bruno 1986b). It is not clear that the production of antibody does anything to help fight off or reduce the effects of bacterial kidney disease.

1. 9. 4. Effect of helper T cells

Helper T cells play an important role in eliminating *R. salmoninarum*. Macrophage activating factor (MAF)-activated macrophages can effectively kill *R. salmoninarum* cells (Hardie et al. 1996), but production of macrophage activating factor in naive helper T-cells may be suppressed at low temperatures. The responses of primed (memory) T cells appears less temperature dependent (Bly and Clem 1992). The proliferation and action of helper T cells in activating macrophages may be the primary successful immune response against BKD. Suppression of T cell action due to immunotolerance, low temperatures, stress or a combination of the three may significantly reduce a salmon's ability to fight off and survive the disease.

1. 10. Immunotolerance

Life-long immunotolerance affects a salmon throughout its life-cycle, whenever it is exposed to *R. salmoninarum*. The immunosuppression referred to here is not an on-off switch for immune response, but rather the loss of a particularly powerful weapon in the immune system's fight against the bacteria. This loss can lead to mortality when the affected salmon is challenged with *R. salmoninarum*, especially when coupled with stress. Immunotolerant fish that survive are more likely to harbor large numbers of bacteria, and are potentially an important source of horizontal infection to other salmon, especially during collection and transportation and on the spawning grounds, thus furthering the effects of BKD on salmon stocks (Evelyn, 1996).

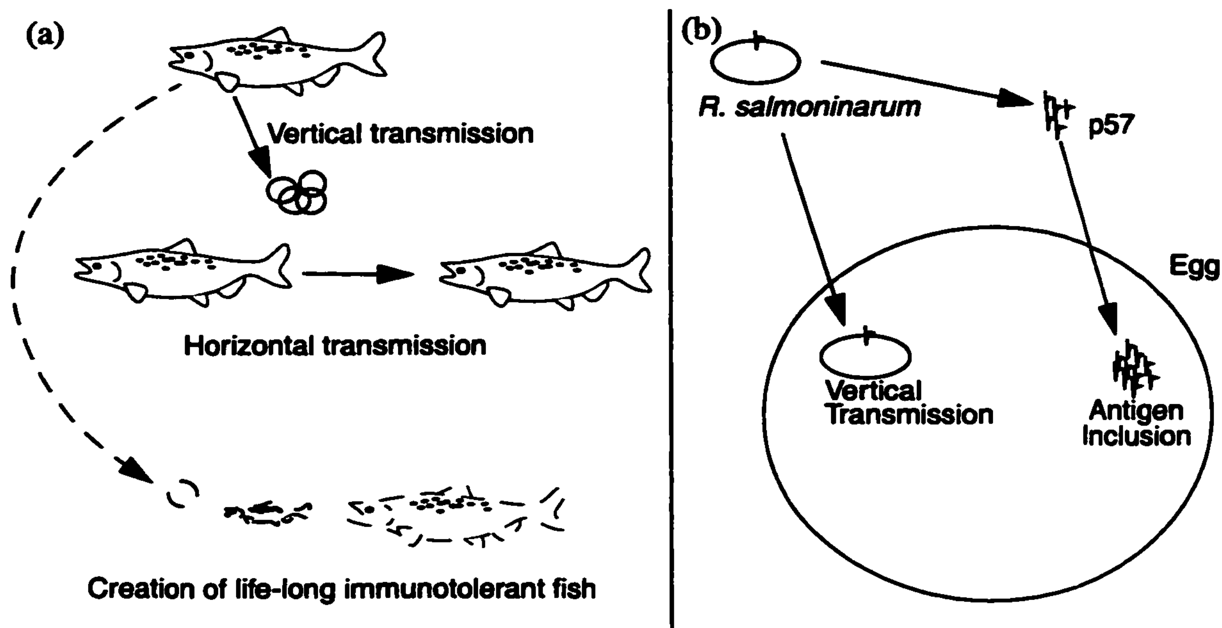

Figure 3 Routes of *R. salmoninarum* transmission or induction of immunotolerance in offspring (a) and micro scale view of vertical transmission and antigen inclusion (b) (not to scale).

Brown et al. (1996) suggested that the inclusion of *R. salmoninarum* antigens in eggs may result in immunotolerance. They showed that in-ovum inclusion of the p57 antigen can result in decreased immune function when resulting fry are exposed to *R. salmoninarum*, even if vaccination occurs between hatch and challenge. 100 ng (ng = 10^{-6} mg) per egg was found to induce this immunotolerance, while 1 ng was found to be insufficient to produce any observable change in immune functioning. Evenden et al. (1993) note that up to 1 mg/ml of p57 and its breakdown products have been found in tissues of infected fish. As an average chinook egg is about 0.2 ml in volume, this translates to up to 200,000 ng p57 per egg. Even if eggs do not accumulate as much of the p57 antigen as tissues, as long as eggs are able to accumulate p57 either during oogenesis from the blood, or later from the ovarian fluid, or both, it appears that the threshold of 100ng of the p57 antigen would be quite attainable. Brown et al. (1996) also found that the immunosuppressive effect of high numbers of killed whole *R. salmoninarum* cells (1000 per egg) on the immune systems of subsequent juveniles was much less than that of 100 ng of p57 per egg.

1. 11. BKD and the salmonid life cycle

1. 11. 1. Pre-spawning Egg development

Factors affecting immunotolerance are established during pre-spawning egg development. Vertical transmission can occur due to the presence of *R. salmoninarum* in ovarian fluid or possibly during oogenesis. Soluble antigens may enter the egg during oogenesis or from the ovarian (coelomic) fluid. Usually only a few bacteria enter the egg, and Brown et al. (1996) showed that even 1000 killed bacteria do not have the same immunosuppressive effect as 100 ng p57. Evelyn et al. (1984) found vertical infection to occur in only 14% of eggs from a single very highly infected female spawner.

1. 11. 2. Fertilization to hatch

Induction of immunotolerance occurs during the period from fertilization to hatch, and during early post-hatch development (Zapata et al. 1996). Prior to hatching, the health of the fish is influenced by environmental conditions such as oxygen availability, temperature, and environmental pollutants. The egg allows for gas transfer, notably oxygen and carbon dioxide, but all wastes are stored in egg until hatching. Infection cannot be initiated during this stage.

1. 11. 3. Alevin to parr

Once the salmon hatches and enters the alevin stage, it is susceptible to horizontal transmission. The immune system is not yet fully developed, so some immunosuppressive effect may still take place. In hatchery fish, rearing conditions are very important for determining survival rates. Increased density and decreased water flow result in reduced percent returns, with greater effects on chinook than on coho (Banks 1992, 1994; Ewing and Ewing 1995).

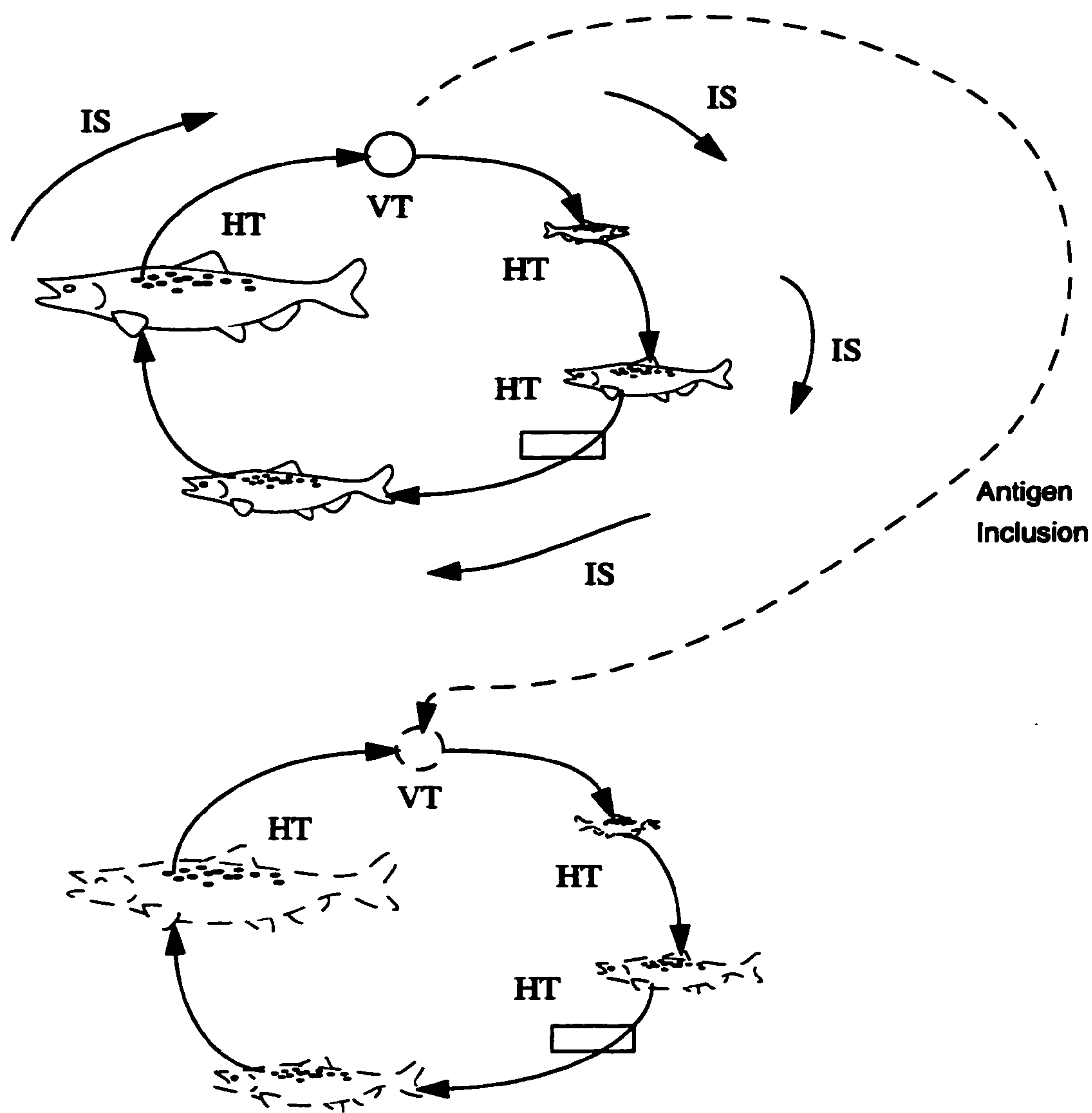

Figure 4 Effects throughout the lifecycle on the transmission and dynamics of BKD. IS = intermittent immunosuppression, caused by stress, temperature or life stage. HT = Horizontal transmission. VT = vertical transmission. Antigen inclusion induced immunotolerant fish are shown in dashed lines.

1. 11. 4. Smoltification, migration and ocean entry

Smoltification involves hormonal, physiological and morphological change and increases in cortisol which suppress internal immune function. Transportation leads to increased stress and proximity which could lead to significant horizontal transmission among salmon and growth of *R. salmoninarum* in stressed salmon. Transition to saltwater involves dramatic physiological change and hormonal action, including increased cortisol levels which reduce immune system function and possibly allow *R. salmoninarum* levels to escalate.

1. 11. 5. Return migration

The stress of return may allow *R. salmoninarum* levels to escalate in fish with latent infections, resulting in vertical transmission, inclusion of antigens in eggs, and horizontal transmission to other spawners possibly inducing vertical transmission to and/or antigen inclusion in their progeny as well. This may be particularly true of spring chinook which spend more time upstream after the return migration waiting to spawn, allowing more time for both the growth of *R. salmoninarum* within a spawner and horizontal transmission to other spawners.

2. Relationship of Antigen Density to Bacterial Load in Tissues and Ovarian Fluid of Spawning Female Pacific Salmon

2. 1. Introduction

In this chapter, using data from three studies that utilized two methods of quantifying the severity of BKD (one that detects the bacteria and another that detects a soluble antigenic fraction thereof), the relationships between bacterial load and antigen density in tissues and fluids are analyzed and mechanisms that might underlie these relationships are proposed. As the p57 antigen and the whole bacteria work in concert to promote the virulence and spread of BKD, knowledge of the relationship between them is critical to a further understanding of the dynamics of the disease.

2. 2. Data and methods

2. 2. 1. Detection methods

The two most rapid and sensitive methods of quantifying the severity of infection in both tissues and fluids are the fluorescent antibody test (FAT), which enumerates *R. salmoninarum*, and the enzyme-linked immunosorbent assay (ELISA), which detects soluble antigen levels. ELISA is more sensitive to minor infections in tissue (Pascho et al. 1987) and FAT is more sensitive in ovarian fluid (Pascho et al. 1998). In a few experiments, both FAT and ELISA have been used to detect infection. Although a relationship between the FAT enumeration and the ELISA value has been shown in the past, this relationship has not yet been formalized, nor has the relationship between bacterial load and antigen density been quantified.

The ELISA and FAT tests give varying amounts of information about tissue and ovarian fluid infection levels. The membrane-filtration FAT quantifies bacteria per ml for ovarian fluid samples (Elliott and Barila 1987), while the FAT for tissue samples reports the number of bacteria per 100 fields of a tissue smear under a microscope. The latter gives a relative infection level, but not an absolute one. The ELISA value is related to density of

antigen (μg ml^{-1}) in solution (Pascho and Mulcahy 1987). In all data examined here, tissues were diluted 1:4 (weight:volume) and ovarian fluid 1:2 (volume:volume) before centrifuging, with the supernatants used for testing.

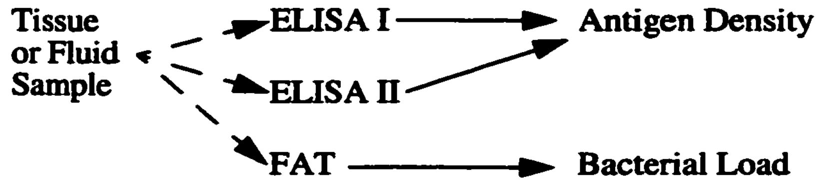

Figure 5 The ELISA diagnostic tests indicate antigen density in solution from tissues or fluids. The ELISA I and ELISA II results can be related to each other using the 1991 data set. The FAT diagnostic tests indicate bacterial load either as an absolute number per ml, as in ovarian fluid, or as a relative measure (number per 100 fields) in tissues.

There is some variation in the sensitivity of individual ELISA tests (even under the same protocol), so that the same ELISA value from two different experiments can reflect different antigen densities in solution. Moreover, there are significant differences between the ELISA I and ELISA II tests, as will be discussed below. The data were thus adjusted to correct for differences in ELISA to antigen relationships among data sets before comparisons of bacterial load and antigen density were undertaken.

2. 2. 2. Data sources

Table 1 Summary of the three data sets.

Publication Date	Species	ELISA test	Kidney Tissue	Spleen Tissue	Ovarian Fluid	n
1987[a]	Coho	I	✔	✔	✔	56
1991[b]	Chinook	I (& II)	✔		✔	30
1998[c]	Chinook	II			✔	37 (of 103)

a Pascho et al. 1987
b Pascho et al. 1991
c Pascho et al. 1998

Three data sets were analyzed for this paper. All three include the results of both the FAT and ELISA tests for tissue and/or ovarian fluid samples. The components of the data sets examined in this paper are summarized in chapter Table 1.

Pascho et al. (1987) tested samples of kidney and spleen tissue, ovarian fluid and blood from 56 sexually mature female coho salmon (*Oncorhynchus kisutch*) for *R. salmoninarum* using five techniques: ELISA, direct or direct-filtration FAT, culture, counterimmunoelectrophoresis, and agarose gel immunodiffusion. ELISA revealed the highest prevalence, finding evidence of infection in all fish (though not in all tissue or fluid samples). The FAT found infection in 86% of fish, with culture finding bacteria in 39%, and the last two techniques finding only 11% and 5% infection, respectively. These results clearly indicate the usefulness of ELISA and the FAT in detecting the presence of *R. salmoninarum*. Here, the ELISA and FAT results are compared for kidney and spleen tissue and ovarian fluid. These data will be referred to as "the 1987 coho data" for the subsequent analysis.

Pascho et al. (1991) tested samples of kidney tissue and ovarian fluid from thirty highly infected sexually mature female chinook salmon (*O. tshawytscha*) at Dworshak National Fish Hatchery (United States Fish and Wildlife Service) for *R. salmoninarum* using both ELISA and FAT. They pre-screened 302 female spawners by kidney and ovarian fluid ELISA, and then tested both kidney and ovarian fluid from candidates for their high infection group via FAT, before settling on thirty for the "high-BKD" group. Thirty female spawners were chosen for their low infection group as well, but all kidney tissues from this group were tested negative via FAT, and therefore only the highly infected group is analyzed here. Kidney FAT values denoted "Too numerous to count" were assigned the value 200,000 for this analysis. While Pascho et al. (1991) used the same ELISA tests as Pascho et al. (1987), they also tested a new, more sensitive ELISA (ELISA II), though the individual results for each sample were not reported for the ELISA II test. These data will be referred to as "the 1991 chinook data" in the subsequent analysis.

Pascho et al. (1998) tested samples of ovarian fluid from 103 spring chinook salmon from Carson NFH for *R. salmoninarum* using both the membrane-filtration FAT and ELISA II tests. The data used below include only the 37 samples for which the FAT enumeration showed a bacterial load of more than ten thousand (10^4) bacteria ml^{-1} in ovarian fluid. Of these samples, 97% (36/37) were also considered positive by ELISA (here: ELISA OD > 0.069) with ELISA values up to 2.512. Only 6% (4/66) of the excluded samples were considered positive by ELISA with a maximum ELISA value of 0.074. This data will be referred to at the "1998 chinook data".

2. 2. 3. ELISA- antigen relationships and correction factors

The study-to-study and even day-to-day variability in the sensitivity of the ELISA test (Pascho et al. 1987) makes the comparison of different data sets and the comparison of FAT to ELISA values difficult. In this paper we consider three data sets, two of which use the ELISA I and a third which uses the ELISA II. The task was not only to standardize the two ELISA I tests to each other, but also to relate the ELISA II test to each of the ELISA I tests, and then to find an absolute relationship between at least one of these tests and antigen density. Ideally, the relationship between antigen density and ELISA values would be recorded with each test by recording results of control samples with a variety of known concentrations. Although this relationship is not given, there is adequate information to make reasonable approximations of the relationships between the ELISA tests and from this to calculate antigen density for each sample.

Pascho and Mulcahy (1987) reported ELISA values for solutions with known concentrations of soluble antigen of *R. salmoninarum*. This relationship appears linear from their second figure which compares the logarithm of known antigen density in solution to ELISA value. Assuming their final standardized procedure which was adopted in the 1987 and 1991 data sets, this relationship can be estimated (from their figure 2) as:

$$ELISA_{1987} = 0.5 + 0.4log_{10}(A) \quad (1)$$

Where A represents the density of antigen (μg ml^{-1}) in solution. This holds for $A > 8.25 \times 10^{-2}$ μg ml^{-1}. For antigen densities below this, the $ELISA_{1987}$ values are indistinguishable from those for the control solutions. The ELISA value corresponding to this threshold antigen density is 0.066 which is denoted the positive-negative cutoff for the $ELISA_{1987}$ test. The value of the positive-negative cutoff varies by experiment as well. Equation 1 was assumed to hold for the 1987 data, which was collected at the same lab and near the same time as the development of the test, and for which day to day corrections were made using a statistical technique developed by the authors (Pascho et al. 1987).

The relationship between the $ELISA_{1991}$ and $ELISA_{1987}$ tests was estimated by assuming the maximum ELISA values to be the same for each data set, and that the calculated positive-negative cutoff should reflect the same antigen density. The former assumption is admittedly somewhat arbitrary. However, the logarithms of maximum countable FAT values are similar for the two data sets, and matching up the average ELISA values over a range of high FAT values would give nearly the same result. We made these comparisons using the tissue data sets since no positive-negative ELISA value threshold for infection was determined for the 1987 ovarian fluid data set. The positive-negative cutoff for tissues was 0.066 for 1987 and 0.058 for 1991. The maximum ELISA value was 1.000 for 1987 and 0.749 for 1991. Assuming a linear relationship between the values of the two ELISA tests and equating their respective maximum and minimum values results in a solvable system of two equations, with the solution being:

$$ELISA_{1987} = 1.35 ELISA_{1991} - 0.12 \tag{2}$$

The ELISA II test was developed before the 1991 data were collected, and Pascho et al. (1991) tested tissue and ovarian fluid by both ELISA I and ELISA II. Using the matched maximum and minimum values of ELISA I and ELISA II from both ovarian fluid and kidney tissue, equation 1 is transformed to reflect these differences. The ELISA I values ranged from 0.041 to 0.709 for both ovarian fluid and kidney tissue, whereas the ELISA II ranged from 0.050 to 2.606 and 0.049 to 2.394, respectively. Assuming a linear

relationship between the ELISA I and ELISA II test values, and taking 2.5 (the average of 2.606 and 2.394) as the ELISA II value equivalent to 0.709 via ELISA I, the resultant relationship for ELISA II is:

$$ELISA_{1991} = \frac{ELISAII_{1998} + 0.1}{3.67} \tag{3}$$

Or, combining Equations 2 and 3:

$$ELISA_{1987} = \frac{ELISAII_{1998} + 0.067}{2.72} \tag{4}$$

2. 3. FAT/ELISA relationship

Relating the numerical results of the ELISA and FAT tests may be useful for comparing studies which use only one of these two methods. ELISA results are typically used to assign fish to Negative, Low, Medium and High infection levels. Relating ELISA to FAT, which indicates the actual bacterial numbers, may help in defining the proper break points between these levels. Few previous direct comparisons of these two tests have been done. Comparisons have rather considered the efficacy of each test in detecting the presence of *R. salmoninarum*.

Pascho et al. (1998) related the logarithm of the ELISA II value to the FAT enumeration in ovarian fluid samples from 103 spawning chinook, finding an R^2 of 0.84. The log transformation of the ELISA II values seems an odd choice, however, as the ELISA value is itself related to antigen density via a logarithmic relationship (see Equation 1). In fact, the ELISA II data appear to increase linearly with FAT enumeration for FAT enumerations greater than one million (10^6) bacterial cells ml^{-1} (see Figure 6). Pascho and Mulcahy (1987) reported that the ELISA value was linearly related to the logarithm of antigen density, for densities above the minimum level of detectability. Given even the simple (though ultimately wrong) assumption that antigen level is linearly related to

bacterial load, the ELISA value would be linearly related to the logarithm of the FAT enumeration.

As mentioned above, the relationship of ELISA value to infection level varies from experiment to experiment. Moreover, the newer, more sensitive ELISA II, which was used for the 1998 data set, gives much higher ELISA values (by a factor of 3 or 4) for the highest infection levels. The original data are presented in Figure 6.

The ELISA to FAT relationship was examined using linear regression. Using the logarithm of the FAT enumeration ($\log_{10}$(FAT + 1) actually) proved to give the best fit for both tissues and ovarian fluid. For the ovarian fluid data, where little increase in the ELISA value was seen until the FAT enumeration exceeded 1 million cells ml^{-1}, the best fit was achieved by dividing the data into two subsets based upon FAT enumeration (less than or greater than 10^6 cells ml^{-1}) and performing two separate regressions.

Table 2 Results of linear regressions of ELISA values versus log Bacterial load via FAT for the raw (uncorrected) data.

Data Set	Subset	Slope	Intercept	R^2	n
1987 coho spleen	N/A	0.167	0.041	0.88	56
1987 coho kidney	N/A	0.189	0.061	0.88	56
1991 chinook kidney	N/A	0.092	0.226	0.83	30
All Tissues	N/A	0.146	0.079	0.83	142
1987 coho ovarian fluid	FAT$<10^6$	0.005	0.071	0.31	50
	FAT$>10^4$	0.252	-1.107	0.92	12
	FAT$>10^6$	0.110	-0.074	0.15	6
1991 chinook ovarian fluid	FAT$<10^6$	0	0.045	N/A	20
	FAT$>10^4$	0.133	-0.551	0.89	17
	FAT$>10^6$	0.095	-0.210	0.86	10
1998 chinook ovarian fluid (ELISA II)	FAT$>10^4$	0.486	-2.453	0.83	36
	FAT$>10^6$	0.706	-4.149	0.89	25

Linear regressions were performed on each data set for the untransformed data, and then for all tissue data together and for all ovarian fluid data together using the transformed

data. Transformed data were generated using the adjustments given in Equations 2 and 4. The results of regressions of ELISA values versus $\log_{10}$(FAT +1) are noted in Table 2. For ovarian fluid samples, the ELISA values do not begin to increase significantly until FAT values reach approximately one million (10^6) bacteria ml^{-1}. Therefore each ovarian fluid data set was divided into two subsets: those samples with less than 10^6 bacteria ml^{-1}, and those with more than 10^6 bacteria ml^{-1}. The 1987 data set contained only six ovarian fluid samples with more than 10^6 bacteria ml^{-1}, so a third regression was run using all samples showing greater than 10^4 bacteria ml^{-1} for all three data sets for comparison. For the 1991 data set, the regression was not significant for the ovarian fluid samples with less than 10^6 bacteria ml^{-1} ($p = 0.23$).

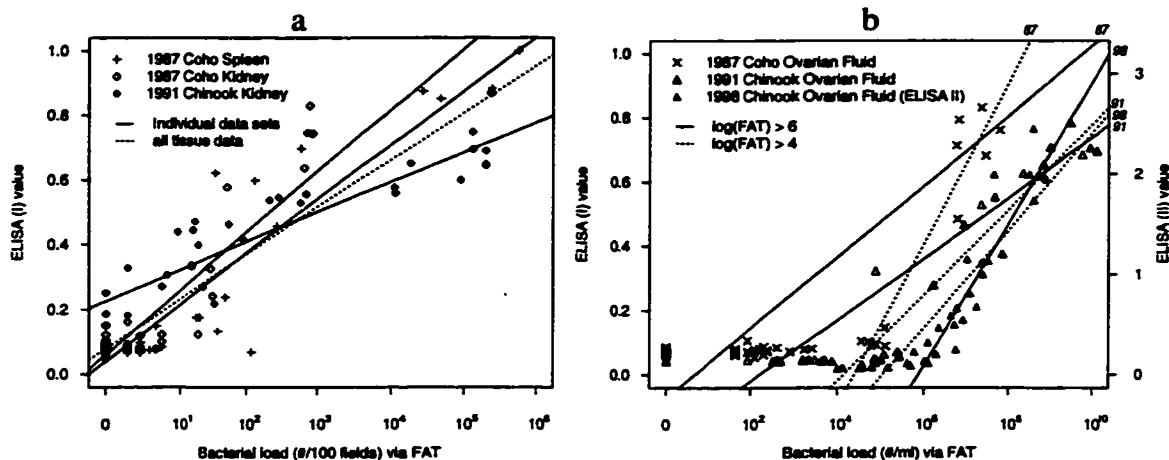

Figure 6 ELISA I or ELISA II (1998 Chinook data) to FAT relationships using the raw (uncorrected) data.

Recall that the tissue data sets are not comparable to the ovarian fluid data sets as the FAT values represent bacteria detected per ml filtered for ovarian fluid samples and bacteria detected per 100 microscope fields of a smear for tissue samples.

The data, corrected for differences in ELISA sensitivities, are plotted in Figure 7. The results of regressions of the corrected ELISA values versus $\log_{10}$(FAT +1) are noted in Table 3. Regressions were done for all tissues together and all fluids together.

Table 3 Results of linear regressions for all tissues or all ovarian fluid data sets combined after the transformations given in Equations 2 and 4.

Data Sets	Subset	Slope	Intercept	R^2	n
Tissues	N/A	0.1721	0.0815	0.85	142
Ovarian Fluid	FAT>10^6	0.2111	-1.0553	0.67	41

The fact that the intercept for tissues is above the negative/positive cutoff (0.066) for the $ELISA_{1987}$ test (to which all other data are standardized) can be explained by two factors. First, ELISA is more sensitive than FAT at low infection levels, (note the high variability of ELISA values at low FAT values), and second, the 1991 data set is biased toward highly infected fish testing positive for BKD in at least one of four tests (kidney or ovarian fluid ELISA or FAT). While infections tend to be most severe in kidney tissue, high level infections in ovarian fluid can lead to higher antigen densities in kidney tissue, which can accumulate antigen from other parts of the body. The original regressions of the 1987 coho kidney and spleen data sets had intercepts below 0.066 (see Table 2).

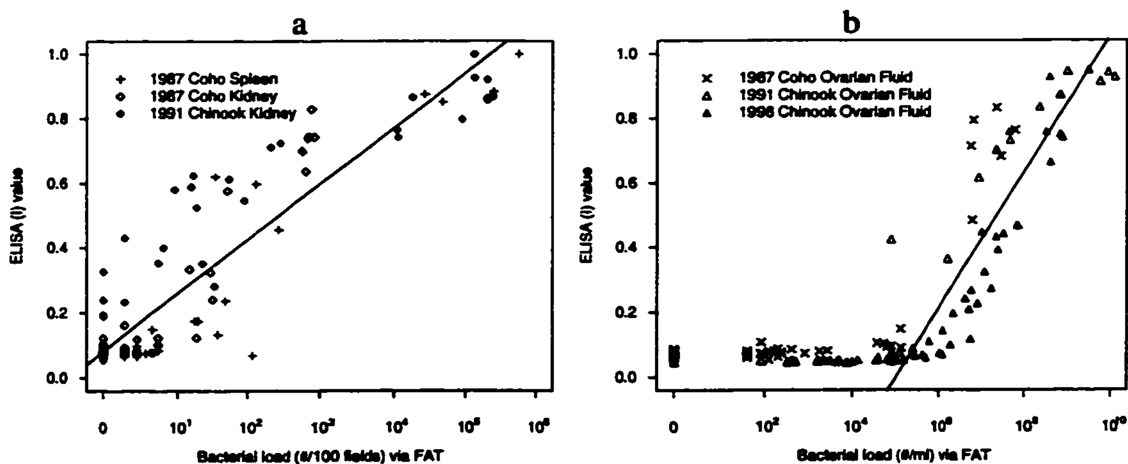

Figure 7 FAT to ELISA I_{1987} relationships. The chinook ELISA values have been recalculated using the transformations given in Equations 2 and 4.

2. 4. Bacterial load- antigen density relationship

The relationship between antigen density and bacterial load was explored after translating the ELISA values into antigen densities. The ELISA values were translated into antigen density in solution via Equation 1. Antigen density in tissues and fluids was then back-calculated from density in solution by multiplying by the dilution factors: 4 for tissue samples and 2 for ovarian fluid samples.

Given the lack of a strong relationship between FAT enumerations and ELISA values in ovarian fluid samples with less than 10^6 cells ml^{-1}, a new measure of ovarian fluid bacterial load was adopted: cells per nanoliter (cells nl^{-1}), found by dividing cells ml^{-1} by 10^6. For tissues, the FAT enumeration represents number per 100 fields, and thus does not give an absolute density of bacteria but rather a relative density. Log bacterial load was used for the bacterial load variable in tissues ($\log_{10}$(#/100 fields + 1)) and fluids ($\log_{10}$(cells nl^{-1} + 1)).

Table 4 Results of regressions of calculated antigen density versus $\log_{10}$ bacterial load (in cells per 100 fields in tissues and cells nl^{-1} in ovarian fluid.)

Data Sets	Slope	Intercept	R^2	n
Tissues	7.1972	-1.8501	0.79	142
Ovarian Fluid	6.3073	-2.9092	0.70	41

The bacterial load to antigen density relationship was modeled applying a few basic assumptions about their relative dynamics. The observed antigen density was assumed to represent the equilibrium state at the observed bacterial level, and this was assumed to be related to the per bacterium rate of antigen production and the rate of antigen breakdown and removal. The results of regressions (Table 4) between antigen density and bacterial load indicate that antigen density increases roughly linearly with the logarithm of bacterial load. This relationship indicates that increases in antigen density result in either a decrease in the per bacterium antigen secretion rate or an increase in the rate of antigen removal and breakdown, or both. An equation representing these

possibilities shows the logarithm of bacterial load to be linearly related to the antigen density and the logarithm of antigen density. Multiple regression was used to fit best curvilinear relationships for all tissue data and for all ovarian fluid data.

The relationship seen between antigen density and bacterial load must be due to underlying mechanisms of secretion, removal and breakdown of antigen. Given the slow dynamics of bacterial growth, we assume the observed relationship represents the equilibrium of a differential equation relating the change in antigen density over time given bacterial load and antigen density. It is clear from the regressions between antigen density and the logarithm of bacterial load (Table 4) that this relationship is not linear. Nor can Michaelis-Menten dynamics, with the antigen acting as the catalyst in its own breakdown, achieve the observed relationship. Equations of the following form were considered:

$$\frac{dA}{dt} = f(A)B - g(A) \tag{5}$$

where A represents antigen density and B represents bacterial load. Equation 5 allows both bacterial secretion of antigen and breakdown or removal of antigen to depend upon antigen density. The relationship of antigen to bacteria has the form:

$$A = c_1 \ln B + c_2 \tag{6}$$

so:

$$B - \alpha e^{rA} = 0 \tag{7}$$

which suggests:

$$\frac{dA}{dt} = aB - be^{rA} \tag{8}$$

However, Equation 8 allows A to be less than zero at equilibrium when B is greater than zero. Considering that both A and its derivative should go to zero as B approaches zero, this implies a loss term which includes A.

$$\frac{dA}{dt} = aB - bAe^{rA} \tag{9}$$

Equation 9 applies to the situation in which antigen is secreted at a constant rate per bacterium, with the rate of removal of antigen increasing exponentially with antigen density. The mechanisms for this increased removal include antigen autolysis (Griffith and Lynch 1991) and increased antibody production. The exponential is surprising even with the two explanations above. One might instead expect instead a power function of A, with the power p most likely 1 or 2, representing either simple mass action or autolysis. The exponential observed in the data can be explained by a decreasing production of antigen by bacteria with increasing antigen densities (autoinhibition), as in the following equation:

$$\frac{dA}{dt} = aBe^{-rA} - bA^{p} \tag{10}$$

The negative exponential of antigen secretion with antigen density may be explained by the rate of antigen production being dependent upon the number of interactions between individual bacteria and antigen or upon the level of saturation of the bacterial cell surface with p57 and its breakdown products. It is possible that both an exponential decrease in antigen production and an exponential increase in antigen breakdown and removal take place simultaneously, resulting in:

$$\frac{dA}{dt} = aBe^{-r_1 A} - bAe^{r_2 A} \tag{11}$$

Equations 9, 10 (with p = 1), and 11 are indistinguishable when considering the equilibrium level of A given any level of B. So all the above can be generalized to:

$$\frac{dA}{dt} = aBe^{-r_1 A} - bA^p e^{r_2 A} \tag{12}$$

with the equilibrium solution being:

$$B = kA^p e^{rA} \tag{13}$$

where $r = r_1 + r_2$ and k is a constant, or, equivalently:

$$\ln B = rA + p\ln A + c \tag{14}$$

or, as the data are in logarithm base 10:

$$\log B = r'A + p\log A + c' \tag{15}$$

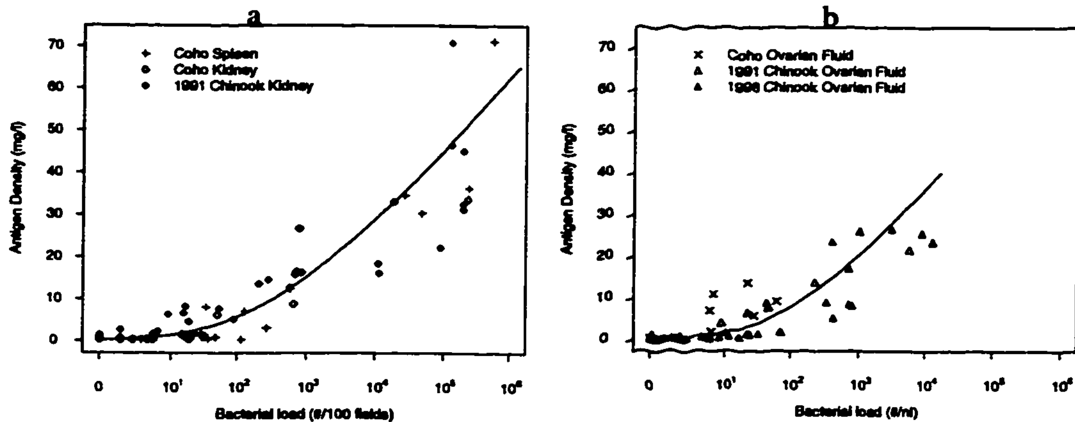

Figure 8 Modeled antigen density to bacterial load curves.

Curves were fit to the data assuming the relationship in Equations 14 and 15 and using the cells nl^{-1} form of the ovarian fluid bacterial load data. The results are shown in Figure 8 and Table 5.

Table 5 Results of regressions based on Equations 14 and 15. The parameters for the bacterial load to antigen density relationship are given for both the natural logarithm and logarithm base 10 forms of the equation.

Data Sets	p	r'	r	c'	c	R^2	n
Tissues	1.282	0.0472	0.109	0.767	1.77	0.89	142
Ovarian Fluid	0.943	0.0485	0.112	0.786	1.81	0.87	123

In the regression results for both tissues and fluids, p, the exponent of A in Equation 12, has a value near 1. This indicates either simple mass action in the removal of antigen with no significant autolysis, or that the autolysis is subsumed in the exponential term. The coefficient of A in the exponential, r, is near 0.11 in both cases, and c is near 1.8. The correspondence in the values of c has no meaning, as the value of c for ovarian fluid depends upon the choice of units, here cells nl^{-1}. The close values of the other two parameters, however, suggest similar processes in both cases with identical multiplicative changes in bacterial load effecting similar increases in antigen density.

Assuming p = 1 and the other results above, the following equation describes the observed equilibrium relationships:

$$B = kAe^{rA} = 6Ae^{0.11A} \tag{16}$$

The change in the equilibrium value of antigen density with bacterial load can then be calculated:

$$\frac{dA}{dB} = \frac{1}{ke^{rA} + rkAe^{rA}} = \frac{A}{B + rAB} = \frac{A}{B + 0.11AB} \cong \frac{9A}{(A+9)B} \tag{17}$$

This can be approximated when A is small (A<<9) by:

$$\frac{dA}{dB} \cong \frac{A}{B} \tag{18}$$

and when A is large (A>>9) by:

$$\frac{dA}{dB} \cong \frac{9}{B} \tag{19}$$

which means that in the limit:

$$A \cong 9 \ln B + C_1 \cong 21 \log B + C_2 \tag{20}$$

This indicates that, according to the model, in the limit, antigen density increases by about 20 $\mu g\ ml^{-1}$ for each tenfold increase in bacterial load. The straight line regressions done on the same data indicate slopes of 6 or 7 (see Table 4), which in contrast approximate the average increase in antigen density for every tenfold increase in bacterial load over the observed range of bacterial loads.

2. 5. Discussion

From data collected to test spawning female Pacific salmon for presence and severity of BKD, a model of the relationship between bacterial load and antigen density in tissues and fluids has been developed in this chapter. The correspondence between two detection methods (ELISA and FAT) was also explored. Strong relationships within individual tissues or fluids were found between the ELISA values and FAT enumerations as well as between the calculated antigen concentrations and bacterial loads.

Bacterial load and antigen density in tissues are strong indicators of fish health, while in the case of ovarian fluid they are predictors of the success of offspring. Bacterial load in tissue is an obvious indicator of severity of disease and predictor of mortality. High bacterial load in ovarian fluid is a precursor to significant vertical transmission to the next generation (Lee and Evelyn 1989). High antigen density in tissues can lead to immunosuppression and tissue destructive granulomas (Bruno 1986b), hastening mortality. High antigen density in ovarian fluid may be a precursor to antigen inclusion in the eggs and subsequent immunotolerance to *R. salmoninarum* infections in offspring (Brown et al. 1996).

The measure of bacterial load used in this paper differed between tissues and ovarian fluid. For ovarian fluid the measure used is cells ml^{-1} or cells nl^{-1}. For tissues the measure is cells per 100 fields. These are not directly comparable. However, the cutoff for observing infection in tissues by this method is about 10^{3}-10^{7} cells g^{-1}. If we assume that this is equivalent to one cell in 100 fields, then 100 fields is equivalent to 10^{-3} to 10^{-7}g or, equating 1 g to 1 ml, 0.1 to 1000 nl. Given the equivalency of the two data sets, it appears that antigen may be about equivalently or perhaps more prevalent in tissues than in ovarian fluid given the same volumetric bacterial load. The increase in antigen density with multiplicative increases in bacterial load appears to be similar in tissues and fluids despite any differences in absolute densities.

Antigen density does not increase linearly with bacterial load, as one might expect, nor is there an apparent maximum antigen density. The dynamics are complex, due to a number of factors. Turaga et al. (1987) noted that up to 1 mg ml^{-1} (1,000 μg ml^{-1}) of p57 and its breakdown products have been found in tissues of severely infected fish. The amounts of soluble antigen calculated here were far less than that amount. There is evidence that p57 is autolytic (Griffith and Lynch 1991, Pascho et al. 1998), yet p57 was found to persist for more than three months in tissues of rainbow trout held at 12 °C (Pascho et al, 1997). Pascho et al. 1998 suggested that ovarian fluid might reduce the detectability of the antigen, but provided no mechanism to explain such an effect. There is evidence of reduced detectability in tissues as well. The breakdown of p57 is accelerated at higher temperatures (Griffiths and Lynch 1991). The relationship between bacterial load and antigen density may change with environmental temperature. Temperature may also have significant impacts upon the efficacy of the immune response (e.g. Sanders et al. 1978) and upon bacterial growth rate, and thus the entire dynamics become more complicated when temperature is allowed to vary. Differences in environmental temperature may account for some of the observed differences between data sets.

The mathematical model developed above indicates that antigen density increases ever more slowly with increasing bacterial load, so that, in the limit, antigen density

increases by no more than about 20 μg ml^{-1} for every tenfold increase in bacterial load. In fact, the model indicates that antigen density per bacterium decreases exponentially with increasing antigen density. The mechanism or mechanisms which control this relationship are not known. Either an exponential increase in antigen breakdown and removal or an exponential decrease in per bacterium antigen secretion with antigen density could account for the observed relationship. The evidence of autolysis of p57 noted above along with the likelihood of increasing antibody production with increasing infection levels supports the possibility of a non-linearly increasing rate of breakdown and removal of antigen with antigen density, though a power function in antigen density has a more obvious mechanistic basis than an exponential in antigen density. Equally compelling, if not more so, is the possibility that the bacteria reduce their production of antigen, thus saving the metabolic cost of such production, when densities of free antigen are high.

One possible mechanism to explain a decreasing antigen secretion rate with antigen density is if an individual bacterium secretes the p57 antigen if and only if it has not interacted with a free p57 antigen molecule over a particular period of time. Assuming complete mixing, the number of interactions of any individual bacterium with free antigen over that time period can be modeled as a Poisson process with the mean $\mu = rA$, with the probability of any individual bacterium having zero interactions over that time period being e^{-rA}. Another possible mechanism is if an individual bacterium secretes the p57 antigen if and only if it has at least one surface attachment site which is not attached to a p57 molecule or one of its breakdown products for a minimum time period. The mean time that any such site would be open would be expected to depend inversely upon the antigen density.

While the cause of the particular form of the relationship between observed bacterial load and observed antigen density is not certain, knowledge of the relationship, regardless of mechanism, is quite useful as antigen concentration in tissue may be of as much or more importance than bacterial load in determining the efficacy of immune response, and antigen concentration in ovarian fluid is likely the prime predictor of

immunotolerance in offspring.

If knowledge of the bacterial load to antigen density relationship and its underlying mechanisms are considered important for the management of the disease, in future publications researchers should indicate the relationship found between antigen density and ELISA absorption for each experiment and report estimated antigen densities as well as ELISA values for each sample. Future research should then also focus upon mechanisms of antigen secretion and breakdown and upon elucidating the underlying dynamics that lead to the observed relationships between bacterial load and antigen density.

3. Vertical Transmission

3. 1. Introduction

BKD is unique in that it is the only bacterial disease of salmonids which is known to be transmitted vertically (from spawner to offspring) (Elliott et al. 1989). *R. salmoninarum* has also been shown to be present in ovarian (coelomic) fluid (Evelyn et al. 1986, Elliott and Barila 1987, Lee and Evelyn 1989). Direct transfer of *R. salmoninarum* cells from ovarian fluid to eggs may be an important route of vertical transmission. It is not clear whether transmission also may occur during oogenesis directly from the blood and ovarian tissue.

That *R. salmoninarum* is successfully transmitted vertically to offspring whereas other bacterial diseases of salmonids are not may be largely due to the fact that salmon are able to harbor and survive high level infections. Other diseases are more likely to kill the infected fish before bacterial loads are high enough to result in significant vertical infection (Evelyn et al. 1986). The ability of *R. salmoninarum* to survive in the presence of the high levels of lysozyme present in salmon eggs, which in contrast is bactericidal to *Aeromonas hydrophila*, *A. salmonicida* and *Carnobacterium piscicola* (Grinde, 1989), is another reason for its successful vertical transmission.

While vertical transmission clearly occurs, it does not appear to occur in all eggs originating from highly infected female spawners. The quantitative relationship between infection levels in female spawners, especially in terms of ovarian fluid bacterial load, and subsequent vertical infection in offspring is the topic of this chapter.

3. 2. Detection of vertical transmission

Typical methods used to detect BKD in the egg stage include culture, gram stain and the fluorescent antibody test (FAT). None of these are sensitive to very low level infections. Brown et al. (1994) used a polymerase chain reaction (PCR) test that detected

the presence of *R. salmoninarum* by amplifying a 501 base-pair region of the gene encoding the p57 antigen. This PCR proved more sensitive than ELISA or FAT techniques and was able to detect infection in all eggs (from spawners testing negative for BKD) injected with as few as 2 *R. salmoninarum* cells. This PCR technique also detected *R. salmoninarum* in 1 of 2 eggs from a single coho spawner whose kidney tissue tested negative for BKD by FAT, ELISA and PCR, in 5 of 7 eggs from chinook spawners whose kidney tissue tested negative for BKD by both FAT and ELISA, and in only 4 of 7 eggs from chinook spawners whose kidney tissue tested positive for BKD via both FAT and ELISA (no kidney tissue PCR was performed on the chinook spawners). They also found infection in only 1 of 69 eggs from 5 coho spawners whose kidney tissue tested positive for BKD via ELISA, but negative via FAT and PCR, and in 0 of 15 from a coho spawner that tested positive for BKD via both ELISA and PCR, though not FAT.

Brown et al. tested spawners' kidney tissue, rather than ovarian fluid, for BKD (via FAT and ELISA, and, in some cases, PCR), so that no direct relationship between ovarian fluid infection levels and percent of eggs found to be infected via PCR can be deduced from this data. It is clear, however, that the PCR test can detect the presence of low level infections in eggs from spawners with undetected infections and that presence of *R. salmoninarum* DNA in the egg does not correlate well with the detectable infections in spawner kidney tissue.

The presence of *R. salmoninarum* bacteria in the egg stage does not necessarily lead to actual vertical transmission. Very low level in-ovum infections may not lead to infection in the offspring, as the few bacteria are unlikely to survive and reproduce. Thus it is not clear whether the infections missed by methods other than PCR actually lead to disease in the offspring, or at what rate. All of the studies examined below used gram stain, culture and/or FAT to detect intra-ovum infections.

3. 3. Data

Evelyn et al. (1984) found no more than a 15% in-ovum infection rate from a single very highly infected female spawner. Lower prevalences of R. salmoninarum infection were noted in eggs exposed to artificially infected ovarian fluid for a period of 12 or 24 hours (Evelyn et al. 1986, Lee and Evelyn 1989). Challenging eggs before fertilization with highly infected ovarian fluid resulted in increased prevalences of BKD in 195-day old smolts (Lee and Evelyn 1989). The observed infection levels (up to 44%) were the result of both vertical and horizontal transmission, and thus possibly an indication, but not a direct test, of vertical infection rates. The higher infection rates observed may be due in part to antigen inclusion induced immunotolerance as discussed in Chapter 5.

Evelyn et al. (1984) tested eggs from a single highly infected female coho spawner using either culture or gram stain to test for *R. salmoninarum*. The ovarian fluid of this female was found to be infected with 4×10^9 *R. salmoninarum* cells ml^{-1}. Evelyn et al. found that the percentage of eggs infected was independent of whether or not the eggs were surface disinfected with iodine and/or treated with erythromycin or penicillin during water hardening. They found 37 of 269 eggs (13.75%) to be internally infected with *R. salmoninarum*. No statistically significant differences were found between the treatment groups, with proportion infected ranging from 10% (5/50) in the group treated with iodine only, with to nearly 20% (13/66) for the group treated with both penicillin and iodine. The 95% confidence interval for percent of all eggs from this female that were vertically infected is 9.65% to 17.85%. This is the only experiment examined here in which all the eggs were infected while within the female spawner rather than under later challenge with artificially infected ovarian fluid.

Evelyn et al. (1986) exposed steelhead and coho to artificially infected ovarian fluid for 24 hours. This resulted in 5.9% of tested steelhead eggs exposed to ovarian fluid with 10^9 cells ml^{-1} being infected and 3.4% of coho eggs exposed to ovarian fluid with 10^{12} cells ml^{-1} being infected. These percentages are lower than those seen by Evelyn et al.

(1984), likely due to the limited exposure time to the artificially infected ovarian fluid. Steelhead eggs exposed to ovarian fluid with 10^3 cells ml^{-1} and coho eggs exposed to ovarian fluid with 10^6 cells ml^{-1} resulted in no detectable infections (0/96 and 0/100 respectively).

Evelyn et al. (1986) also considered the infection levels of 97 day old coho fry which, as eggs, had been exposed to ovarian fluid with 10^{12} cells ml^{-1} for 24 hours. The overall proportion of infection was 5.2% (26/500), ranging from 0 to 19% among the five groups tested. Water hardening in solutions of various forms of erythromycin did not reduce the infection levels in these fry. While horizontal infection is a possibility in this case, dechlorinated city drinking water was used for rearing and was not a possible source of the disease. Rearing temperature ranged from 13-15 °C.

Lee and Evelyn (1989) exposed chinook salmon to artificially infected ovarian fluid for 12 hours. Infection levels in ovarian fluid were approximately 0, 10^3, 10^6, 10^9, and 10^{12} cells ml^{-1}. Natural infections levels in the female spawners which were the source of the experimental eggs were low (10^1-10^2 cells ml^{-1}). Pools of 10 eggs each (occasionally 5-14) were tested via culture and the fluorescent antibody test (FAT). No infection was detected in pools of eggs infected in the 0 or 10^3 cells ml^{-1}groups. One of 37 or 38 pools (2.7 or 2.6% indicating about 0.3% of individual eggs infected) was found to contain *R. salmoninarum* cells in each of the 10^5 and 10^7 cells ml^{-1}groups, and 8 of 38 pools (21% indicating about 2.3% of individual eggs infected) were found to be infected in the 10^{12} cells ml^{-1} group.

Lee and Evelyn (1989) also reared duplicate subsets of juveniles from each exposure group in dechlorinated drinking water until they reached the smolt stage when, at 195 days after hatch, they were tested for infection via the FAT. They found 0 to 34% average infection as indicated in Table 6 (both duplicate points at each level are shown in Figure 10). These infection levels were due to both vertical transmission and subsequent horizontal transmission. Infections were found in 1-2% of smolts which were not exposed

to artificially infected ovarian fluid while in the egg stage, but whose female spawners harbored low level infections (28-113 cells ml^{-1}). The infections in these smolts probably arose from intra-ovum infections which were either undetectable via culture and FAT tests or in a very small percentage of eggs, as no vertical infection was detected when eggs from these females were tested.

The very low prevalence of infection (1-2%) in smolts from mildly infected (28-113 bacteria ml^{-1} detected in the ovarian fluid) female spawners observed by Lee and Evelyn (1989) may be due to fairly low level intra-ovum infections undetectable by FAT. Alternatively, they may be due to very rare FAT-detectable intra-ovum infections which were simply not among the sample tested.

Table 6 Infection prevalences in eggs and progeny when exposed to infected ovarian fluid in the egg stage.

<table>
<tr><th>Year of publication</th><th>Species</th><th>Ovarian fluid infection level cells ml^{-1}</th><th>Exposure time</th><th>Percent of eggs infected</th><th>Post-hatch rearing time</th><th colspan="2">Percent of fish infected</th></tr>
<tr><td>1984</td><td>Coho</td><td>4x10^9</td><td>Natural</td><td>13.75</td><td></td><td colspan="2"></td></tr>
<tr><td rowspan="4">1986</td><td>Coho</td><td>1.3x10^6</td><td>24 hours</td><td>0.0</td><td></td><td colspan="2"></td></tr>
<tr><td>Coho</td><td>1.3x10^{12}</td><td>24 hours</td><td>3.4</td><td>97 days</td><td colspan="2">5.2</td></tr>
<tr><td>Steelhead</td><td>1.3x10^3</td><td>24 hours</td><td>0.0</td><td></td><td colspan="2"></td></tr>
<tr><td>Steelhead</td><td>1.3x10^9</td><td>24 hours</td><td>5.9</td><td></td><td colspan="2"></td></tr>
<tr><td rowspan="7">1989</td><td>Chinook</td><td>28</td><td>Natural</td><td>0.0</td><td>195 days</td><td colspan="2">1</td></tr>
<tr><td>Chinook</td><td>56</td><td>Natural</td><td>0.0</td><td>195 days</td><td colspan="2">1</td></tr>
<tr><td>Chinook</td><td>113</td><td>Natural</td><td>0.0</td><td>195 days</td><td colspan="2">2</td></tr>
<tr><td>Chinook</td><td>1.7x10^3</td><td>12 hours</td><td>0.0</td><td>195 days</td><td>8</td><td>10</td></tr>
<tr><td>Chinook</td><td>1.7x10^5</td><td>12 hours</td><td>0.3</td><td>195 days</td><td>9</td><td>20</td></tr>
<tr><td>Chinook</td><td>1.7x17^7</td><td>12 hours</td><td>0.3</td><td>195 days</td><td>15</td><td>29</td></tr>
<tr><td>Chinook</td><td>1.7x10^9</td><td>12 hours</td><td>2.3</td><td>195 days</td><td>24</td><td>44</td></tr>
</table>

3. 4. Analysis

The three studies reviewed above differ greatly in the amount of time eggs were exposed to infected ovarian fluid. Evelyn et al. (1984) examined eggs from a naturally infected female coho salmon with an ovarian fluid bacterial load of over 4 billion per ml. Evelyn et al. (1996) artificially infected coho and steelhead ovarian fluid with up to 1.4 billion (coho) or 1.4 trillion (steelhead) bacteria per ml. Unfertilized eggs were immersed in this fluid for 24 hours before either water hardening or fertilization and water hardening. There was no significant difference in infection rates between fertilized and unfertilized eggs. Lee and Evelyn (1989) immersed unfertilized chinook salmon eggs in infected ovarian fluid for 12 hours before fertilization and water hardening.

Detectable in-ovum infection does not appear to occur from exposure to ovarian fluid with a bacterial load of less than 10^5-10^6 cells ml^{-1}. No significant infection is seen until an ovarian fluid density of 10^9 cells ml^{-1}, yet an increase to above 10^9 cells ml^{-1} did not seem to increase the infection rate. However, as the data represents three species of salmon and three experimental protocols, it is difficult create an overall picture. What is clear is that at ovarian fluid infection levels between 10^5 and 10^7 cells ml^{-1}, vertical infection is still quite rare. At 10^9 cells ml^{-1}, vertical infection may be important, but it is not clear that even a natural infection level of 10^{12} cells ml^{-1} would result in an egg infection rate higher than the nearly 14% observed by Evelyn et al. (1984).

The presence of *R. salmoninarum* in smolts from the control treatment indicates that vertical infection can occur from natural infections where there are only low levels of bacteria in the ovarian fluid. This does not necessarily mean that these eggs were infected by the ovarian fluid as it is possible that the infection occurred during oogenesis. The infection level in each group is much higher than the infection level seen by testing eggs (Table 6). This is probably due to both the presence of very low density undetectable infections in some eggs and horizontal transmission during rearing. Antigen inclusion induced immunotolerance may have increased the susceptibility of some of the fry to

infections resulting from horizontal transmission.

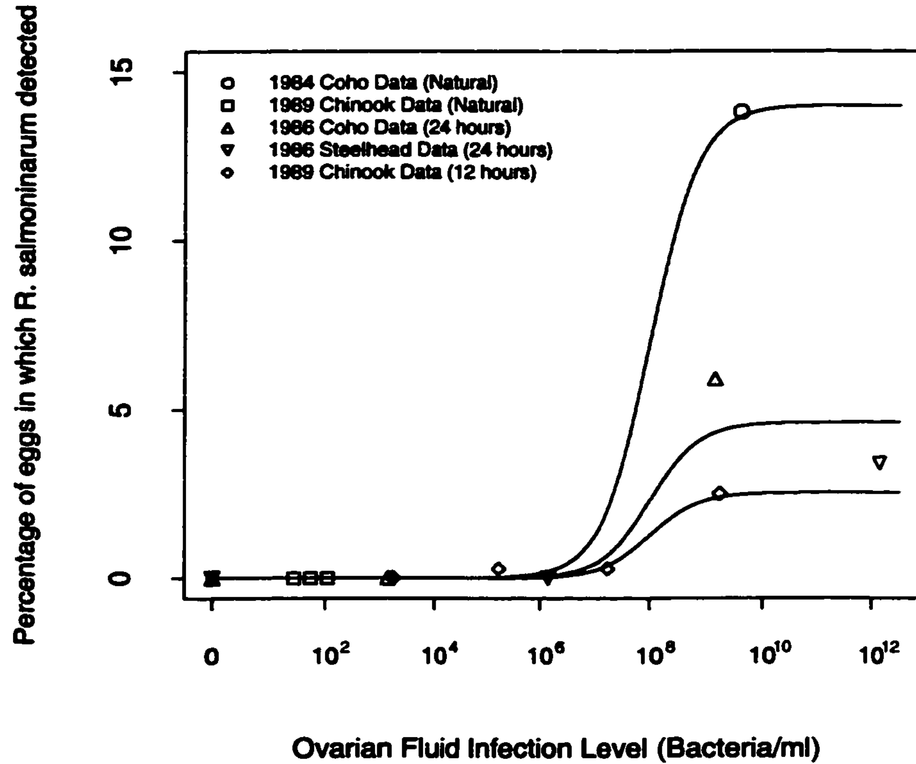

Figure 9 Percent detected vertical infection from the three experiments. Lines represent model fits for natural, 24 hour and 12 hour exposure to ovarian fluid at each infection level. Data are from Table 6. Curves are from Equation 21.

The following model fits both the data points which represent the effect of long term contact with infected ovarian fluid in vivo and those that the relative levels of infection seen from the experiments using short exposure times in vitro.

$$VI = 0.14\left(\frac{B}{B + 10^8}\right)(1 - e^{-t/2.5}) \qquad (21)$$

Where VI is the percent of detectable vertical infection (using the FAT, gram stain or culture tests), B is the ovarian fluid bacterial load in cells ml^{-1}, and time (t) is in days.

Equation 21 results in the curves seen in Figure 9, with natural infection (t >> 1 day, $e^{-t/2.5} \cong 0$), 24 hour exposure (t = 1 day) and 12 hour exposure (t = 0.5 days).

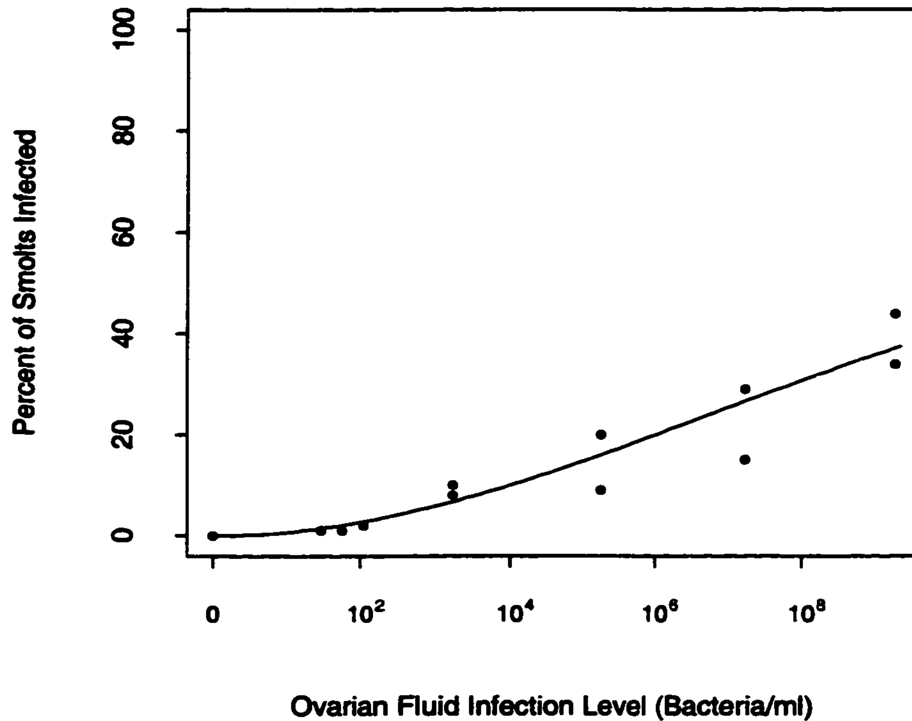

Figure 10 Percent of 195 day old smolts infected exposed to various natural (unpaired points) or artificial ovarian fluid infection levels. Data are from Table 6. Curves are from Equation 22.

Infection levels in smolts increase with exposure to ovarian fluid with naturally or artificially high bacterial loads, as reported by Lee and Evelyn (1989) (Figure 10). Lee and Evelyn also reported that no BKD-related mortality occurred in the first 195 days, before the sample was taken. The percent infection in smolts can be approximated by:

$$P = \frac{(\log(B))^2}{144 + (\log(B))^2} \qquad (22)$$

Equation 22 was chosen simply because it fits the data well. It does not appear to have close mechanistic ties to Equation 21. The mechanisms which lead to a particular

proportion infected at smoltification are not the same that lead to a proportion of eggs with detectable *R. salmoninarum* via FAT, gram stain or culture. The latter requires the entry of a significant number of bacteria into the egg. Infection at smoltification simply requires the successful horizontal transmission of the disease from a co-habitating fish. Individual juvenile salmon which experienced rather severe vertical infection are more likely horizontally infect others in their cohort. Thus increases in infection at smoltification reflect both the prevalence and severity of vertical transmission, as well as the susceptibility of the cohort to the disease. Susceptibility might be increased in many salmon due to antigen-inclusion induced immunotolerance (see Chapters 1.10 and 5.4), which is correlated with incidence of vertical infection due to the correlation between bacterial load and antigen density explored in Chapter 2. The result of all this is that the equations describing vertical infection in eggs and infection in smolts have no obvious strong relationship.

3. 5. Horizontal transmission model

The infection levels seen in Figure 10 are due to vertical transmission and subsequent horizontal transmission. This can be modeled using the S-I-R (susceptible, infected, recovered) model (Anderson and May 1991, Murray 1989, Kermack and McKendrick, 1927). In this case, we can only considered infected those fish with detectable infection, and assumed that infection levels at 195 days represented the cumulative effect of horizontal transmission over the course of that time.

$$\frac{dI}{dt} = \alpha IS \tag{23}$$

where $I + S = 1$, so that:

$$\frac{dI}{dt} = \alpha I(1-I) = \alpha(I-I^2) \tag{24}$$

The solution to this equation is:

$$I(1-I) = I_0(1-I_0)e^{\alpha t} \qquad (25)$$

where I_0 is the initial infection level, resulting from vertical transmission.

If we assume that the actual vertical transmission is slightly greater than that observed by the FAT test (given in Table 6), and set average vertical infection for the two groups exposed to the highest infect ovarian fluid at 3%, the best bit is achieved with the following value for alpha:

$$\alpha = 0.0105 \qquad (26)$$

Now Equation 25 can be solved for I_0 for each group, which is done in Table 7:

Table 7 Calculated percent of eggs infected assuming the S-I-R model.

Ovarian fluid infection level cells ml^{-1}	Exposure time	Observed percent of eggs infected	Percent of 195 day old smolts infected	Calculated percent of eggs infected
28	Natural	0.0	1	0.13
56	Natural	0.0	1	0.13
113	Natural	0.0	2	0.25
1.7×10^3	12 hours	0.0	8	1.0
1.7×10^3	12 hours	0.0	10	1.2
1.7×10^5	12 hours	0.3	9	1.1
1.7×10^5	12 hours	0.3	20	2.1
1.7×17^7	12 hours	0.3	15	1.7
1.7×17^7	12 hours	0.3	29	2.7
1.7×10^9	12 hours	2.3	24	2.4
1.7×10^9	12 hours	2.3	34	3.3

The calculated percent of eggs originally infected under this model differ greatly from the observed percent of eggs infected. This is partially due to inability of the FAT, gram stain and culture tests to detect the lowest level infections. However, the SIR model is based upon the concept of random individual contacts which result in transmission of the disease and the dynamical equations. However, in the aqueous environment, while some transmission may be due to individual contact, such as through ingestion of feces, other infections are due to the bacterial load in the water. Thus the S-I-R model may not accurately represent the dynamics in this case. Water flow rate and rearing density are likely to affect both routes of infection, as they affect density of bacteria in the water and residence time of feces.

Another complication touched upon earlier is the induction of immunotolerance in some individuals due to the entry of substantial amounts of soluble antigen of *R. salmoninarum* into the egg before fertilization. While it is not clear if the artificially infected ovarian fluid would have similar amounts of antigen as seen in naturally infected ovarian fluid, this is one possible explanation for the increasing infection levels in the smolts that were exposed to highly infected ovarian fluid as eggs. If, in fact, it is not the rate of infection which is limiting, as is assumed in the S-I-R model, but rather the limiting factor is susceptibility to the disease, then the infection levels seen in the 195 day old smolts largely reflect rates of immunotolerance induction. Some infection would be expected even without any immunotolerant fish, as long as the requisite vertical transmission occurs.

Since the exposure to infected ovarian fluid was limited in time (12 hours) and the ovarian fluid was artificially infected, so that the antigen density cannot be assumed to be related to bacterial load by Equation 16 (in Chapter 2), this does not provide a basis for modeling the relationship between bacterial load and proportion of immunotolerant offspring.

3. 6. Discussion

Vertical transmission of BKD does occur, but is, for an unknown reason, relatively rare even among eggs and offspring of extremely highly infected female spawners. The PCR detection technique is useful for detecting low level infections in eggs, but it is not clear if these low level in-ovum infections lead to actual infection in offspring. Given the weak correlation between kidney infections and vertical transmission, either ovarian fluid or eggs themselves should be tested for *R. salmoninarum* infection when broodstock segregation or culling is undertaken.

The modeling undertaken here to explain infection levels in smolts due to both vertical and subsequent horizontal transmission only applies to situations in which a treated water supply is used for rearing. In most artificial rearing facilities, as well as certainly in the wild, this condition is not met. Under many actual rearing conditions, infection levels are much less dependent upon vertical transmission rates and more dependent upon external sources of *R. salmoninarum*.

4. Effects of Environmental Temperature on the Progression of BKD

4. 1. Introduction

There are two observable effects of temperature on mortality rate of salmonids infected with *R. salmoninarum*. Higher temperatures often lead to lower total mortality, while decreasing the mean time to mortality in those fish that do die due to the effects of the disease. The relationship of temperature to total BKD-related mortality and to time to mortality are examined in this chapter.

Teleosts, being ectothermic animals, must contend with internal changes in temperature that would be lethal to endotherms. These temperature changes provide an extra challenge for the ectotherm immune system and for the maintenance of homeostasis. Teleost immune systems have adapted so as to be optimally competent near normal summer temperatures for each species (Manning and Nakanishi 1996). In general, temperatures well above the optimum result in stressed fish and increased disease-induced mortality. Temperatures significantly below the optimum, however, may be immunosuppressive. Low temperatures may seriously impair the action of teleost helper T cells, reducing the production of cytokines and thus eliminating an important route of phagocyte stimulation (Hardie et al. 1994). Helper T cells involved in the stimulation of B cells for the production of antibodies are affected as well (Bly and Clem, 1992).

Researchers have recognized the immunosuppressive effects of low environmental (and thus body) temperatures on ectotherms for over three decades (e.g. Avtalion 1969). The primary mechanism of this low-temperature immunosuppression in teleosts has been shown to be suppression of virgin T cell activation (Manning and Nakanishi 1996) rather than suppression of B cells or primed T cells. Immunosuppressive temperatures have been established in some teleost species with more or less certainty. These include 14 °C for carp (*Cyprinus caprio*), 22 °C for bluegill (*Lepomis macrochirus*), 17-22 °C for channel catfish (*Ictalurus puctatus*), and 4 °C for salmonids (Bly and Clem 1992). As will be made evident below, there is evidence that temperatures well above 4 °C may significantly affect

the efficacy of the salmonid immune response to *R. salmoninarum* infection.

Continued or increased functioning of components of the non-specific immune system at low temperatures may help to make up for the concurrent weakness of the specific immune system in fighting off many diseases. Phagocytes acclimatized to cold temperatures have higher respiratory burst activity than those acclimatized to warmer temperatures when stimulated with macrophage activating factor (MAF) (Le Morvan et al. 1998). Production of MAF, however, has been shown to be suppressed at low temperatures (Hardie et al. 1994). The complement system continues to function at low temperatures, though with less efficacy (Yano 1996).

The effectiveness of the immune reaction to BKD may be particularly sensitive to low temperatures. If the primary immune system mechanism for defeating BKD is the activation of phagocytes by helper T cells via MAF, this alone would explain the sensitivity of BKD-infected salmon to the effects of low temperature immunosuppression. Moreover, since opsonization (tagging) of *R. salmoninarum* by antibody, complement, or both has been shown to aid *R. salmoninarum* in surviving entry into and reproducing within phagocytic cells (Bandin et al. 1995), rather than increasing the bactericidal activity of phagocytes, continued complement activity may actually accelerate the progression of *R. salmoninarum* infection rather than hinder it.

The major soluble antigen of *R. salmoninarum*, often referred to as p57, has been shown to be both a significant virulence factor of BKD, and an immunosuppressive agent in its own right. Griffiths and Lynch (1991) found the p57 antigen to be autolytic, with instability increasing with temperature. High temperatures may thus reduce antigen loads and thereby diminish the tissue damaging and immunosuppressive effects of the p57 antigen.

Temperature affects the growth rate of bacterial parasites. The growth rate of *R. salmoninarum* peaks in the range of 15 to 18 ^{o}C (Weins and Kaattari, 1999). In general,

temperatures above the optimum for cold water fish species, such as salmonids, result in higher bacterial growth rates that, coupled with the stress of high temperatures, often spell doom for infected fish. Likely due to the reasons stated above, the opposite was found to be true for three salmon species artificially infected with BKD and raised at seven different temperatures between 3.9 °C and 20.5 °C (Sanders et al. 1978). These data are reanalyzed below.

Pascho et al. (1991) followed two groups of spring chinook salmon during 17 months of rearing from hatch to release. The two groups were the result of a broodstock segregation experiment at Dworshak National Fish Hatchery begun in 1988. While the purpose of the study was to compare mortality rates and infection levels of offspring of highly infected female spawners to offspring of female spawners with little or no evidence of infection, the data also provide evidence for the influence of temperature on the course of BKD infection.

Murray et al. (1992) report on the time to death of four groups of juvenile chinook either artificially infected with varying doses of *R. salmoninarum* or challenged by cohabitation with the artificially infected fish. These data, along with data from Sanders et al. (1978), provide a basis for modeling the bacterial growth rate and the infected salmon's rate of progression towards mortality as a function of environmental temperature.

4. 2. Total mortality with temperature

4. 2. 1. Sanders data

Few experiments have been undertaken with the goal of elucidating the effect of temperature on the progression and outcome of BKD. One experiment did follow coho, sockeye and steelhead for 3-4 months after artificial infection. Sanders et al. (1978) artificially infected juvenile coho and sockeye salmon and steelhead trout with *R. salmoninarum*. These fish were then reared in freshwater for 90 days (sockeye) or 112 days (coho and steelhead) at seven temperatures ranging from 3.9 to 20.5 °C. Two groups

of infected fish and two groups of control fish were reared for each species and temperature. Each group consisted of approximately 25 fish.

The three species differed in average weight at the beginning of the experiment. Coho, averaging 6.5 g, were injected with 2.5×10^8 bacteria, or 4×10^7 bacteria/g. Steelhead, averaging 18 g, were injected with 2.9×10^8 bacteria, or 1.6×10^7 bacteria/g. Sockeye, averaging 38 g, were injected with 3.3×10^8 bacteria, or 8.7×10^6 bacteria/g. This difference in size (and possibly developmental stage) cannot be ruled out as a cause of observed differences in mortality rates and time to mortality among the three species. The difference in inoculation dose is most likely insignificant.

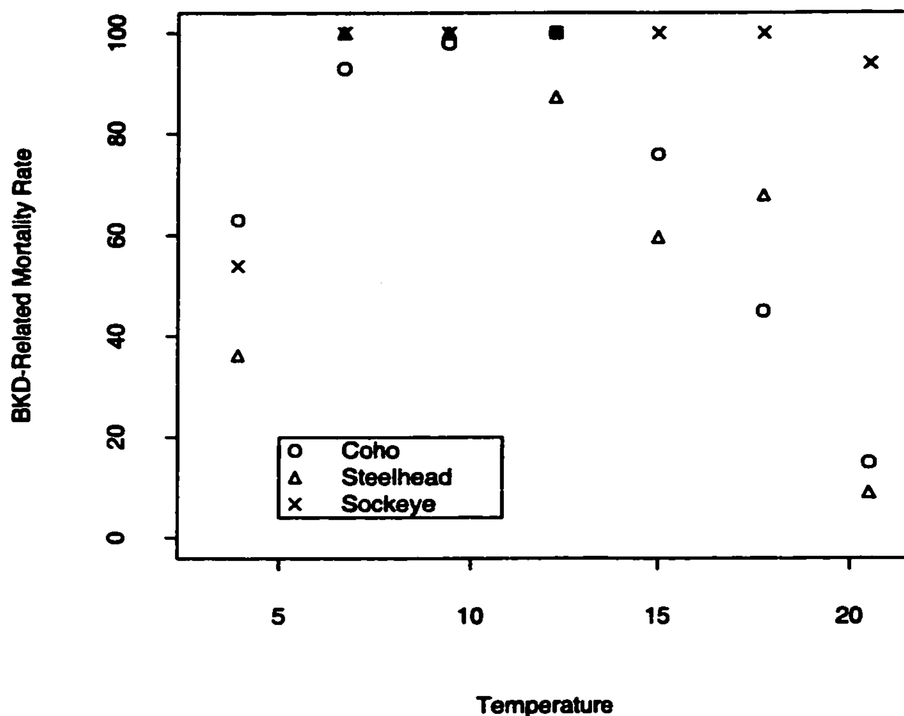

Figure 11 Calculated BKD-related mortality rates for each species at each temperature. The points at 3.9 °C underestimate the true rates due to the limited time of the experiments and the slow rate of mortality at that temperature.

For those fish that died during the experiment, the level of infection at death was determined by Sanders et al. using the fluorescent antibody test (FAT). Those mortalities that could not be attributed to BKD were not included in their analysis. Let total fish in each species at each temperature be represented by N_{TOT}, those that die due to BKD by n_{BKD} and those that die due to other causes by n_{OTH}. Sanders et al. calculated the BKD-related mortality rate as:

$$\mu_{BKD} = \frac{n_{BKD}}{N_{TOT}} \tag{27}$$

This, however, ignores the mortalities that were determined not to be due to BKD. This is not a problem for sockeye, for which all mortalities were determined to be BKD-related, or coho, for which only a few mortalities were determined to be due to other causes. For steelhead, however, the majority of mortalities at the two highest temperatures were not due to BKD, with smaller proportions of mortalities at other temperatures being due to other causes. In these cases, determining the true BKD-related mortality rate is complicated by the other mortality as it is not know how many of those fish would have died due to BKD if they had not died first of other causes. The time to mortality for these other mortalities is not given by Sanders et al. (1978), so it is not possible to place the other mortalities as occurring before, after or simultaneously with the BKD-related mortalities. All that can be done is to assume compensatory mortality. In equation form, then, total survivorship (S_{TOT}) is:

$$S_{TOT} = \frac{N_{TOT} - n_{BKD} - n_{OTH}}{N_{TOT}} = (1 - \mu_{BKD})(1 - \mu_{OTH}) \tag{28}$$

Where μ represents the rate of mortality due to BKD or other causes. Under this assumption, the ratio of the mortality rates should be equal to the ratio of the observed mortalities:

$$\frac{\mu_{OTH}}{\mu_{BKD}} = \frac{n_{OTH}}{n_{BKD}} \tag{29}$$

or:

$$\mu_{OTH} = \frac{n_{OTH}}{n_{BKD}}\mu_{BKD} \tag{30}$$

So that, if we let:

$$k = \frac{n_{OTH}}{n_{BKD}} \tag{31}$$

Then:

$$S_{TOT} = (1-\mu_{BKD})(1-k\mu_{BKD}) \tag{32}$$

which can be solved using the quadratic formula:

$$\mu_{BKD} = \frac{1+k-\sqrt{(1+k)^2-4k(1-S_{TOT})}}{2k} \tag{33}$$

Equation 33 was used to calculate the BKD-related mortality rates seen in Figure 11.

For all three species, the highest BKD-related mortality rates were observed in the middle of the temperature range studied, with decreased BKD-related mortality at the extremes (see Figure 11). The reduced mortalities at lower temperatures are at least partially (and perhaps completely) explained by the limited duration of the experiment (90 days for sockeye and 112 days for coho and steelhead) coupled with the slower progression of the disease at low temperatures. The decrease in BKD-related mortality rates at higher temperatures can best be explained by increased resistance to and recovery from the disease due to temperature related increases in immune system functioning and/or temperature related reduction in the virulence of *R. salmoninarum*.

BKD was the only bacterial disease, of four studied by Sanders, Fryer, Pilcher and their colleagues, for which the highest temperatures (17.8 and 20.5 °C) resulted in decreased total mortality. BKD also produced the highest mortalities at low temperatures

(3.9 and 6.7 °C) among all diseases tested. Results for the other diseases studied are reported in Holt et al. (1975) and Groberg et al. (1978).

For coho, BKD-related mortality was highest in temperatures in the range of 6.7-12.2 °C, with nearly linearly decreasing mortality levels between 12.2 and 20.5 °C. At 12.2 °C, all 42 test fish died. Similar results were seen at 9.4 and 6.7 °C with 98% (47/48) and 93% (41/44) mortality respectively. At 3.9 °C, only 63% (26/41) mortality was observed, but due to the slow rate of events at this low temperature, complete mortality may not have been observed before the end of the experiment. This may also be the case at 6.7 and 9.4 °C, with complete mortality expected to occur in a longer experiment. All mortalities at these temperatures were determined to be due to BKD, by the presence of severe infection in all dead fish.

Mortality rates for coho declined above 12.2 °C. Only 76% mortality occurred at 15.0 °C, 50% (though only 41% conclusively due to BKD) at 17.8 °C, and 23% (though only 14% conclusively due to BKD) at 20.5 °C. Using Equation 33 for data at 17.8 and 20.5 °C reveals BKD-related mortality rates of 76%, 45% and 15% at 15, 17.8 and 20.5 °C. This can be modeled linearly and exactly by assuming that, given the dose administered and the initial size of the coho, complete mortality due to BKD will occur at temperatures up to 12.8 °C with an 11% drop in mortality for every 1 °C above that, at least up to 20.5 °C.

Coho model:

$$\begin{aligned} mort &= 1 && T < 12.8°C \\ mort &= 1 - 0.11(T - 12.8°C) && 12.8°C \le T \le 20.5°C \end{aligned} \qquad (34)$$

Zero to seven percent mortality was observed in coho controls at all temperatures, with only 10 of 333 control coho dying during the experiment. Only two of these control coho were determined to have died of BKD, and those at 6.7 and 9.4 °C. Thus, the incidence of naturally occurring BKD was low. Eight coho in the control group died from

causes other than BKD. Similarly, eight of the test coho were found to have died from causes other than BKD.

Temperature had a smaller effect on BKD-related mortality levels in sockeye (Figure 11). For artificially infected sockeye held at temperatures between 6.7 and 17.8 °C, complete mortality was observed. A slight decrease was observed at 20.5 °C, where 94% (46/49) died. Only 54% (28/32) died at 3.9 °C, but again, complete mortality was certainly not observed before the end of experiment, in this case only 90 days in duration. Only 3% of control sockeye died. It appears that, at least under the conditions of this experiment, sockeye are particularly susceptible to BKD at all temperatures.

BKD-related mortalities among steelhead followed a pattern similar to that of coho (Figure 11) however, total mortality was high at all temperatures. Other factors resulted in mortality that could not be attributed to BKD. Relatively high mortality rates were observed in control fish at 17.8 and 20.5 °C as well as at 3.9 °C. The presence of significant and highly variable non-BKD related mortality in both the control and treatment groups reduces the ability to accurately calculate BKD-related mortality rates. However, considering the data in chapter Figure 11, if we ignore the "outlier" at 17.8 °C, where half of all steelhead died due to non-BKD related causes, a linear model fits quite closely, similar to the coho model (Equation 34), with the decrease in mortality beginning at an lower temperature, as follows:

Steelhead model:

$$\begin{aligned} mort &= 1 & T < 10.8°C \\ mort &= 1 - 0.094(T - 10.8°C) & 10.8°C \le T \le 20.5°C \end{aligned} \quad (35)$$

The overall pattern of BKD-related mortality indicates that higher temperatures reduce total mortality due to the disease in both coho and steelhead, with little effect being observed in sockeye in this experiment.

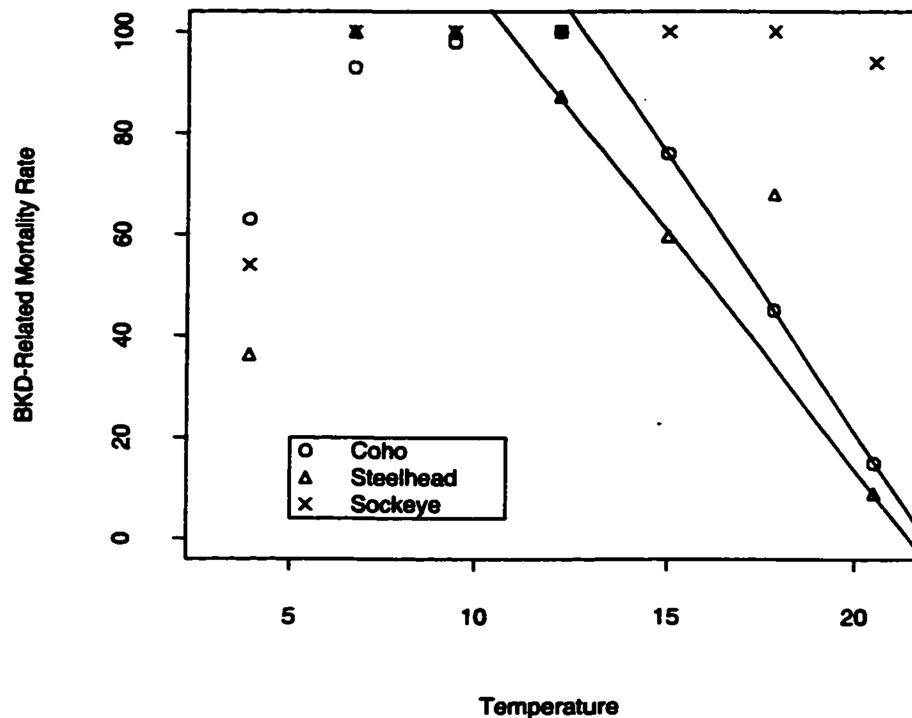

Figure 12 Calculated BKD-related mortality rates for each species at each temperature (Figure 11) with coho and steelhead model lines.

4. 2. 2. Broodstock segregation data

Further evidence for an inverse relationship between temperature and the success of *R. salmoninarum* infections is seen in the 1988 broodstock segregation experiment performed at Dworshak NFH (Figure 13). In this experiment, the eggs and offspring of 30 females with little or no evidence of infection and the eggs and offspring of 30 females with high levels of infection in ovarian fluid and/or kidney tissue were kept separate through hatch and then raised in separate raceways. These two groups were then used to study survival and BKD infection levels (via ELISA) in hatchery, relative recovery to McNary dam, and survival under salt water challenge. Pascho et al. (1991, 1993) and Elliott et al. (1995) report a strong affect of the infection level of female spawners on the in-hatchery infection levels and multiple life stage survival rates of their progeny. In Chapter 5 the time course of mortalities and infection levels are considered and evidence is

presented that the observed differences are most likely caused by antigen-inclusion induced immunotolerance, the existence of which was suggested by Brown et al. (1996).

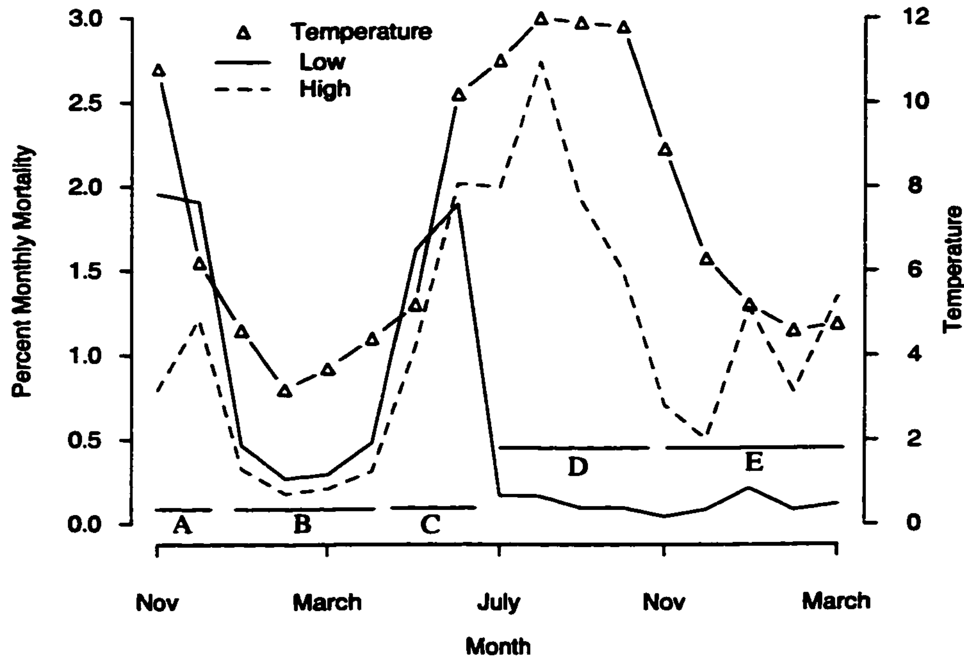

Figure 13 Monthly mortality rates for the low- and high-BKD groups from November 1988 through March 1990. Data from Pascho et al. (1991) with recalculated monthly mortality rates from the original data. Temperatures are mean monthly water temperatures. Note that mortality includes both BKD and non-BKD related mortalities.

The first significant differences in mortality and infection levels occur in July, 8 months after hatch, and just after average temperatures have reached above 10 °C for the first time in the lives of the young chinook salmon (see Figure 13). It appears from this that prior to the warmer temperatures seen with the advent of summer, all fish were immunosuppressed due to the low temperatures. After this point the immune systems of a portion of the salmon, largely among offspring of the high-BKD broodstock, were still compromised due to the effects of in-ovum antigen-inclusion induced immunotolerance. It appears, moreover, that immunotolerance and low temperature immunosuppression have compounding effects, as although many of the high-BKD fish did die in summer months,

likely due to increased bacterial growth rates coupled with immunotolerance, it is also true most individuals that survived in both groups had low levels of infection in November at 12 months after hatch (see Figure 23, Chapter 5).

The time course of the mortalities can be explained as follows. The mortality in November and December of 1998 (Marked "A" in Figure 13) are early losses unrelated to BKD. The higher mortality rates observed among the low BKD offspring are due to relatively high mortality in a single nursery tank. The low levels of mortality seen in both group in January through April 1989 ("B") reflect the slow growth of the bacterium in those infected either due to outside sources or (for a very small percentage) vertical infection. Bacterial loads and associated damage reach critical levels in a small percentage of both groups in May and June of 1989 as temperatures begin to rise ("C"). During June, temperatures reach the level at which virgin helper T-cells begin to function, stimulating macrophages to kill the bacteria. This saves nearly all the non-immunotolerant fish, even those with fairly high, but not yet extreme, levels of infection. The continued high mortality throughout the summer in the high-BKD group ("D") is due to immunotolerance, which is discussed in Chapter 5. The high temperatures appear to reduce bacterial loads in immunotolerant fish that survive as well (see Figure 14). The small peaks in mortality in January and March of 1990 in both groups ("E") are likely due to handling stress in the first instance and the stress of smoltification in the second.

It may seem somewhat contradictory that temperatures above 10 °C appear to reduce mortality of these salmon when little or no increased survival was seen by Sanders et al. (1978) below 15 °C. This just serves to further point out the complexity of the picture. Sanders' data were not consistent in that some increased survival appeared for steelhead at 12.2 °C, the first temperature over 10 °C tested, where as no gain was seen for sockeye until 20.5 °C, and even then over 90% mortality was observed. Species, size, the temperature to which the fish are acclimatized, and the level of infection all may alter the effect of environmental temperature on the progression of the disease. In this case, it appears that temperatures above 10 °C did aid in the survival of these chinook salmon.

Evidence that these temperatures materially affect the progression of the disease is seen in the differences in infection levels at 8 and 12 months post hatch in both groups (Figure 23, Chapter 5).

4. 3. Mean time to BKD-related mortality

4. 3. 1. Sanders data

The mean time to BKD-induced death was dependent upon temperature. For coho, this ranged from 23 days at 20.5 °C to 81 days at 3.9 °C (see Table 8). As a first step in characterizing the response, it is tempting to assume that the crucial rates, including bacterial growth and the salmonid physiological responses to infection follow the "Q_{10}" relationship, and double with every increase of 10 °C (for the purposes of this chapter, this is equivalent to T_c = 10 °C). Regressing the inverse of average time to death versus $2^{T/10}$ (where T = temperature in °C) results in quite a good fit ($R^2 = 0.95$).

The Q_{10} (or T_c = 10 °C) model, where t_μ = mean time to death in days, is:

$$t_\mu = 86.2(2^{-T/10}) \tag{36}$$

Sanders et al. regressed the logarithm of time to BKD-induced mortality versus temperature for all three species (omitting the 20.5 °C data for steelhead due to the very few incidents of BKD induced mortality). This results in estimates of the temperature change necessary for halving time to death (T_c) being 8 °C (coho), 6.6 °C (steelhead), and 10 °C (sockeye). Similar regressions using only the mean time to death data reported in Sanders et al. (1978), results in similar values of T_c, 8.5 °C, 7.1 °C and 10 °C for coho, steelhead and sockeye respectively. The original data were not reported in the paper nor were the authors able to find the original data upon request. Using the same technique, the logarithm of mean time to death for all species was regressed against all temperatures. This regression gives a T_c of 8.6 °C (see Figure 14).

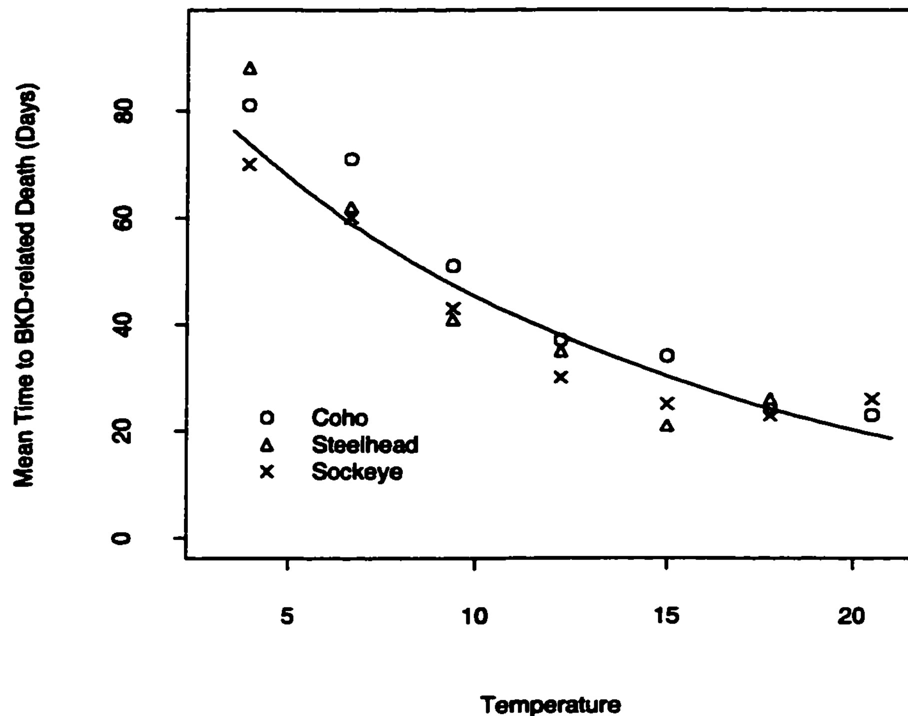

Figure 14 Mean time to BKD-induced death for the artificially infected salmon in Sanders et al. (1978). The curve represents the best fit of log(mean time to death) vs. temperature, resulting in a halving of the time to death every 8.6 °C.

The T_c = 8.6 °C model is:

$$t_{\mu} = 101(2^{-T/8.6}) \tag{37}$$

However, this model does not fit all that well, missing above or below all data points for three of the seven temperatures, and generally appearing too low at the extremes and too high in the middle of the temperature range (see Figure 14).

Considering all the data may not be the best approach in this case. The mean time to death data at 3.9 °C are likely consistently too low due to the limited time of the experiment (90 or 112 days). This results in an incomplete record of BKD related mortality at the lowest temperature due to the possibly substantial number of individual fish still

dying from the disease at the end of each experiment. In the following regression these data were not included. Moreover, at high temperatures, 15 °C and above for this experiment, the salmon experienced reduced BKD related mortality in at least two of three species when compared to 6.7 - 12.2 °C. At and after 15 °C the mean time to death does not appear to change but averages 24-25 days.

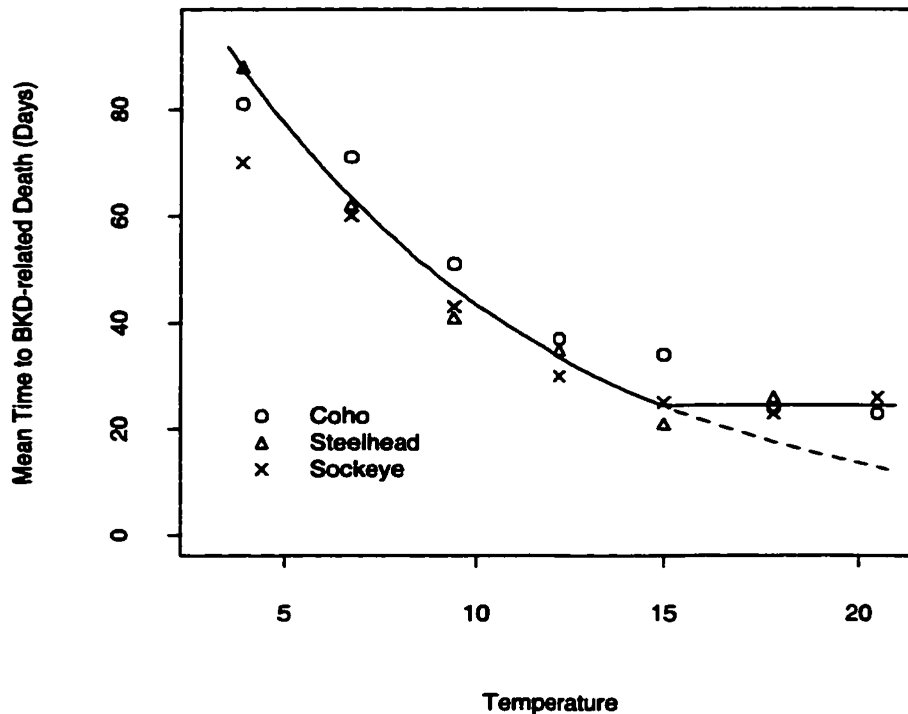

Figure 15 Best fit achieved by regression the log of time to death versus temperature only for temperatures between 6.7 and 12.2 °C, and then allowing for a leveling out above 15 °C.

The fact that the mean time to death does not continue to decrease with increased temperature over 15 °C may be due to the same processes that reduce BKD-related mortality at those temperatures. Those processes include increased immune functioning and/or decreased virulence of *R. salmoninarum*, due at least in part to increased breakdown of the p57 antigen at high temperatures. The growth rate of R. salmoninarum peaks at 15-18 °C as well (Weins and Kaattari, 1999).

A regression of the logarithm of mean time to death versus temperature was done for the data at 6.7 °C, 9.4 °C and 12.2 °C. Assuming that the mean time to death levels out to 24.5 days when the modeled curve reaches that level, a much better fit is achieved. The curve fits very well in the three 6.7-12.2 °C range, as would be expected, but also provides a reasonable fit at 15 °C and hits the highest of the three points at 3.9 °C, which are suspected to be underestimates of the true mean time to BKD-induced death at this temperature (Figure 15).

Table 8 Modeled versus actual mean time to BKD-related mortality for data from Sanders et al. (1978). *Underestimates due to limited time of experiment. ** Too few BKD-related mortalities to calculate an accurate estimate of mean time to death.

T °C	T_c Models			Observed Data			Combined Data	
	10 °C	8.6 °C	6 °C	Coho	Sockeye	Steelhead	Mean of Three Species	Median of Three Species
3.9	66	74	88	81*	70*	88*	80*	81*
6.7	54	59	63	71	60	62	64	62
9.4	45	47	46	51	43	41	45	43
12.2	37	38	34	37	30	35	34	35
15.0	30	30	24.5	34	25	21	27	25
17.8	25	24	24.5	24	23	26	24	24
20.5	21	19	24.5	23	26	--**	24.5	24.5

The T_c = 6 °C model, where again, t_μ = mean time to death in days, is:

$$\begin{aligned} t_\mu &= 137.5(2^{-T/6}) \quad && T < 15°C \\ t_\mu &= 24.5 && T \geq 15°C \end{aligned} \tag{38}$$

which implies halving of the mean time to BKD-related death every 6 °C until about 15 °C when the mean time to death levels out at about 24-25 days. This model only applies to salmon in the size range and dose (here all 3 x 10^8 bacteria fish^{-1}) included in the experiment. However, the doubling of the rate or halving of the mean time to death of

around 6 °C may be assumed to be more universal, as might the break in this trend at around 15 °C. This model is compared to the models with other T_c's in Table 8.

It is compelling that despite differences in total BKD-related mortality experienced by each tested species at 17.8 and 20.5 °C, the mean time to BKD-related mortality is quite similar among all species (Figure 15 and Table 8). Since the length of the experiment was multiple times longer than the mean time to BKD-related death, it is unlikely that any BKD-related mortalities due to the artificial infection were missed. Thus it appears that those fish that die progress towards death at essentially the same rate, independent of species. This implies that there is an early division between those artificially infected fish that die and those that survive at each temperature. Otherwise, one would expect the mean time to death to increase with decreasing total mortalities as some fish struggled to fight off the disease for longer periods of time before finally succumbing. The surviving fish can be assumed to begin suppressing the infection relatively early on, while the fish that die are ineffective at fighting off the injected dose from the beginning. The survivorship curves are thus likely similar to those represented in Figure 16.

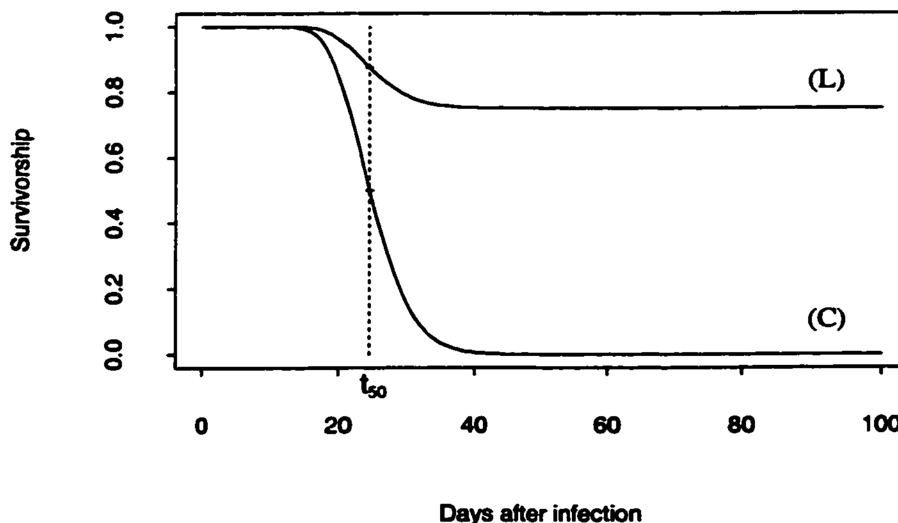

Figure 16 Hypothesized shape of survivorship curves for low (L) and complete (C) mortality with the same time to 50% mortality (t_{50}).

4. 3. 2. Murray data - rate of progression of the disease

R. salmoninarum reproduces at a very slow rate even under the best of conditions, with a generation time approximating 1 day at typical experimental temperatures (10-15 ^{o}C). In comparison, *Aeromonas salmonicida*, the causative agent of furunculosis, has a generation time of well under 4 hours and can result in mortality in 2 or 3 days.

There are no studies that have followed the progression of BKD in terms of severity of infection in individual fish. However, there have been studies that recorded the time to death of artificially infected salmon under laboratory conditions. Murray et al. (1992) infected three groups of 500 seawater adapted chinook (mean weight = 48.9 g) with three different inoculation doses (B_0) of 10^3, 10^5, or 10^7 bacteria per fish. These chinook, held at an average temperature of 9.9 ^{o}C, all died within 5 months (a fourth group of "crossover fish" also all died, and these data are considered later in the chapter). Mean time to death (t_μ) was 104.1, 82.5 and 60.2 days post infection for the three groups, indicating that a hundred fold increase in inoculation dose decreases life expectancy by about 22 days. If time to mortality is directly related to growth of bacteria and a critical average bacterial load (B_c) is reached at the time of death, this can be expressed as follows:

$$B_c = B_0 e^{\gamma t} \tag{39}$$

or:

$$\ln B_c = \ln B_0 + \gamma t \text{ or } \ln B_0 = \ln B_c - \gamma t \tag{40}$$

B_c and γ can then be found by regressing t_μ versus the natural logarithm of B_0.

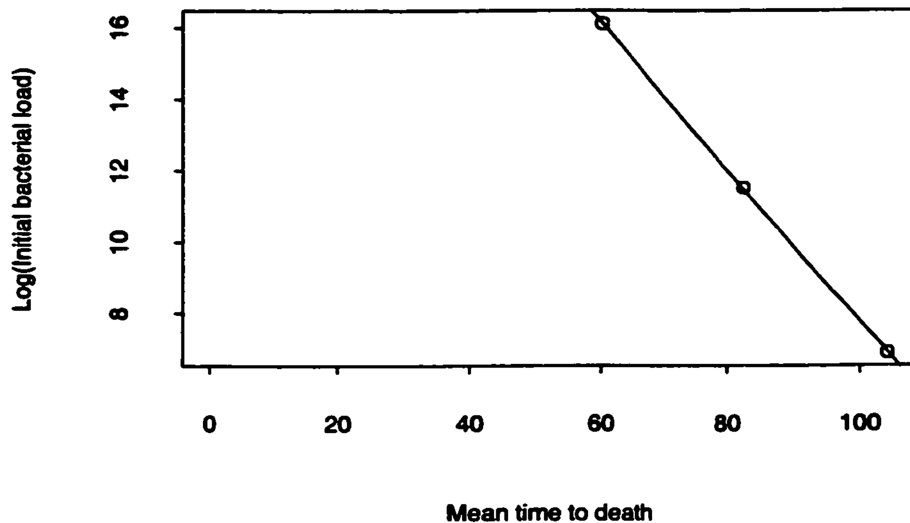

Figure 17 Regression of the natural logarithm of initial bacterial load versus mean time to death in days. The regression is significant with an R^2 of 0.9999 (p =.006).

The regression reveals the following relationship:

$$\log B_0 = 28.8 - 0.21t \tag{41}$$

which translates to:

$$B_c = 3x10^{12} \text{ bacteria.} \tag{42}$$

B_c actually represents total bacteria produced, rather than total bacterial load at mortality. Actual bacterial load is reduced due to the loss of bacteria to the environment through skin, gills and in feces, and the death of individual bacteria. Other physiological processes that progress at differential rates depending upon temperature might be partially responsible for the observed relationship between initial bacterial load and time to death. For simplicity, in this model mortality is assume to be related to bacterial growth alone.

The exponential growth rate of the bacteria from Equation 41 is $\gamma = 0.21$ day^{-1}.

Using the rate doubling temperature change found above ($T_c = 6$ °C) leads to the following equation of the growth of *R. salmoninarum* assuming constant temperature and less than optimal conditions for immune system functioning (due to low temperatures, high levels of p57 or stress):

$$B = B_0 e^{0.0667(2^{T/6})t} \tag{43}$$

This is equivalent to the bacterial load doubling about every 3.25 days at 10 °C (and therefore every 1.6 days at and above 15 °C and 6.5 days at 4 °C accordingly).

The mean time to death t_μ given any initial bacterial load B_0 can be calculated by solving for t in Equation 43 using the B_c given in Equation 42. This is (in days):

$$t_\mu = \frac{\ln B_c - \ln B_0}{0.0667}(2^{-T/6}) \cong 15(\ln B_c - \ln B_0)(2^{-T/6}) \tag{44}$$

When $B_0 = 3 \times 10^8$, the original dose in all three species studied by Sanders et al. (1978), the mean time to death is:

$$t_\mu = 138(2^{-T/6}) \tag{45}$$

which is nearly identical to Equation 38 for temperatures below 15 °C. Thus the data from both papers, for temperatures up to 15 °C, can be explained by a single model given by Equations 42, 43, and 44.

4. 3. 3. Dynamic analysis

Equation 43 can be extended for non-constant temperatures as follows:

$$B = B_0 e^{0.0667\int\left(2^{\frac{T(\tau)}{6}}\right)d\tau} \tag{46}$$

for temperatures below 15 °C. To see how well average temperature works for this model, assume that fish were held in a tank which averaged 12 °C, with a third of the time being at

each of 8°C, 12 °C, and 16 °C. The bacterial growth experienced within the fish would be the same as if they were held at a constant temperature of 12.42 °C. For the Sanders experiment, assuming that mean time to death is expressed by Equation 45, the difference between calculated mean time to death assuming the mean temperature (12 °C) and using Equation 46 is 34.5 days versus 32.2 days. For a more realistic example with continually varying temperatures between 8 °C and 16 °C, the effect would be less.

Even this model does not account for the delay in adaptation when salmonids are exposed to changing temperatures, which takes a variable amount of time depending upon the magnitude, direction and rapidity of the change as well as other stressors on the salmon. It also does not account for the recovery of many individual salmon.

Applying Equation 36 to the brood stock segregation data (shown in Figure 13), and assuming an initial dose of 1 bacterium at the day of hatch results in 3×10^{10} bacteria after the first 8 months, in July (C-D in Figure 13), reaching 3×10^{12} bacteria (B_c) in early August. Significant BKD related mortality begins earlier than this in both groups, in May and June (C in Figure 13). This is not surprising, as the fish are infected from a very early age, and the damage to the fish is ongoing even at initial low levels of infection. Bruno (1986b) found more extensive internal damage in salmon that died of natural BKD infections than those that died from artificial infection with initially high doses of bacteria. Equation 46 does not necessarily apply well to low level infections in very young fish. The variation in timing and severity of initial infection coupled with variations in size, stage, temperature, and stress level of individual fish at and after initial infection, for which there is no data, preclude more detailed application or revision of this model.

While bacterial load is a convenient measure to relate to mortality timing, it is actually the internal processes that lead to high bacterial loads which induce mortality. Bacterial growth results in uptake of nutrients, destruction of phagocytes and other cells, secretion of antigens and the creation of antibody-antigen complexes which contribute to granulomas, and changes in the immune and endocrine system outputs. The damage

associated with these processes, rather than the instantaneous bacterial load, are the underlying causes of death.

4. 3. 4. Vitality model analysis

Another approach to modeling time to mortality data, is the vitality model (Anderson 1992, 2000), which takes into account the damage done due to stressors, including diseases, over time. The vitality model is a mechanistic survivorship model based upon the abstract quantity "vitality", which is a composite measure of the health or condition of an individual in relation to its ability to survive. The vitality model has three parameters: "r", the average or expected rate off loss of vitality; "s" a measure of the variability of the vitality loss rate, and "k", the rate of accidental mortality, independent of vitality. The vitality portion of the model, parameterized by r and s, describe a directed random walk in vitality towards zero for each individual. An individual dies when vitality reaches zero. The composite result of the vitality dynamics of the entire population is approximated by an advection diffusion equation in vitality with an absorbing boundary at zero. This is described mathematically by the inverse gaussian equation. The accidental mortality portion of the model simply tacks an exponential decay term on the end of the inverse gaussian equation, representing the possibility of death as a random event at any time. Thus the probability, P, of surviving to time t, or equivalently the expected proportion of individuals surviving to time t, is:

$$P = \left[\Phi\left(-\left(\frac{rt-1}{s\sqrt{t}}\right)\right) - e^{\frac{2r}{s^2}}\Phi\left(-\left(\frac{rt+1}{s\sqrt{t}}\right)\right)\right]e^{-kt} \tag{47}$$

where Φ is the cumulative normal function.

For the laboratory experiments described by Sanders and Murray it can be assumed that accidental mortality, as defined by the vitality model, was insignificant. Under these circumstances r is approximately equal (though slightly smaller than) to $1/t_{50}$ where t_{50} = time to 50% mortality. Here r is approximated by the inverse of mean time to death ($1/t_{\mu}$)

which, on the other hand, slightly underestimates r. However, mean time to death is the only applicable statistic given in Sanders et al. (1978). An optimization routine (Salinger et al. in preparation) was used to find r, s and k values for the Murray data. All k values were zero, and all r values were all within 1% of the $1/t_{\mu}$ approximation. The model fits for the Murray data, including the mortality in the crossover fish, are shown in Figure 18, with the parameters given in Table 9.

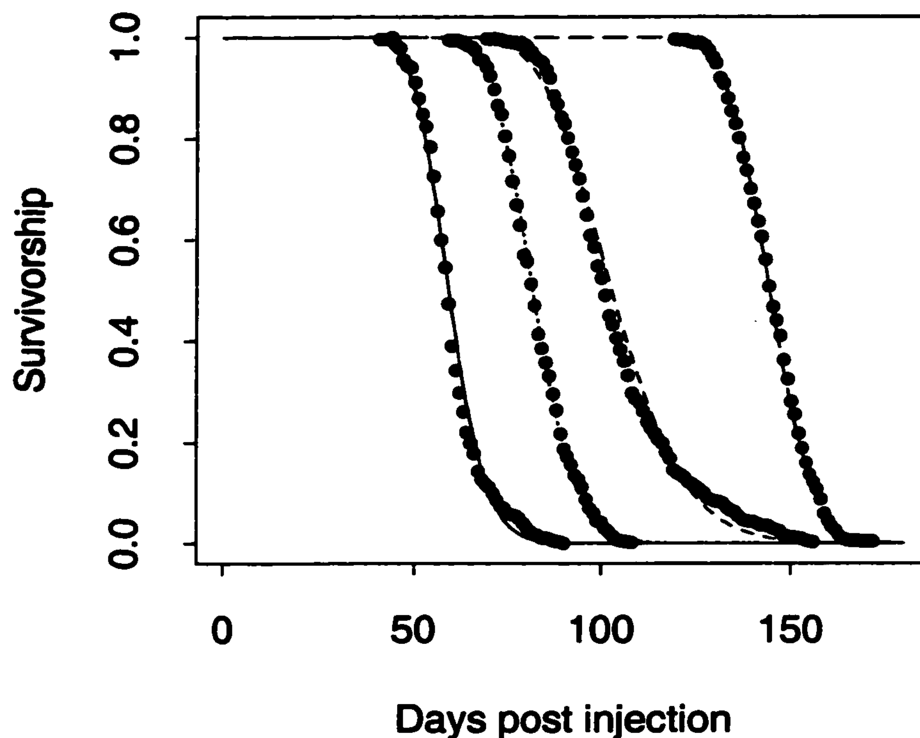

Figure 18 Vitality model fits to the Murray data (approximated from Figure 2 in Murray et al. (1992)). The curves represent, from left to right, survivorship at day post artificial injection for those chinook injected with 10^7, 10^5, 10^3 R. salmoninarum cells and crossover fish, infected due to cohabitation with the injected fish.

The crossover fish have a mean time to death of 145 days. This is equivalent, via Equation 41, to an initial bacterial load of 0.2 bacteria per fish, or of having an initial dose of 1 bacterium on day 6. The truth is more complicated by the continuous pressure from

the bacterial load in the aqueous environment, which is ever increasing, at least over the first two months of the experiment.

Table 9 Vitality model parameters for Murray data

Initial Dose	r	s	k
10^7 bacteria	0.0168	0.0174	0
10^5 bacteria	0.0122	0.0125	0
10^3 bacteria	0.0096	0.0146	0
Crossovers	0.0069	0.0054	0

Anderson (2000) showed that r, s, and k vary in a consistent and often linear manner with changes in stressors. For this application, r can be related to bacterial load and temperature. Typically, then, r would be related to B and T as follows:

$$r = r_0 + r_1 B + r_2 T \tag{48}$$

In this case, however, temperature is not so much a stressor as a co-factor affecting rate. Therefore, the following form is more applicable to this particular situation:

$$r = r_0 + r_1 BT \tag{49}$$

This is in multiplicative form rather than additive form because temperature affects the rate of progression towards mortality due to BKD, rather than having an intrinsic negative influence on the salmon, at least within the range of temperatures tested.

Previously, the vitality model has generally been applied to situations in which the stressors have been held constant over the course of the experiment, such as dose-response studies. In this application the bacterial load is increasing up until the point of death in all individuals that experience BKD-related mortality. It is assumed, however, that temperature is held constant in both experiments, even though this is not strictly the case

for the Murray data, where temperature varied over a narrow range over the course of the experiment, with a mean temperature of 9.9 °C. Given a constant temperature, the mean rate of vitality loss is simply:

$$r = r_0 + r_1 \bar{B} T \tag{50}$$

Where, assuming the bacterial growth model (Equation 46),

$$\bar{B} = \frac{1}{t_\mu} B_o \int_0^{t_\mu} e^{0667(2^{T/6})\tau} d\tau = \frac{B_o(e^{0667(2^{T/6})t_\mu} - 1)}{0.0667(2^{T/6})t_\mu} \tag{51}$$

Even though complete mortality is not seen in some species at all temperatures, here it is assumed that those fish that die due to BKD form a separate group from those that survive or die without high levels of *R. salmoninarum* in their system. In other words, their vitality dynamics diverge once the infection becomes successful. This does not violate the assumptions of the vitality model, but rather assumes that even though all individuals in a particular dose and temperature group are initially exposed to the same conditions, the subsequent conditions are different depending upon whether or not the infection is successful in each individual. The model given in Equation 49 still fits well for those fish that fight off the disease. As the bacterial load decreases, the vitality loss rate "r" approaches zero and those fish that end up with low bacterial loads are therefore unlikely to reach zero vitality and die due to the effects of BKD.

The vitality model is applied below to data including all three artificial infection level groups from the Murray data and all species in four of the seven temperatures from the Sanders data. The data from the lowest temperature (3.9 °C) are not included due to the fact that the experiments ended before complete mortality could occur, and the data from the two highest temperatures (17.8 and 20.5 °C) are removed as the dynamics change above 15 °C as discussed above. The mean bacterial loads as calculated by Equation 51 for those groups included in the vitality fitting are presented in Table 10.

Table 10 Data from Murray et al (1992) and Sanders et al. (1978) used to calibrate the vitality model for artificially infected salmon held under laboratory conditions with mean calculated bacterial load from Equation 51 and r calculated as $1/t_\mu$.

Paper	Species	B_o	$\bar{B}$	T °C	t_μ	r
Murray	Chinook	10^3	$1.40x10^{11}$	9.9	104.1	0.0096
	Chinook	10^5	$1.90x10^{11}$	9.9	82.5	0.0121
	Chinook	10^7	$2.42x10^{11}$	9.9	60.2	0.0166
Sanders	Coho Sockeye Steelhead	$3x10^8$	$3.25x10^{11}$	6.7	71 60 62	0.0141 0.0166 0.0161
Sanders	Coho Sockeye Steelhead	$3x10^8$	$3.25x10^{11}$	9.4	51 43 41	0.0196 0.0233 0.0244
Sanders	Coho Sockeye Steelhead	$3x10^8$	$3.25x10^{11}$	12.2	37 30 35	0.0270 0.0333 0.0286
Sanders	Coho Sockeye Steelhead	$3x10^8$	$3.25x10^{11}$	15	34 25 21	0.0294 0.0400 0.0476

The initial regression, for the murray data, of "r", the average vitality loss rate versus "B", the bacterial load ($R^2 = 0.98$, $p = 0.09$) revealed the y intercept to not be significantly different from zero ($p = 0.92$), with a negative fitted value. The vitality model does not allow for negative values of r. The subsequent regression with the intercept forced to zero results in the following fit ($R^2 = 0.98$):

$$r = 6.9x10^{-14}\bar{B} \text{ at } 9.9\,^{\circ}\mathrm{C}, \text{ or } r = 7.0x10^{-15}\bar{B}T \quad (52)$$

assuming that temperature is multiplicative. This assumption was tested by regressing "r" versus temperature for all three species studied by Sanders for temperatures between 6.7 °C and 15 °C. The initial regression ($R^2 = 0.81$, $p < 0.0001$) revealed the y intercept to not be significantly different from zero ($p = 0.47$), with a negative fitted value. Forcing the fit

through zero as above results the following fit ($R^2 = 0.80$):

$$r = 7.7x10^{-15}\bar{B}T \qquad T \le 15°C \tag{53}$$

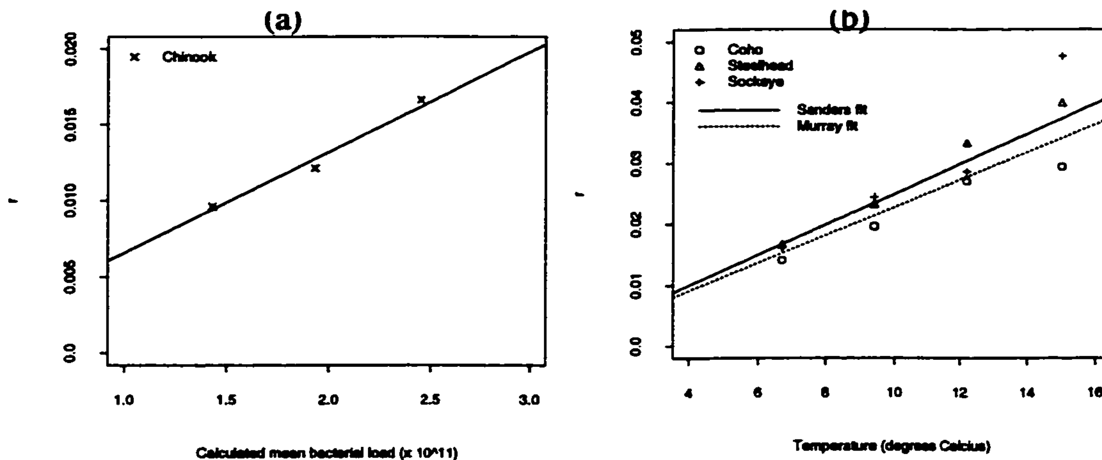

Figure 19 Plots of r versus (a) calculated mean bacterial load, when all groups experienced the same temperature regime (mean temperature = 9.9 °C), using data from Murray et al. (1992), and (b) temperature, when all experience the same mean bacterial load ($3.25x10^{11}$ bacteria ml^{-1}), using data from Sanders et al. (1978).

The modeled relationships are plotted in Figure 19. The Murray model fit (Equation 52 and dotted line in Figure 19b), which is based upon data concerning chinook, is closest to Sanders' coho data. Since chinook is more closely related to coho than to the other two species, it is not surprising that coho and chinook appear to have the most similar response to the disease.

Clearly, the additive model given in Equation 48 does not work for these data. Both the regressions reported above, holding either temperature or bacterial load constant, result in intercepts of zero or less. The best regression fit using the additive Equation 43 results in a negative intercept (r_0) which is significantly different from zero ($p = 0.0007$) Thus for Equation 43 to hold, r_0 would have to be negative, which violates the assumptions of the vitality model.

To test for curvilinear fits, the logarithm of r was regressed against the logarithm of B and the logarithm of T for both data sets. The resulting fit significant (R2 = 0.93, p = 0.0000001). The statistics are given in Table 11. The fit is given in Equation 54:

$$r = 4.9x10^{-15}\bar{B}^{1.1}T^{1.1} \tag{54}$$

Table 11 Results of regression of log(r) against log(B) and log(T)

Coefficient	Value	SE	t	p
Intercept	-7.6193	0.3110	-24.4975	0
log(B)	1.1328	0.1342	8.4403	0
log(T)	1.1091	0.1216	9.1246	0

However, neither of the exponents are significantly different from 1 (see Table 11). Forcing the exponents to 1, the best fit is:

$$r = 7.3x10^{-15}\bar{B}T \tag{55}$$

which is illustrated in Figure 20.

While the vitality model fits are based upon the bacterial growth model, any monotonically increasing model of bacterial growth would be expected to result in a reasonably good fit to the data, and the fit to the Sanders data is independent of the bacterial growth model as the initial infection levels and calculated mean bacterial loads used were identical for all temperatures and all species studied by Sanders et al. (1978).

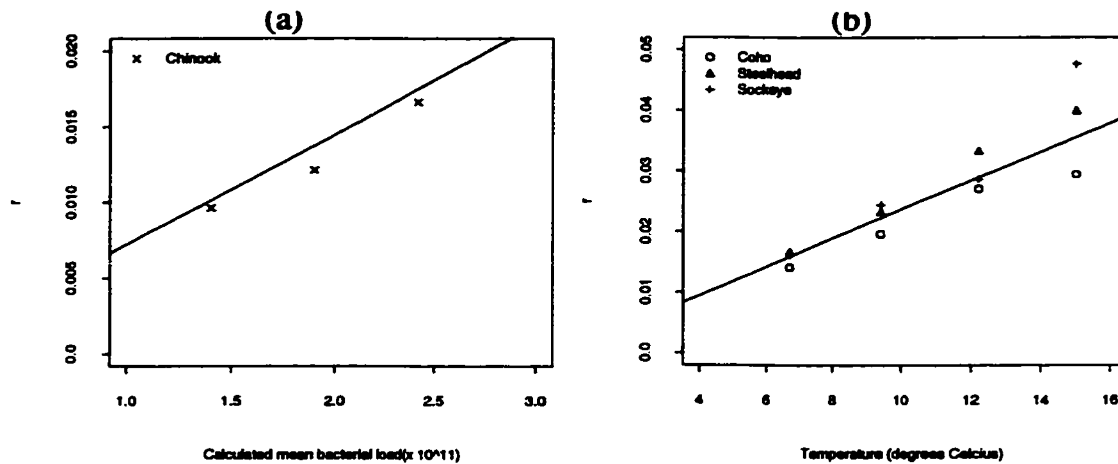

Figure 20 Plots of r versus (a) calculated mean bacterial load, when all groups experienced the same temperature regime (mean temperature = 9.9 °C), using data from Murray et al. (1992), and (b) temperature, when all experience the same mean bacterial load (3.25×10^{11} bacteria ml^{-1}), using data from Sanders et al. (1978). Lines are from Equation 55.

4. 4. Discussion

The threshold for low temperature immunosuppression in salmonids does not appear to be the 4 °C that is quoted in Bly and Clem (1992), at least not when the disease in question is BKD. It appears that a threshold of 10 or 12 °C is more representative of the data, and in fact, the effect of temperature on the immune system may be a continuously varying one with ever worsening immunological problems as temperatures drop, with an optimal anti-BKD temperature being near the top of the physiological temperature range for salmonids. The decreased mortality rates seen among the offspring of low-BKD broodstock, when temperatures reached around 10 °C (Pascho et al. 1991) might only occur when bacterial loads are still relatively low. The high mortality rates seen at up to 12.2 °C in salmon inoculated with 3×10^8 *R. salmoninarum* cells, and the increasing survivorship with increasing temperatures at 15 °C and above (Sanders et al. 1978), suggests that the relative success of the immune response to *R. salmoninarum* infection varies continuously with both dose and temperature.

Mean time to death, when BKD-induced death occurs, is much easier to model. It appears that the mean time to death halves with every 6 °C increase in temperature below around 15 °C. If mortality is directly related to bacterial growth, then the model indicates a doubling time near 3 days at 10 °C. Above 15 °C the bacterial growth rate levels off, likely due to increased ability of the immune system, even if compromised, to fend of the bacterium, though possibly due to direct effects of temperature on the growth or virulence of *R. salmoninarum*. Whether or not the bacteria continue to spread within individual salmon at each temperature depends the state of the immune system, which is affected by no only temperature but also stress, life-long immunotolerance, and the p57 antigen. High bacterial and antigen loads alone may cause the immune system to be unable to fight off the disease.

For these laboratory data, the vitality model fits well to the time to death data when BKD mortalities are treated as a separate population from survivors and non-BKD mortalities. It is difficult, on the other hand, to apply the vitality model to the broodstock segregation data as an assumption of the vitality model is that all individuals are experiencing the same external stressors (which in this case includes the internal bacterial load). This rule can be bent, as done earlier in this chapter in the case of Sanders' data, where it was assumed that after artificial infection, the fish either recovered or the infection succeeded, resulting in two subgroups within each temperature level. However, for the broodstock segregation data there is simply too much variation in conditions and too little overall mortality during hatchery rearing. Sources of variation include variation in the timing and severity of initial infection, including vertical infection, and variation the immune response to disease once it establishes, depending upon stage, size, stress level, temperature and immunotolerance status. This last results in a bimodal response to the disease, which is discussed in Chapter 6.

5. Re-evaluation of the 1988 Brood Stock Segregation Experiment at Dworshak National Fish Hatchery: Evidence for In-Ovum Antigen Inclusion Induced Immunotolerance.

5. 1. Introduction

A broodstock segregation experiment at Dworshak National Fish Hatchery was undertaken in 1988 to observe the effects of vertical transmission of bacterial kidney disease (BKD) on subsequent infection and mortality rates of progeny (Pascho et al. 1991).

Pascho et al. (1991) concluded that "segregation of brood stock by the ELISA and the MF-FAT can be used to reduce the prevalence and levels of BKD in hatchery-reared spring chinook salmon..." The evidence they put forth, however, more strongly suggests that segregation only separates offspring with different susceptibilities to the disease without, in fact, changing the overall prevalence and levels of BKD in the first 8 months post hatch, and perhaps without changing total disease-induced mortality. Their 1991 paper and subsequent papers concerning the same segregation experiment (Pascho et al. 1993, Elliott et al. 1995) are reviewed here. This gives a picture of survival of the two groups throughout the lifecycle and, based upon these and other data sources, a new hypothesis is proposed for the underlying dynamics leading to the observed results.

5. 2. Review of broodstock segregation data

5. 2. 1. Overview

Thirty spawning female chinook salmon with little or no evidence of BKD infection and thirty spawning female chinook salmon with high levels of infection were chosen for a broodstock segregation experiment at Dworshak National Fish Hatchery in the fall of 1988. The offspring of these sixty female spawners will be henceforth referred to as the low-BKD group and the high-BKD group respectively, following the original paper (Pascho et al. 1991). Male spawners were tested as well to ensure that none of the eggs of low-BKD spawners were fertilized by highly infected male spawners, though there

is no evidence of male spawner to offspring vertical transmission or antigen transfer. The offspring were held in nursery tanks and then in six raceways such that there were three high-BKD raceways and three low-BKD raceways, each containing the progeny of 10 female spawners. The juvenile chinook salmon were reared until smoltification 17 months after hatch, and then released. Mortalities in each nursery tank or raceway were recorded for each month, and samples were taken every three of four months to test for prevalence and levels of *R. salmoninarum* infection via the enzyme-linked immunosorbent assay (ELISA).

Upon release, approximately 750 smolts from each low-BKD raceway and 1500 smolts from each high-BKD raceway were tagged, and recaptures for transportation were noted at Lower Granite, Little Goose and McNary dams. A small number of smolts were not released but transferred to a remote site and subjected to a salt water challenge.

5. 2. 2. Ovarian fluid infection levels in female spawners

Pascho et al. (1991) tested female spawners for the presence of R. salmoninarum in kidney tissue and ovarian fluid using the FAT and ELISA diagnostic tests. All female spawners in the low-BKD group had less than 100 bacteria per ml measured in their ovarian fluid with 60% (18 of 30) having no measurable infection. All but one of the high-BKD group female spawners had a measurable infection, with up to 13 billion cells ml^{-1}. Raceway 20 held the offspring of the two most highly infected females, with 6 of 10 female spawners having over 1 million bacteria ml^{-1} in their ovarian fluid. Raceway 21 contained the offspring of the 3rd and raceway 19 the 4th most highly infected females, with only 2 of 10 spawners in each raceway having over 1 million bacteria ml^{-1} of ovarian fluid. One female spawner whose offspring were reared in raceway 19 had no evidence of infection and was included in the high-BKD group in error.

Soluble antigen was detected by ELISA in the ovarian fluid of two female spawners whose offspring were reared in raceway 19, seven female spawners whose

offspring were reared in raceway 20, and five female spawners whose offspring were reared in raceway 21. Antigen densities (as calculated in Chapter 2) correlated well with bacterial loads (Table 12).

Table 12 Bacterial loads (cells ml^{-1}) (BL) and calculated antigen densities ($\mu g\ ml^{-1}$) (AD) in the ovarian fluid of spawning female chinook salmon in the high-BKD group. Within each raceway the spawners are ranked by increasing ovarian fluid bacterial load.

Raceway	Unit	Female Spawner Number (Ranked by Bacterial Load)									
		1	2	3	4	5	6	7	8	9	10
19	BL	0	85	341	427	512	1,664	2,176	7,552	1,664,000	1,024,000,000
20	BL	1,621	4,480	104,064	247,467	8,917,333	22,613,333	230,400,000	704,000,000	9,002,660,000	13,184,000,000
21	BL	2,304	3,115	5,077	72,320	75,648	78,336	123,008	273,067	47,360,000	5,888,000,000
19	AD	0	0	0	0	0	0	0	0	0.92	25.94
20	AD	0	0	0	0.18	3.89	6.35	13.71	17.05	25.54	23.44
21	AD	0	0	0	0	1.29	0	0.17	0.16	7.66	21.52

5. 2. 3. In hatchery survival rates

Mortality rates were recalculated from the original data for this analysis, taking into account those fish removed for testing (Figure 21). Note the peak in mortality in the low-BKD raceways during the parr stage, and the subsequent sustained mortality in the high-BKD raceways. Overall mortality during hatchery rearing, as noted by Pascho et al. (1991), was approximately 10% for the low-BKD group and 20% for the high-BKD group (ignoring a single large mortality event in the first ten days after hatch in one high-BKD group nursery tank destined for raceway 19; this event was deemed to be unrelated to BKD. Note, however, that a much smaller anomalous mortality rate in one low-BKD nursery tank was included, accounting for the higher mortality rate in the low-BKD group in November and December of 1988.

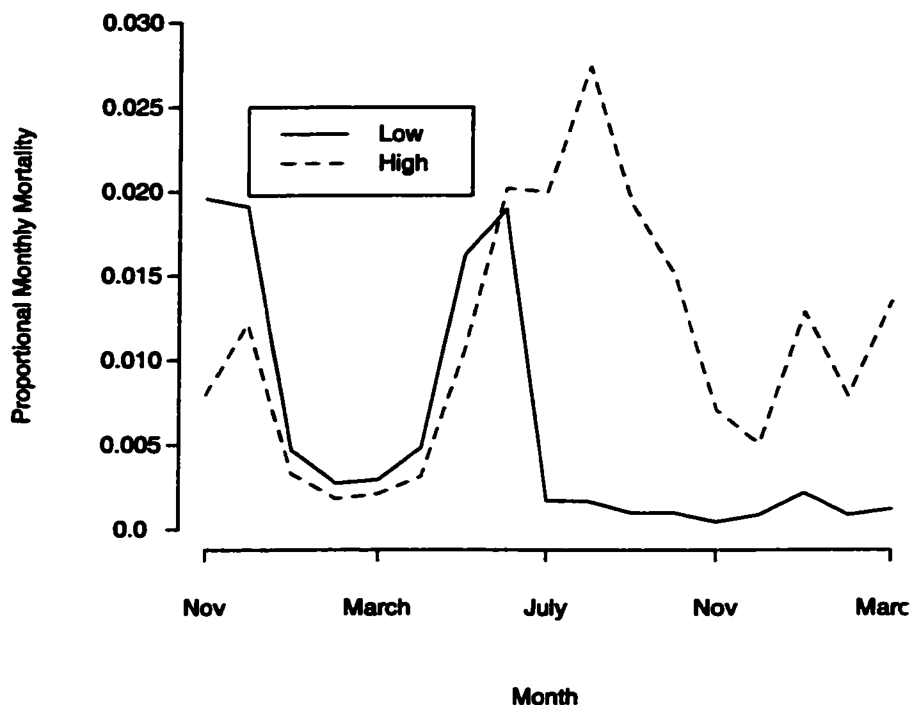

Figure 21 Monthly mortality rates for the low- and high-BKD groups from November 1988 through March 1990.

The mortality data for the six individual raceways are plotted in Figure 22. The pattern for the three low BKD raceways are very similar except in the first two months, due to the anomalous mortality in the one nursery tank. The pattern among the three high BKD diverge somewhat, with raceway 20 experiencing the highest mortality and raceway 19 the lowest mortality during the summer and fall, with the pattern reversing during the second winter of hatchery residence. The small peak in mortalities in January 1990 was due to handling stress and the increase in mortalities in March 1990 may have been due to the effects of smoltification on the immune system.

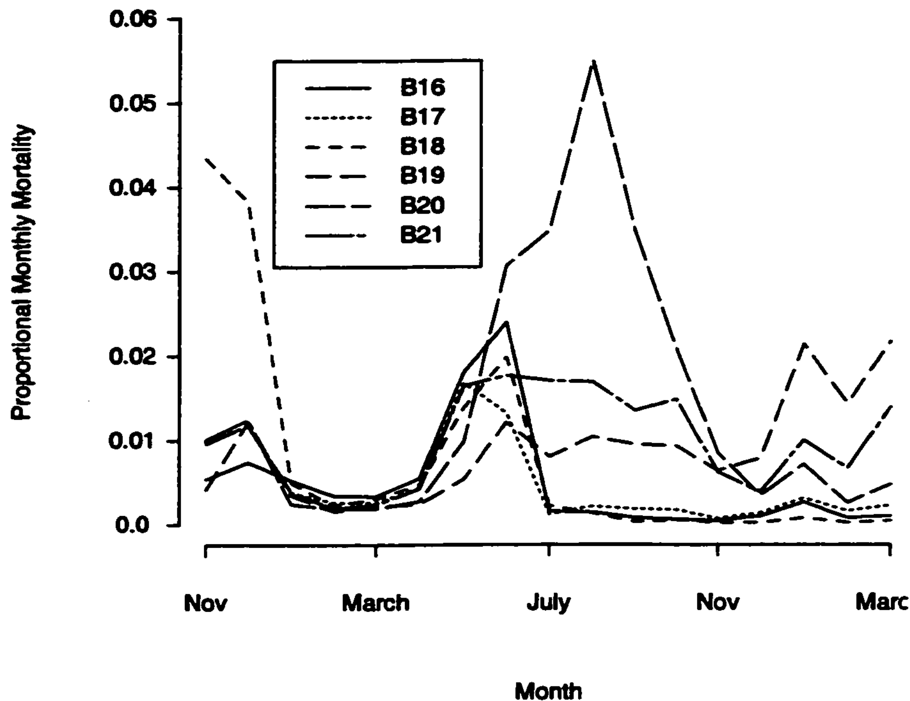

Figure 22 Monthly mortality rates for the six raceway groups from November 1988 through March 1990.

5. 2. 4. In hatchery infection levels

Sample fish were tested to determine the distribution of infection levels at 1, 5, 8, 12, and 16 months post-hatch. The ELISA values are related to the logarithm of bacterial counts. Fry, parr and pre-smolts were pooled in the first four samples taken due to the small size of the juvenile chinook at these times. The logarithm of the average of only a few data points (3 or 5 in these cases) is dominated by the largest value if it is significantly larger than the other numbers. The actual infection levels of individual fish has a distribution with a long right tail, similar to the log-normal distribution. Therefore, the infection level (negative, low, medium or high) given by the ELISA test was assumed to represent the infection level of the most highly infected individual in that pool. This also simplified the statistical analysis. In particular, under this assumption, the distribution of negative, low, medium and high level infections among the population can be calculated

by finding the maximum likelihood estimator (MLE) of the proportion p of individual fish within each infection level.This is done as follows:

For each level of infection starting with "high", let x equal the number of pools with that level of infection, n equal the total number of pools with less than or equal to that level of infection and m equal the number of fish per pool (5, 3 or 1 for this data). Then,

$$P(x|n, m) = \binom{n}{x}\phi^{x}(1-\phi)^{n-x} \tag{56}$$

Where $\binom{n}{x} = \frac{n!}{x!(n-x)!}$ (*n choose x*) and

$$\phi = 1-(1-p)^{n} \tag{57}$$

is the probability of any given pool being positive, given p.

The MLE of ϕ is x/n, and the MLE of p is:

$$MLE(p) = 1-\left(\frac{n-x}{n}\right)^{1/m} \tag{58}$$

This technique was used in the subsequent analysis. It was not possible to statistically separate out proportions of negative and low-level infections for the samples taken at 5, 8 and 12 months post-hatch.

At one month after hatch, twenty pools of five swim-up fry apiece were tested from each group of two nursery tanks destined for a particular raceway. A total of 13 of 120 (11%) of these pools indicated low levels of infection. There was no evidence of medium or high levels of infection; the remaining 107 pools tested negative. There was no significant difference in number of infected swim up fry between the low and high BKD groups, though, interestingly, the majority (69% (9/13)) of the pools testing positive for BKD were from the low-BKD group, the opposite of what would be expected if vertical transmission is assumed to be the cause of these infections (see Figure 23).

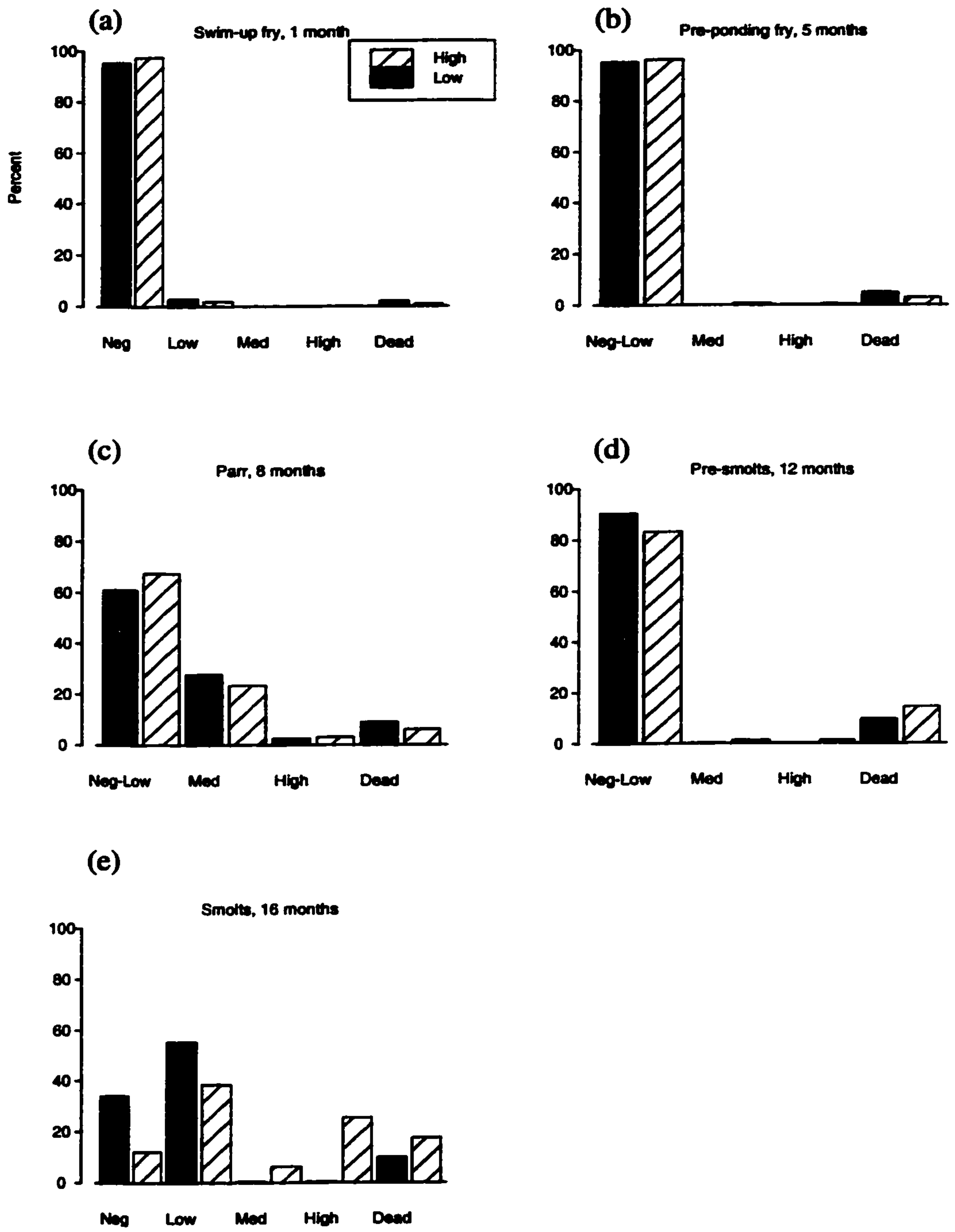

Figure 23 Calculated percent of offspring in the low and high-BKD groups with different levels of BKD infection at 1, 5, 8, 12, and 16 months post hatch. "Dead" refers to cumulative mortalities in each group.

At five months after hatch, twenty pools of five fry apiece were tested from each nursery tank group before transfer to the raceways. All but one of these 120 pools showed at least a low level of infection. This indicates that at least 60% of the individual fry were actually infected. One pool had a medium level of infection, and two showed high levels of infection, all from the high-BKD group. However, there is no significant statistical difference between the results for the two groups at this stage. Two of the pools with medium or high levels of infection came from precursors to raceway 20, and one from a precursor to raceway 21. In fact, these fish came from nursery tanks 49, 50 and 66, which contained the eggs from the 2nd, 1st and 3rd most highly infected females via ovarian fluid FAT (2nd, 3rd and 4th by ovarian fluid ELISA). This strongly suggests the possibility of vertical infection in individual fish from these pools. It may be then that the observed 1% medium to high infection rate among the high-BKD group is representative of vertical infection. This is similar to the expected vertical transmission rate using the vertical transmission model (See Chapter 3).

At the parr stage, in July, 1989, 8 months after hatch, 60 pools of 3 fish each were tested from each of the raceways. Both groups and all raceways showed indications of high levels of infection. The high-BKD group contained the pools with the highest levels of infection. However, the low-BKD group had more pools in the medium and high categories. All pools from both groups had evidence of infection and there were no statistically significant differences between the two groups or individual raceways (Figure 23c). According to the statistical analysis, and given the assumptions stated above, in both groups approximately 27% of the individual parr at this stage had medium levels of infection, and 3% had high levels of infection.

At 12 months after hatch, 60 pools of 3 fish each were tested from each of the raceways. The overall infection rate in both groups was much lower at this stage than at the parr stage. Most pools in both groups showed low levels of infection, with only 16 of 360 pools showing medium or high levels of infection, 15 of which were from the high-BKD group. The high-BKD group had significantly more pools and individual fish with medium

and high infection levels ($p<0.02$). However, the overall percentage of individuals with medium or high infection levels had by this time dropped to 1.5%, with about 3% in the high-BKD group and 0.5% in the low-BKD group at these levels. No significant difference was seen between raceways within a group, though again, raceway 20 had the most pools (8) in the medium to high categories, followed by raceway 21 (4) and raceway 19 (3).

At 16 months after hatch, in March 1990, just before release, 70 individuals from each raceway were tested for BKD. 74% (310 of 420) of all individual fish tested were positive for BKD, with 16% (66) having high levels of infection and 4% (17) having medium levels of infection. However, 39% (81/210) of the high-BKD group had medium to high levels of infection, versus only 1% (2/210) of the low-BKD group. This difference is highly significant, both biologically and statistically. Between raceways, raceway 20 had the highest number of medium to high levels of infection at 49% (34/70), while raceway 21 had the lowest level at only 26% (18/70), and raceway 19 had 41% (29/70).For the Chi-square test, $\chi^2 = 4.96$, $\chi^2_{.05,2} = 5.99$, $0.05 < p < 0.10$, so even here the null hypothesis that there is no difference in infection level among the three high-BKD raceways cannot be rejected.

5. 2. 5. Migration

Upon release in April, 1990, PIT tags were used to monitor the downstream migration of both high and low-BKD group smolts. Approximately 1500 smolts from each of the high-BKD raceways and 750 smolts from each of the low-BKD raceways were PIT-tagged. Recaptures for transportation were noted at Lower Granite, Little Goose and McNary dams (Pascho et al. 1993, and Table 13).

Second observations of a few multiply recaptured fish (that were not transported after the first recapture) were ignored by removing the second observation from the data base and thus assuming all recaptures were transported. This allowed for the calculation of the percentage of tagged fish from each raceway that were recaptured at each dam without

replacement. Relative recaptures at each dam, especially McNary, give an indication of relative survival between each dam, and a better indication of total survivorship to the third dam. Assuming equal recapture efficiencies at each dam, which the data limit to values between 32 and 45%, estimates of absolute survivorship can be obtained. Here a recapture rate of 35% is assumed. This is done by assuming that the number of fish observed is exactly 35% of the fish passing each dam, so that the number which pass the dam without being recaptured (and thus continue downstream) is (0.65/0.35) = 1.86 times the number observed. More complex methods of estimating survivorship, such as the Jolly-Seber method, require multiple recaptures of the same fish, and thus are not applicable to these data.

Table 13 Recaptures for transportation t at Lower Granite Dam (LGR), Little Goose Dam (LGO), and McNary Dam (MCN).

Rearing Raceway	Number Tagged and Released	Recaptures at			Total Recaptures
		LGR	LGO	MCN	
B16	763	226	107	55	388
B17	767	235	96	44	375
B18	745	260	99	67	426
Total Low	2275	721	302	166	1189
B19	1514	468	148	84	700
B20	1525	479	179	104	762
B21	1510	398	117	53	568
Total High	4549	1345	444	241	2030

Table 14 gives estimates of survival between each dam, assuming a 35% recapture rate at each dam. Other possible recapture rates (between 32% and 45%) give similar overall and relative percent survivals. The one value over 100% in the table is due to the strict assumption of exactly 35% recapture at each dam and the small sample size (number of fish still in river) at McNary Dam. The assumption that all dams have the same

recapture rate is probably a poor one. The assumption that both groups have similar recapture rates at each dam, on the other hand, is reasonable, despite possible minor effects of BKD on location in the water column. Thus these estimates are most useful for examining relative survival between the two groups, rather than absolute survival.

Table 14 Assuming 35% recapture at each dam, this table represents the calculated percent survival from release to Lower Granite Dam (LGR), between Lower Granite and Little Goose Dams (LGO), between Little Goose and McNary Dams (MCN), and total calculated percent survival of non-transported fish to McNary Dam. The one value over 100% is due to the assumption of constant recapture rate.

Rearing Raceway	Number Tagged and Released	Estimated survivorship from			
		Release to LGR	LGR to LGO	LGO to MCN	Release to MCN
B16	763	84.6%	72.8%	79.1%	48.7%
B17	767	87.5%	62.8%	70.5%	38.8%
B18	745	99.7%	58.6%	104.1%	60.8%
Total Low	2275	90.6%	64.4%	84.6%	49.3%
B19	1514	88.3%	48.7%	87.3%	37.5%
B20	1525	89.7%	57.5%	89.4%	46.1%
B21	1510	75.3%	45.2%	69.7%	23.7%
Total High	4549	84.5%	50.8%	83.5%	35.8%

5. 2. 6. Salt water challenge

Twenty five (25) smolts from each raceway were retained and subjected to a 14 week salt-water challenge. In both the high and low-BKD groups, 11-12% of the fish that did not die from BKD died from other causes. A total of 37% of the high BKD group died of BKD.

There were large differences between the raceways (Figure 24). Total mortality experienced was 4, 8 and 24% in the fish from the three low-BKD raceways 18, 17 and 16

respectively, and 24, 32 and 76% among the fish from the three high-BKD raceways 21, 19 and 20 respectively. Mortality was highest among fish from raceway 20, which also posted the greatest mortality during rearing and had the highest ovarian fluid levels among its female broodstock.

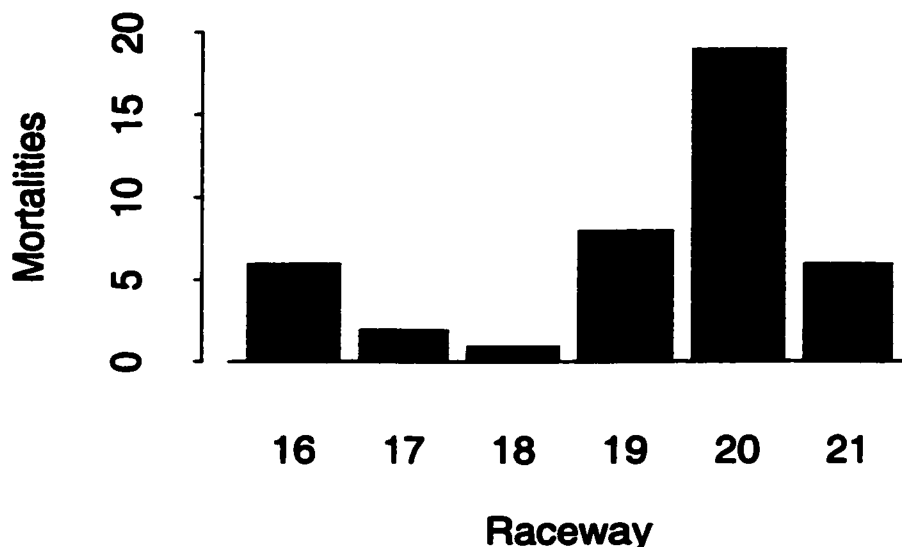

Figure 24 Mortalities observed among 25 chinook smolts from each raceway subjected to salt water challenge.

5. 2. 7. Returns

The overall returns amounted to 60 out of 148,563 tagged fish released, with 41 out of 76,537 (0.054%) high-BKD group and 19 out of 72,028 (0.026%) low-BKD group salmon returning. The relative rate of return (high-BKD to low-BKD) was 49%. Among the individual raceways, returns were as follows: Raceway 16: 0.051%, Raceway 17: 0.031%, Raceway 18: 0.078%, Raceway 19: 0.026%, Raceway 20: 0.016%, Raceway 21: 0.037% (Figure 25).

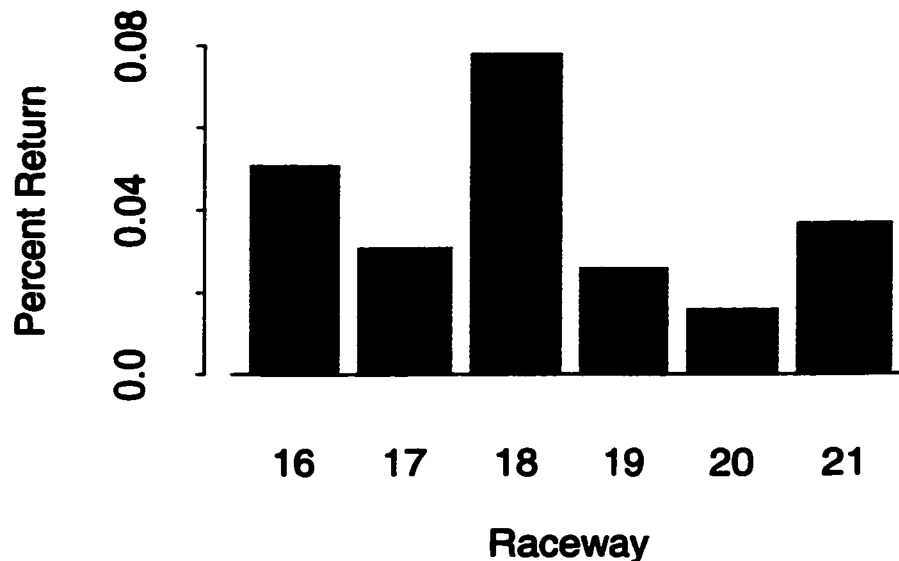

Figure 25 Percent returns (SARs) of tagged smolts from each raceway.

5. 3. Analysis

5. 3. 1. Hatchery rearing

The lack of distinction between the disease levels in the two groups in the first three samples in hatchery (at 1, 5, and 8 months after hatch) indicates lack of significant vertical transmission. The 1% highly infected fry in the high-BKD group at 5 months after hatch may indeed be due to vertical transmission. This is too small a rate of vertical transmission to account for later differences in survival and infection levels between the two groups. Nor does this small percentage of fish with very high infection levels appear to result in significant horizontal transmission between juveniles, at least when compared to, or coupled with, an external source, which seems the only possible explanation for the high levels observed in all raceways at the parr stage, 8 months post-hatch.

There is a significant difference in prevalences and levels of infection at 12 months, but both groups have lower infection levels than at 8 months. There is a rebound in infection at smoltification, at 16 months after hatch, in the high-BKD group but not in the

low-BKD group. Higher temperatures between 8 and 12 months post hatch may have allowed for significant recovery in many of these pre-smolts (see Chapter 4), though more so in the low-BKD group than in the high-BKD group. The reasons for this difference are discussed later in this chapter.

5. 3. 2. Migration

The relative recapture rate between the two groups is 93.3% at Lower Granite dam (116 km from the release point). This drops to just 73.5% at Little Goose dam (176 km) and 72.6% at McNary dam (342 km) (Table 14). It appears that the highest difference in survivorship occurs between Lower Granite and Little Goose dams, despite the fact that this is the shortest stretch with only 60 km between the dams. It also appears that the highest overall mortality during migration in both groups occurs in this section of river. The significant drop in the relative recapture rate of the two groups at Little Goose supports the finding that the greatest problems for the smolts occur in this stretch.

Thus for chinook smolts not transported between Lower Granite and McNary dams, the relative survivorship of the high-BKD group to the low-BKD group to McNary dam is under 73%. The relative survivorship to the estuary is likely also less than, and possibly much less than, 73%.

While the overall trend of recaptures at the three dams during migration reveals lower survivorship among the high-BKD group, the differences among the raceways are not as strong. There is little difference if raceways B19 and B20 are compared to B16 and B17 (calculated relative survival of 95%). Recaptures from raceway 18 were much higher than the others, whereas recapture numbers from raceway 21 were much lower (calculated relative survival (raceway 21 vs. raceway 18) of 39%).

5. 3. 3. Saltwater challenge

After 3.5 weeks acclimatization and 14 weeks in saltwater the total mortality rate

was 12% (9/75) in the high-BKD group and 44% (33/75) in the low-BKD group. None of the mortalities in the high-BKD group could be attributed to BKD nor could five (5) mortalities in the high BKD group. Thus 37% (28/75) of the high-BKD group died from BKD in this period while 11% (5/47) of the remaining fish died of other causes. The rate of non-BKD mortality was similar (12% vs. 11%) in both groups. Relative survival under saltwater challenge was 64%.

Under salt water challenge, mortality closely mimicked patterns seen in the hatchery. Highest mortality was seen in smolts from raceway 20. Mortality was 76% for this raceway, whereas it was 32% and 24% for raceways 19 and 21 respectively. Mortalities ranged from 4% to 24% in the low-BKD raceways, the lowest mortality seen in raceway 18, which also experienced the lowest mortality rate under migration.

Banner et al. (1983) found similar levels of mortality in chinook smolts from three hatcheries in Oregon. These hatcheries released juveniles which had reached adequate size in the fall, and held the rest until spring. A portion of both release groups were held in salt water for 100 days (fall) or 200 days (spring). Total mortality in the first 100 days ranged from 10-12% for the fall release group and 17-49% in the spring release group. After 200 days, mortalities in the spring release group ranged from 45% to 81%.

Mortalities for one low-BKD raceway and two high-BKD raceways fall within the range seen by Banner at 100 days (approximately 14 weeks) for the spring release group (17-49%). A mixed low and high-BKD population would be expected to have mortalities between the values experienced by the low-BKD raceways and those experienced by the high-BKD raceways. Banner's data indicate that significant mortalities continue to occur after 14 weeks.

The results reported by Banner et al. (1983) reinforce the fact that BKD is slow to manifest itself and mortality can continue for long periods of time after the initiation of stressful events. Moreover, the difference in mortality rates between the fall and spring

releases in this study is further evidence for the importance of relatively high temperatures to allow for recovery from the disease before salt water entry. The fall releases had the benefit of relative high temperatures over the summer before release. The chinook in the brood stock segregation experiment in contrast were placed under seawater challenge in April, before the freshwater had reached 10 °C.

5. 3. 4. Overall Survivorship

Overall returns amounted to 60 out of 148,563 tagged fish released, with 41 out of 76,537 (0.054%) low-BKD group and 19 out of 72,028 (0.026%) high-BKD group salmon returning (Elliott and Pascho 1995) (note that this reflects survivorship only after release from the hatchery). The relative rate of return was 49%. Considering all data, about 73% relative survivorship during migration to McNary and 64% relative survival under salt water challenge were observed. If these mortality effects are multiplicative, overall relative survivorship is calculated to be 47% which is just below the observed value of 49%. As other stressful events occur in the life-cycle, including return migration, there is likely to be some degree of compensation between mortality rates in these two life stages. Many fish were transported, reducing direct mortality during migration, but perhaps resulting in greater compensatory mortality upon salt water entry.

Considering individual raceways, returns rates were as follows: Raceway 16: 0.051%, Raceway 17: 0.031%, Raceway 18: 0.078%, Raceway 19: 0.026%, Raceway 20: 0.016%, Raceway 21: 0.037% (Figure 25). Raceway 18 also had the highest survivorship under both migration and salt water challenge. Multiplying the calculated migration survivorship rates (Table 14) and saltwater challenge survivorship rates (Figure 24) together for each raceway gives estimates of 37%, 36%, 58%, 25%, 11%, 18% survivorship through migration and saltwater entry in the six raceways (16-21).

The observed return rates were regressed against these calculated survivorship rates from release through early ocean residence (Figure 26). Despite the low numbers of

fish tested in the salt water challenge and the use of recaptures as a surrogate for survivorship in migration, this relationship indicates that these effects do propagate all the way to return numbers.

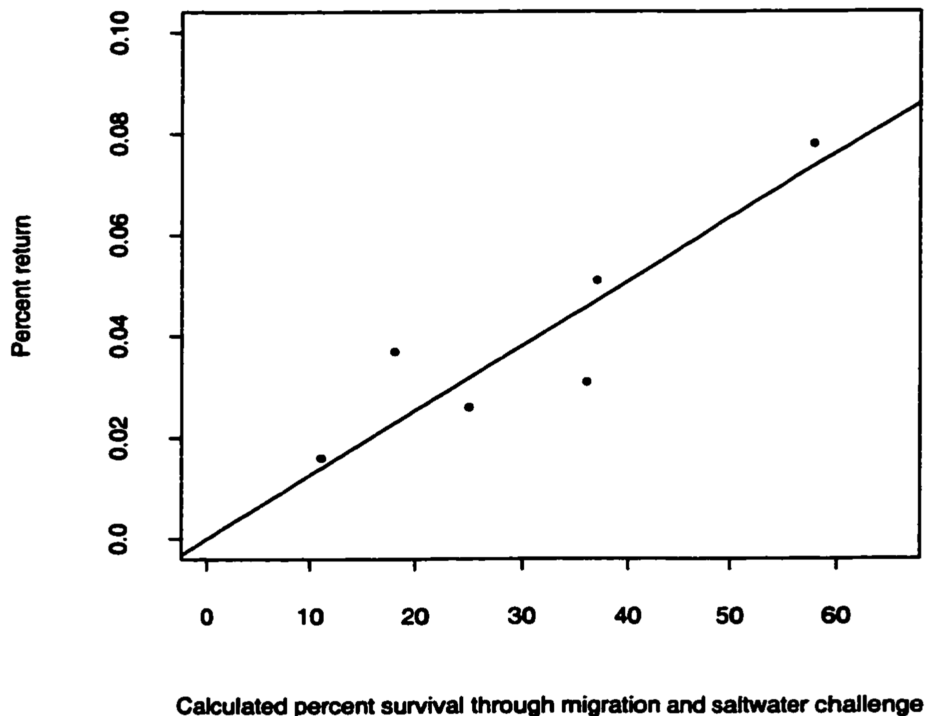

Figure 26 Calculated percent survival through migration and saltwater entry from the tagging and saltwater challenge studies, compared with the actual percent return from each raceway. The line is the best fit regression line through the 6 points and forced through (0,0) as the intercept was not significantly different from zero in the first regression. $R^2 = 0.80$.

5. 4. Antigen-inclusion induced immunotolerance

As Pascho et al. (1991) note, there was a higher overall mortality rate throughout raceway rearing, as well as higher prevalence and levels of BKD at release in the high-BKD group. However, the data do not suggest that vertical transmission is the cause of these differences. No significant differences between the high and low-BKD groups occur until after the parr stage, more than 8 months after hatch. If neither group had

significant infection levels before this point, the increased infection levels and mortality in the high-BKD group could be explained by vertical transmission, but this is not the case. Both groups have fairly high prevalences and levels of BKD at the parr stage. Thus it appears that there is a difference in the immune functioning of at least a portion of the high-BKD group which causes subsequent increased prevalence and levels of the disease, as well as increased mortality throughout the life-cycle.

The supposed difference in immune system functioning is unlikely to be genetic due to the strong selection against immunotolerant salmon. This is likely an acquired trait, as was suggested by Brown et al. (1996), caused by antigen inclusion in the pre-fertilized egg. According to their paper, 100 ng (0.1 μg) of soluble antigen in an egg is more than sufficient to induce immunotolerance in the offspring, while 1 ng was insufficient to induce any observed change in immune functioning against BKD. Antigen densities in the ovarian fluid of the high-BKD group spawning female chinook ranged up to over 25 μg ml^{-1} (Table 12). Chinook eggs are generally have volumes in the of 0.2 - 0.4 ml. If free antigen is more readily taken up than whole bacteria by salmon eggs, then ovarian fluid antigen densities of well under 1 μg ml^{-1} might be sufficient to result in antigen inclusion induced immunotolerance in offspring.

An advantage of the antigen hypothesis is that while the likelihood of immunosuppression in progeny is still related to the severity of infection in female spawners, actual vertical transmission is not necessary for immunosuppression to occur. The low levels of infection seen by Evelyn et al. (1984, 1986) and the lack of evidence for greater prevalence and severity of infection after hatching (Pascho et al. 1991) are not in conflict with the supposition of widespread immunotolerance among progeny of high-BKD broodstock.

Given the hatchery survival data, the overall return data, and the average survival data through migration or saltwater challenge, the percentage of the high-BKD group offspring that would have to be immunotolerant to result in the observed data can be

estimated. About half of the offspring would have to be immunotolerant to explain the observed differences in total returns. Only 14 of the 30 high-BKD female spawners had detectable antigen levels in their ovarian fluid (Table 12). While lower (undetectable) levels may result in some antigen inclusion high enough to result in immunotolerance, let us assume that, in fact, all eggs from those 14 spawners resulted in immunotolerant (IT) offspring, while the other 16 spawners produced only normal or non-immunotolerant (non-IT) offspring. The breakdown by raceway is such that 20% of the raceway 19 spring chinook, 70% of the raceway 20 spring chinook, and 50% of the raceway 21 spring chinook would have been immunotolerant under the above assumption. Assuming, moreover, that all immunotolerant fish die, and thus are irrelevant in predicting returns, is equivalent to beginning with only 80%, 30%, and 50% of the actual fish hatched and reared in raceways 19, 20 and 21 respectively.

Hatchery mortalities are accounted for by assuming that approximately 10% of the non-immunotolerant chinook died in hatchery rearing (the level seen in the low-BKD group) and 30% of the immunotolerant chinook died during hatchery rearing to achieve the observed 20% mortality in the high-BKD raceways. This assumes 86%, 76% and 80% survival in raceways 19, 20 and 21, very close to the actual calculated values (accounting for periodic removals of fish for testing) of 84.7%, 75.6% and 82.9% survival observed in the three high-BKD Raceways.

The result of correcting for hatchery mortality is that 16%, 64% and 44% of the fish actually released from raceways 19, 20 and 21 were immunotolerant under the assumptions stated above. Percent returns, corrected by ignoring the proportion of releases from each raceway that are assumed to be immunotolerant are given in Table 15 and Figure 27. This represents the percentage of non-immunotolerant (non-IT) chinook salmon released from each raceway that returned. After this correction the returns from the low and high-BKD raceways are indistinguishable, and with quite similar variation among raceways within each group.

Table 15 Percent returns calculated based upon all releases and calculated non-immunotolerant (non-IT) releases described in the text. Return data from Elliott and Pascho (1995).

Raceway	% IT at hatch	% IT at release	Total Releases	Non-IT Releases	Returns	Overall % Return	Non-IT % Return
16	0	0	25369	25369	13	0.051	0.051
17	0	0	25483	25483	8	0.031	0.031
18	0	0	25685	25685	20	0.078	0.078
19	20	16	22645	18959	6	0.026	0.032
20	70	64	24801	8811	4	0.016	0.045
21	50	44	24582	13827	9	0.037	0.065

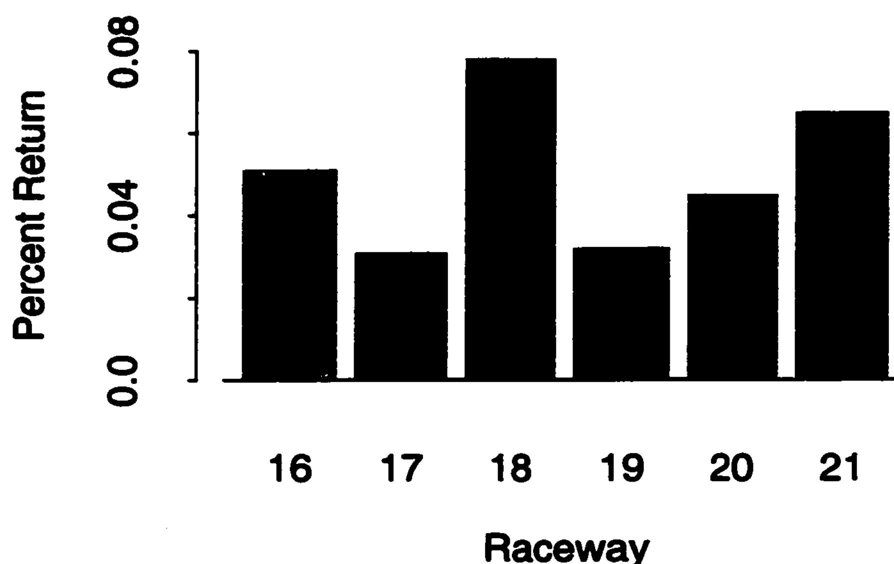

Figure 27 Estimated return rates (SARs) of non-immunotolerant spring chinook released from each raceway given assumptions in text. Compare to Figure 25.

The high variation in the percent returns among raceways within both the low and high-BKD groups is an artifact of the low numbers. Approximately 25,500 fish were released from each of the three low-BKD raceways. Given the very low number of fish returning, returns can be modeled as Poisson, with mean and variance $\lambda = 14$. The 95%

confidence interval for this λ is (6.5,21.5) which contains all three data points.

5. 5. Discussion

Pascho et al.'s (1991) original hypothesis was that vertical transmission from the more highly infected females would result in higher levels of *R salmoninarum* infection and mortality in their progeny. The data presented in their paper refutes this hypothesis. Differences in prevalences and levels of infection are not observed until after 8 months, a peak of infection levels for both groups. Yet subsequent mortality and infection levels were higher among the offspring of highly infected female spawners. Pascho et al. (1991) attributed the pattern of infection and mortality to some "unknown mechanism", while considering tolerance induction one possible explanation. However, they did not take the next step and consider that broodstock segregation might only be separating two groups of offspring with different probabilities of survivorship, without changing the overall survivorship of the entire group.

The differences in survivorship observed among the three high-BKD raceways can be directly related to the level of infection (bacterial load or antigen density) observed in the ovarian fluid of the ten female spawners whose progeny ended up in a particular raceway. The more highly infected females the higher the rate of mortality in the raceways and under salt water challenge. The results did not hold up perfectly under migration, where recaptures were used as a surrogate for relative survival. In particular, raceway 20 had the highest concentration of extremely highly infected female spawners, raceway 21 came in second in this category and raceway 19 had the fewest highly infected females among the high-BKD raceways. Mortality during both raceway rearing and saltwater challenge was highest in raceway 20.

The observed patterns of infection and mortality can be explained by differences in immune system functioning of some of the offspring of the highly infected female spawners. This specific immunotolerance to BKD may be caused by the inclusion of a

soluble antigen (p57) of *R. salmoninarum* in the salmonid egg before fertilization. This mechanism, coupled with effects of stress and temperature, can explain the differences in infection and mortality described in all three papers (Pascho et al. 1991, 1993, Elliott et al. 1995). Other mechanisms seem less likely. While genetic differences are a possibility, those genetically less able to survive BKD would likely have died out quickly given the observed differences in survivorship.

Brown et al. (1996) found that the inclusion of the antigen p57 in the egg at levels of 100 ng per egg induced immunotolerance in fry. This seems a likely hypothesis to explain the observed differences in response to the disease. But there is still the conundrum of the lack of differences in the first 8 months post hatch. This can be explained, however, by the cold temperatures (below 10 °C) experienced by the fry during the first seven to eight months after hatch (see Chapter 4). These low temperatures may impair the development and functioning of the immune system, particularly helper T cells (Bly and Clem, 1992), thus preventing effective response by most individuals of either group. Higher subsequent temperatures allow individuals in the low-BKD group to fight off the disease, whereas some members of the high-BKD group are still impaired even at higher temperatures due to antigen inclusion induced immunotolerance.

6. Summary of Analysis

6. 1. Overview of factors affecting BKD and salmonid survival

As stressed in Chapter 1, BKD is unique in that it is a chronic disease, caused by a slow growing, obligate salmonid pathogen. In Chapters 3 through 5, a number of important factors affecting infection and progression of the disease were examined in detail. In Chapter 2 the relationship between bacterial numbers, an obvious measure of infection, and antigen density, the p57 antigen being an important virulence factor, was examined. Here a synthesis of all the above results is made in terms of the lifecycle of the spring chinook salmon. An overview of the major factors influencing the progression of the disease is displayed in Figure 28, and Figure 29 gives an overview of the life-cycle with a focus on the long term effects of vertical transmission and antigen inclusion.

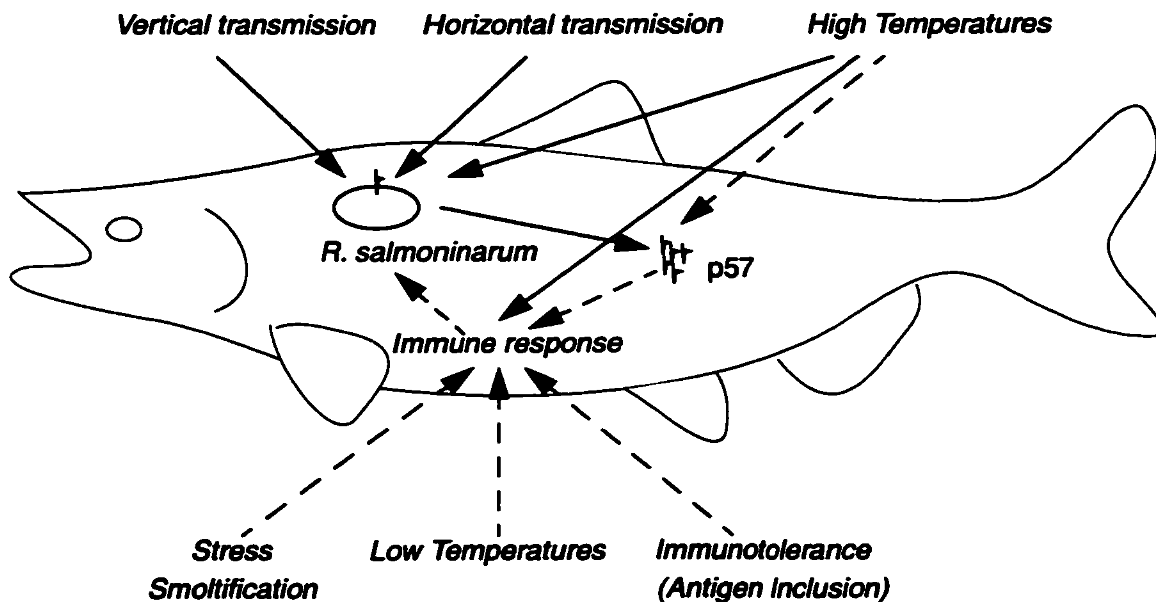

Figure 28 Factors influencing bacterial load, antigen density and the immune response in infected salmon. Solid arrows indicate promotion of object and dashed arrows indicate negative effects on object.

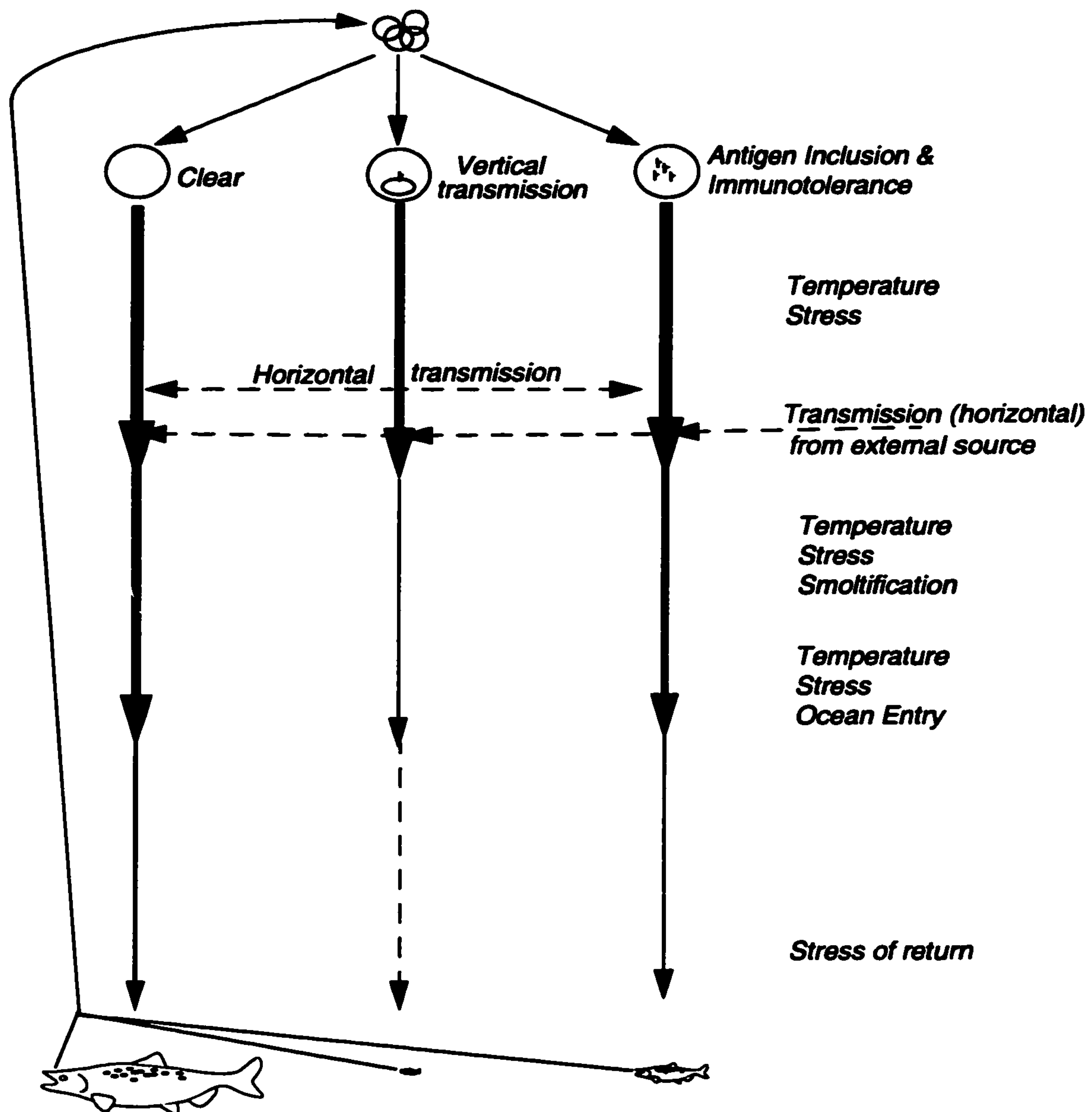

Figure 29 Visualization of relative survivorship and factors affecting survival of offspring that experience no interaction with the bacterium or antigen in the egg stage ("clear") and those experiencing either vertical transmission or antigen inclusion. Line widths and spawner sizes are general indications, but by no means quantitative predictions, of relative survivorship. Horizontal transmission can occur throughout the lifecycle but is most likely during rearing, transportation, and pre-spawning holding.

6. 2. Vertical transmission

Individual salmon which are exposed to the *R. salmoninarum* bacterium in ovum, before fertilization and water hardening, may hatch with the bacterium still viable and, as the immune system is still in development at hatch, appear likely to fail at fighting off the disease and, in many cases, die within the first few months post hatch. In the process, they are a source of infection for others in their cohort.

Vertical transmission is a fairly rare event as detailed in Chapter 3. Natural vertical infection rates were approximated by:

$$VI = 0.14\left(\frac{B}{B + 10^8}\right) \qquad (59)$$

where B represents the ovarian fluid bacterial load (cells ml^{-1}). So that even at very high levels of infection only about 1 in 7 eggs is infected vertically. This may result in much higher infection prevalences by the smolt stage due to subsequent horizontal transmission. However, if the disease is already endemic in the area, horizontal infection from other salmonids in the rearing areas may be the dominant factor in determining smolt infection prevalences.

6. 3. Antigen inclusion and immunotolerance

Brown et al. (1996) hypothesized that antigen-inclusion induced immunotolerance could occur in offspring of female spawners with high antigen densities in their ovarian fluid before spawning. While there are no published studies which address the rate of immunotolerance induction in offspring of female spawners with different antigen densities, evidence presented in Chapter 5 suggests that it may be quite prevalent in offspring of spawners with moderate to high densities of antigen in their ovarian fluid. In that chapter it was assumed that all offspring of any female with detectable levels of antigen in the ovarian fluid would be immunotolerant. While this is obviously an oversimplification, it fits the data as well as any model. Given that the antigen p57 is

responsible for the agglutinating properties of R. salmoninarum, it would not be surprising if the antigen p57 in soluble form is readily taken up by cells, including eggs.

The implications of antigen inclusion are severe for the subsequent offspring. The immune system of immunotolerant salmon are much less likely to suppress the infection in the short term and much more likely to die due to the disease at some point in the life-cycle. In the case of salmon which are both vertically infected and immunotolerant, the probability of survival past the first few months of life is presumably far reduced relative to unaffected fry.

6. 4. Temperature effects

Temperature affects the ability of helper T cells to function and to stimulate phagocytic cells to kill *R. salmoninarum*. Temperature affects the breakdown rate of the p57 antigen as well, increasing that rate at high temperatures, so that high temperatures not only aid immunocompetence but also may decrease the virulence of the infection. Temperature affects the growth rate of the bacterium as well. An estimate of the growth rate of the bacteria, as developed in Chapter 4, is:

$$\frac{dB}{dt} = 0.0667B\left(2^{\frac{T'}{6}}\right) \tag{60}$$

where:

$$T' = T \qquad T \leq 15°C$$
$$T' = 15 \qquad T \geq 15°C$$

Hypothesized forms of the relationships between temperature and the immunocompetence of the host, the virulence of *R. salmoninarum* or the exponential bacterial growth rate are plotted in Figure 30.

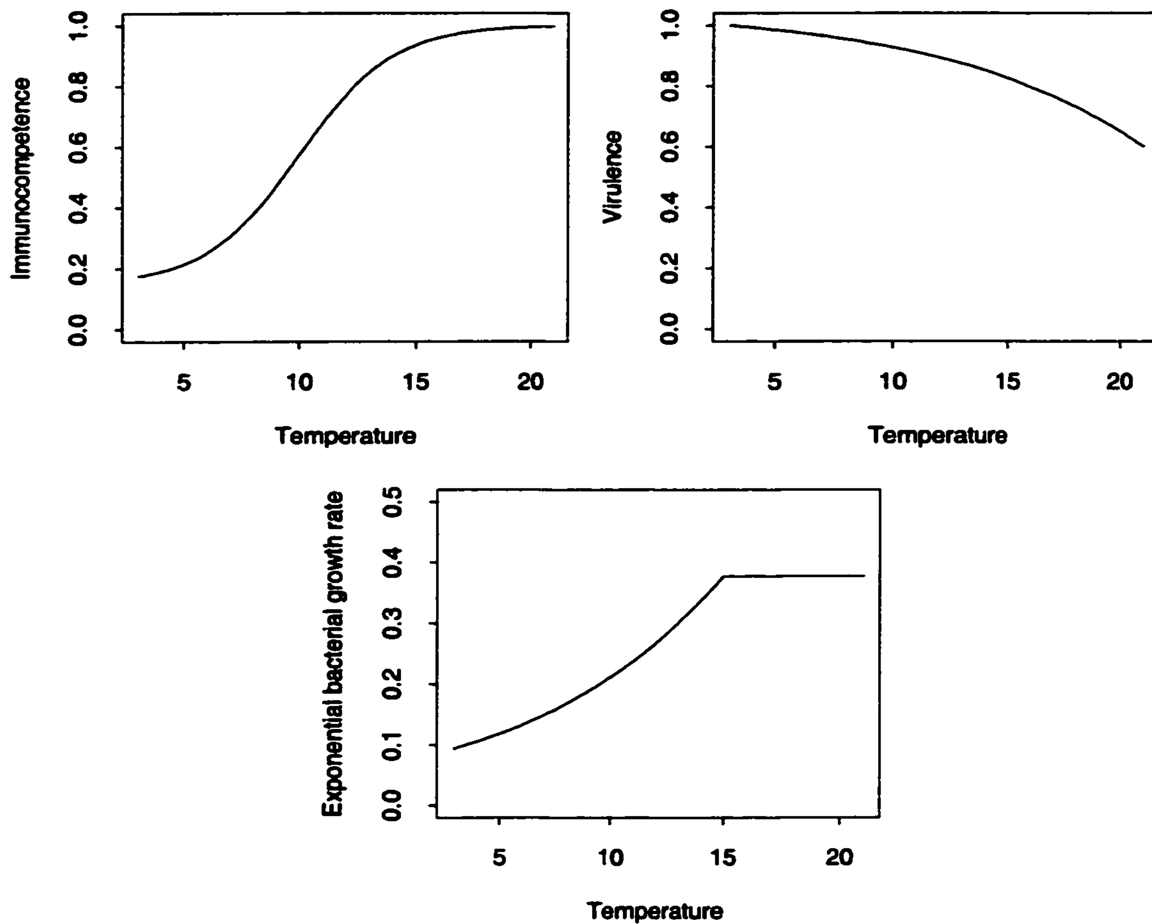

Figure 30 Hypothesized forms of relationships between temperature and other important variables. Bacterial growth rate from Equation 43.

The effect of temperature is more severe at first infection as low temperature immunosuppression is know to affect virgin helper T cells more than primed T cells, so that, in fact, two different relationships exist between temperature and immunocompetence, depending upon the previous experience of the immune system.

6. 5. Stress effects

Chronic or frequent intermittent stress can cause immunosuppression, partially due to the effects of cortisol. Thus stress, like low temperatures, immunotolerance, or high

concentrations of the antigen p57 can allow for the growth of *R. salmoninarum*, and hasten mortality. Significant stress is associated with saltwater entry and the accompanying osmoregulatory conditions.

6. 6. Divergence of infections

Sanders et al. (1978) found that at high temperatures, groups of coho artificially infected with *R. salmoninarum* had low mortality rates, while sockeye similarly infected had high mortality rates. Despite this difference, the mean time to death, for those fish that died of BKD, was similar for both groups (these data were explored in Chapter 4). Survivorship curves which match this situation are plotted in Figure 16. It appears from this that the coho in this experiment can be placed in two groups, those that succumbed to the disease, as in the case of the sockeye, and those that recovered from the infection.

After initial exposure the infection progresses or is suppressed depending upon the immune system competence (which will be referred to as *immunocompetence*) versus *R. salmoninarum*. Immunocompetence at the time of infection varies among fish in a population, which explains why some fish could recover and others not. Immunocompetence is dependent upon temperature, stress, smoltification and other life stage effects, the density of the p57 antigen, life long immunotolerance, and other factors such as diet. A suppressed infection can be released by immunosuppression (reduced immunocompetence) due to any of the above factors or a combination of such factors.

Once an *R. salmoninarum* infection takes hold, the antigen density increases, resulting in decreased immunocompetence from the effects of that factor alone. In Chapter 4, bacterial growth was estimated to occur at an exponential rate in uncontrolled infections:

$$B = B_0 e^{0.0667(2^{T/6})t} \tag{61}$$

The equilibrium level of the antigen density associated with any bacterial load was

estimated in Chapter 2:

$$B = 6Ae^{0.11A} \tag{62}$$

With the increases in bacterial load and antigen density, the infection becomes more difficult to suppress. Thus even if other conditions improve (e.g. decreased stress, increased temperature, or the end of smoltification), the immune system may not regain enough competence to fight off the infection. Those fish, on the other hand, that initially are able to control the disease are likely to survive unless other factors, such as stress or temperature, become worse before the bacterial load has been substantially reduced. For the controlled laboratory experiments undertaken by Sanders et al. (1978), these other factors would have been fairly constant. Thus, there appears to be a bimodal response to infection. Initially, the infection either takes hold or it does not. Since high infection levels reduce immune system function, the probability of recovering becomes less as the infection becomes greater.

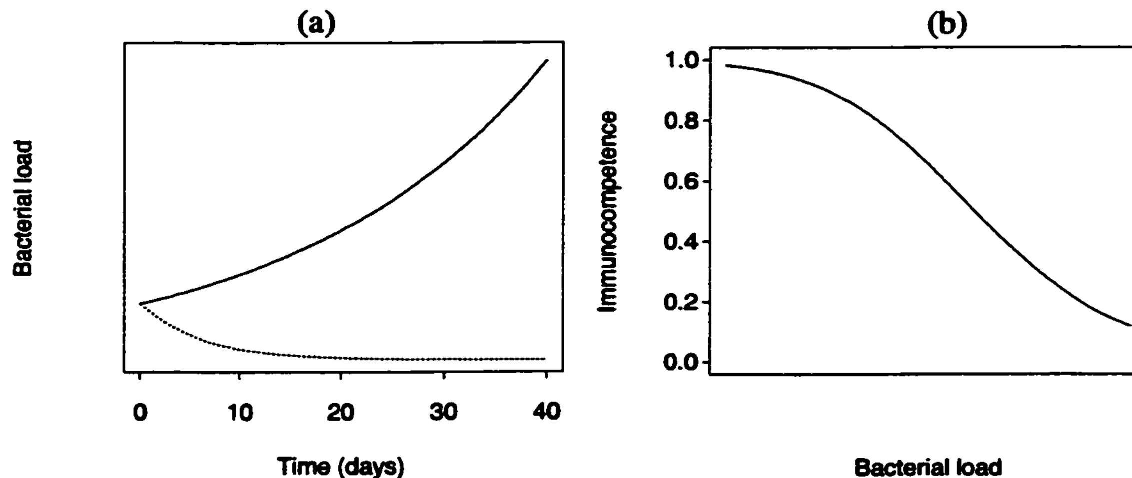

Figure 31 Divergence of bacterial load over time in two individual fish with different initial immunocompetence versus *R. salmoninarum* (a), and the effect of bacterial load on immunocompetence, ignoring other factors (b).

6. 7. Vitality model

The vitality model (Anderson 2000, and discussed in Chapter 4) does not apply to the entire population when disease is a stressor, at least in the case of BKD. The bimodal response discussed above results in diverging bacterial loads among subsets of the population. Since r, the mean vitality loss rate and a measure of progression toward mortality, is a function of bacterial load (see Equation 63), r diverges as bacterial load diverges. This results in different survivorship curves for each subset of the population. The relationship, found in Chapter 4, between bacterial load (B), temperature (T), and r is:

$$r = 7.3x10^{-15}BT \qquad (63)$$

Given the hypothetical changes in bacterial load above (see Figure 31), the effect on r and median vitality can be deduced. Representative temporal changes in r and median vitality are plotted in Figure 32.

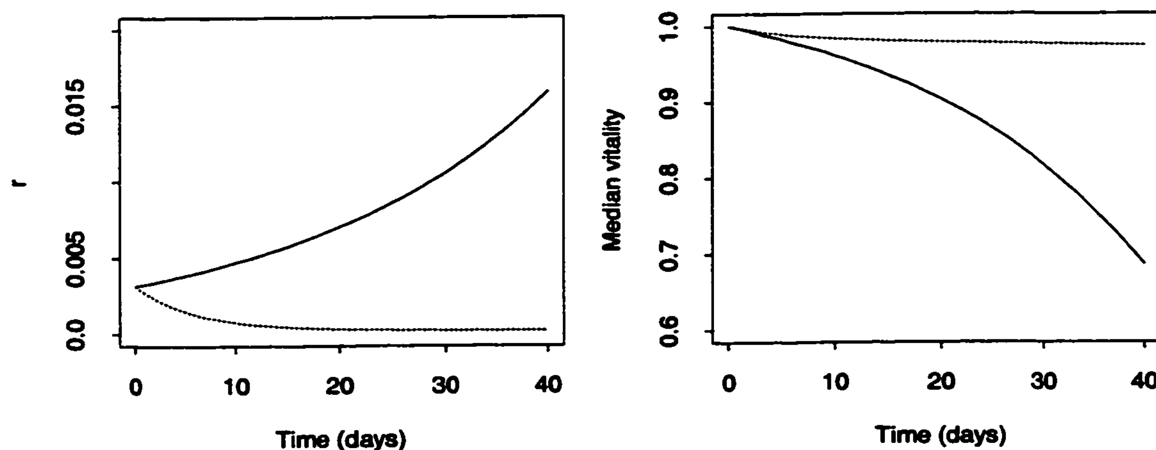

Figure 32 The progression in values of r and median vitality given bacterial growth or suppression.

This use of the vitality model, essentially transforming it into a dynamic model with r changing with time as bacterial load and temperature (and possibly other factors)

change, is different than previous applications. The model in this form, however, can well explain the divergence of two groups within the population in terms of vitality.

6. 8. Implications for timing of ocean entry in relation to river temperature

At low water temperatures in river, the immune system is suppressed at some level. Eventually the water temperature reaches a level at which helper T cells are sufficiently active, which, coupled with a slightly lower virulence of the bacterium, enables the individual to fight off the infection. The more severe the infection, the warmer the temperature necessary for successful suppression of the disease. Saltwater entry at time "a" will likely lead to the demise of the salmon, whereas at time "b" the salmon is much less likely to die of BKD. Barge or truck transportation, which moves smolts more quickly downstream resulting in earlier salt water entry might be counter-indicated for *R. salmoninarum* infected salmon before the river has warmed significantly (to about 10 °C).

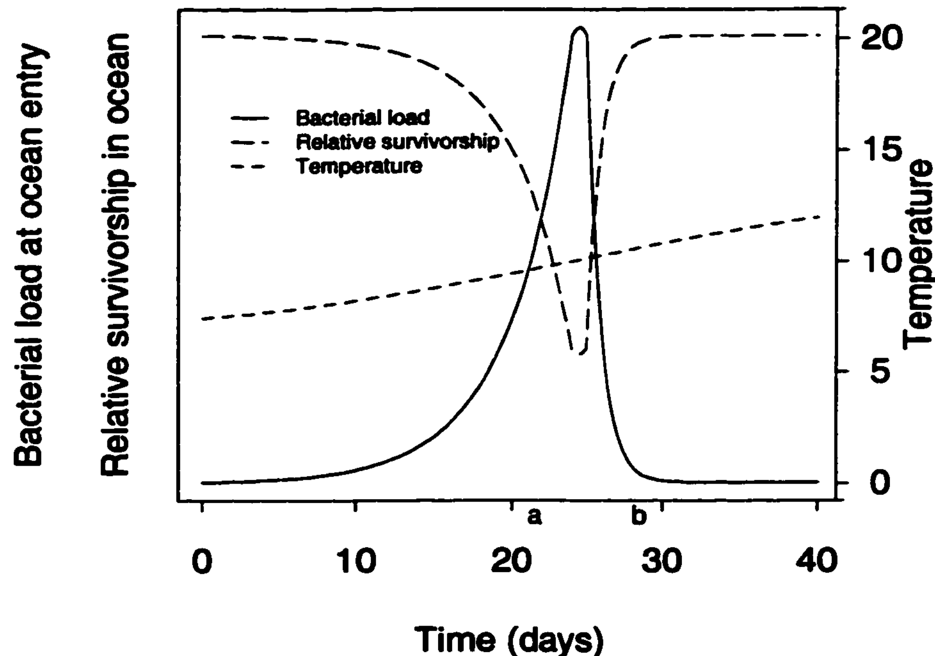

Figure 33 Hypothesized shape of mean bacterial loads during migration and relative survivorship upon ocean entry as river water temperatures increase in the spring.

The rate at which *R. salmoninarum* cells are cleared has not been quantified, but it certainly occurs at a very rapid rate relative to the growth of the bacterium.

A barge transportation study conducted by the National Marine Fisheries Service in 1995 found that in April, SARs (smolt to adult return rates) of transported Snake River yearling chinook salmon were fairly low (around 0.25%) and no higher than the SARs of non-transported yearling chinook for that month. In May through early June, SARs of transported yearling chinook increased to around 0.7%, 2 to 5 times the SARs of the non-transported yearling chinook for that time period.

The differences in observed survival of transported Snake River yearling (spring) chinook in April and May of 1995 may have been in part or in whole due to the differences in temperature regime experienced by the different groups of transported fish and the immunosuppressive effects of low temperatures on salmon with *R. salmoninarum* infections.

6. 9. Conclusions

The survival and return rates of Snake River spring chinook salmon depend upon many factors, of which BKD appears a crucial one. The effect of BKD itself depends upon many factors. Whether or not an individual chinook is infected with the disease would seem a primary consideration, but when nearly all individuals are affected with at least a minimum infection level by the time of downstream migration, other factors become more interesting. These include vertical infection, antigen inclusion, temperature and stress effects, as well as the external pressure of continuous or intermittent challenge with waterborne or ingested *R. salmoninarum* bacteria.

In this dissertation, the probability of vertical infection and antigen inclusion have been considered in relation to spawning female infection levels; the effects of low temperature immunosuppression coupled with other temperature effects on mortality rates have been investigated; and the effects of an individual chinook salmon's history

(including vertical and horizontal transmission, antigen inclusion, temperature regime and stress) on its survivorship has been contemplated. Also, the internal dynamics and equilibrium relationships between bacterial load and antigen density in tissues and fluids have been investigated.

Conclusions that can be drawn from the above investigations are as follows: (1) Temperature regimes are quite important for the survivorship of young salmon infected with *R. salmoninarum*. Warm temperatures appear to help in fighting off infections due to increased immune system functioning and also increased breakdown rate of the p57 antigen. This is especially important before stressful events such as smoltification or ocean entry. (2) The ovarian fluid infection level of female spawners is a predictor of the success of their offspring, with the primary factor appearing to be antigen inclusion induced immunotolerance rather than vertical transmission or genetic effects. Culling of highly infected female spawners may be effective in reducing mortalities and horizontal transmission after release from the hatchery. Broodstock segregation may only be successful in preventing horizontal transmission if a clean water source in available for rearing purposes. (3) The probability of survivorship throughout the lifecycle is related to multiple life history events. Spring chinook that experience vertical transmission and/or antigen inclusion are far less likely to survive than those that do not, but equally important are periods of stress or low temperature and the amount of time between such events for recovery to occur. As the antigen p57 is immunosuppressive, once a certain infection level is surpassed recovery is unlikely even with otherwise favorable conditions.

7. Mathematical Models of Disease Effects and Dynamics

7. 1. Introduction to modeling BKD

Applying mathematical models to the dynamics and effects of BKD in Snake River spring chinook salmon is a complex and difficult task. The ultimate goals include modeling the total effect of BKD on the survival to spawning (returns) of particular salmon stocks, and assessing the effect of hatcheries and hatchery stocks in maintaining or exacerbating the disease problem in wild stocks. To begin to address these questions, simple models of survivorship and disease transmission are adapted to the problem of BKD, and more complex models of bacterial growth and immune system response within individual salmon are proposed. Finally, a numerical dynamical model of disease transmission with a carrier state is used to analyze the disease dynamics for the broodstock segregation experiment reviewed in Chapter 5.

The following analyses will make use of matrix algebra. In particular, *eigenvalues* will be used to determine whether a population is growing or shrinking, in the case of Leslie matrices, or whether an equilibrium point is stable or unstable, in the case of stability analysis using Jacobian matrices. An eigenvalue λ of a square matrix $\mathbf{M}$ is a scalar, such that for a particular *eigenvector* ξ, $\mathbf{M}\xi = \lambda\xi$. For a n by n matrix, there can be at most n unique eigenvalue/eigenvector pairs. In the analysis below, $\mathbf{M}$ represents a population transition matrix such that with a unit increase in time, $\mathbf{x(t+1) = Mx(t)}$, given any initial population vector $\mathbf{x(0)}$. In the limit, in each time step the population will increase (or decrease) by a factor of λ^*, the dominant (largest) eigenvalue (except for exceptional initial population structures). If λ^* is greater than one the population increases.

The Jacobian matrix is used in stability analysis of equilibria or steady states for a system of differential equations. Its components are the instantaneous rates of change of each of the differential equations with an increase in each of the variables. In this case, if λ^* is greater than zero, then small departures from the steady state (which are not orthogonal to the associated eigenvector) will grow, and the steady state is unstable.

7. 2. Leslie matrix model of population survival

Ultimately, the goal of modeling disease dynamics is to be able to determine the effects of the disease on population survival and fecundity. In gross, this can be addressed by a Leslie matrix population model (Leslie 1945). The age-structured matrix model considered here describes survivorship in each year and fecundity for female spawners in each year. No density dependence is assumed, such that the matrix only applies to populations well below carrying capacity.

$$M = \begin{bmatrix} 0 & 0 & s_r s_1 r_3 f_3/2 & s_r s_1 r_4 f_4/2 & s_r s_1 r_5 f_5/2 \\ s_2 & 0 & 0 & 0 & 0 \\ 0 & s_3 & 0 & 0 & 0 \\ 0 & 0 & (1-r_3)s_4 & 0 & 0 \\ 0 & 0 & 0 & (1-r_4)s_5 & 0 \end{bmatrix} \qquad (64)$$

where:

s_i = survival in the ith year of life

s_r = survival during return migration and holding to spawning

r_i = proportion of females of age i which return to spawn in that year

f_i = the average number of eggs per female of age i (fecundity)

Again, given an initial population vector **x** which represents the population numbers in each age class, **x(t+1) = Mx(t)**. The unit of time is one year.

Taking the values similar to those used in the cumulative risk initiative (National Marine Fisheries Service) analysis for Snake River spring chinook salmon stocks and ignoring year to year variability, the Leslie matrix adopted for this analysis is:

$$M = \begin{bmatrix} 0 & 0 & 0.4 & 12 & 30 \\ 0.044 & 0 & 0 & 0 & 0 \\ 0 & 0.8 & 0 & 0 & 0 \\ 0 & 0 & 0.79 & 0 & 0 \\ 0 & 0 & 0 & 0.45 & 0 \end{bmatrix} \quad (65)$$

For this matrix, the dominant eigenvalue ($\lambda^* = 0.93$) indicates a population drop of 30% every 5 years.

If the elimination of the disease were to result in a 20% increase in survival in both years one and two, a 10% increase in survival during return migration, and a 1% increase in average fecundity, the resulting matrix would be as follows:

$$M = \begin{bmatrix} 0 & 0 & 0.533 & 16 & 40 \\ 0.0528 & 0 & 0 & 0 & 0 \\ 0 & 0.8 & 0 & 0 & 0 \\ 0 & 0 & 0.79 & 0 & 0 \\ 0 & 0 & 0 & 0.45 & 0 \end{bmatrix} \quad (66)$$

For this matrix, the dominant eigenvalue ($\lambda^* = 1.03$) indicates a population increase of 18% every 5 years until density dependence begins to be a significant factor. This simple analysis shows that if, in the presence of BKD, survivorship is reduced by one sixth in each of the first two years of life and one eleventh upon return, and average fecundity is reduced by 1% for those that do spawn, then BKD could be responsible for the decline of this hypothetical stock.

7. 3. The internal bacterial dynamics model

The following makes use of stability analyses of equilibria (or "steady states"). Stability is important as a stable steady state is one that is likely to persist under perturbation, whereas unstable steady states will not persist, and therefore are not indicative of states that are likely to be found in nature. Stable steady states can represent

extinction or stable coexistence between two or more species or states.

At the other extreme in terms of detail and scale from the Leslie matrix model, which considers population survival, is a model of the dynamics of the bacteria within individual salmon. This can be modeled assuming a growth (R) and a dormant (H) phase where bacterial reproduction (α) occurs only in the growth phase, and the immune response (I) only affects the growth phase. In the model, the μ_is represent immune-independent mortality of bacteria and β and γ the rates of transfer from one phase to the other.

$$\begin{aligned} \frac{dR}{dt} &= (\alpha - \beta - \mu_1 - I)R + \gamma H \\ \frac{dH}{dt} &= \beta R - (\gamma + \mu_2)H \end{aligned} \tag{67}$$

Assuming the immune response I is a constant and letting $\alpha_1 = \beta + \mu_1 + I - \alpha$ and $\gamma_2 = \gamma + \mu_2$, then the above equations can be simplified to:

$$\begin{aligned} \frac{dR}{dt} &= -\alpha_1 R + \gamma H \\ \frac{dH}{dt} &= \beta R - \gamma_2 H \end{aligned} \tag{68}$$

If $\alpha_1 < 0$, then there is no limit to bacterial growth (R), and the dynamics are not interesting. If $\alpha_1 > 0$, it is useful to perform phase plane analysis. To do this we examine the values of the derivatives at particular points in phase plane. Note that R and H are restricted to be non-negative.

When $R = 0, H \neq 0, \frac{dR}{dt} > 0, \frac{dH}{dt} < 0$.

When $R \neq 0, H = 0, \frac{dR}{dt} < 0, \frac{dH}{dt} > 0$.

When $R = \frac{\gamma}{\alpha_1}H$, $\frac{dR}{dt} = 0$. This is referred to as line 1 in Figure 34.

When $R = \frac{\gamma_2}{\beta}H$, $\frac{dH}{dt} = 0$. This is referred to as line 2 in Figure 34.

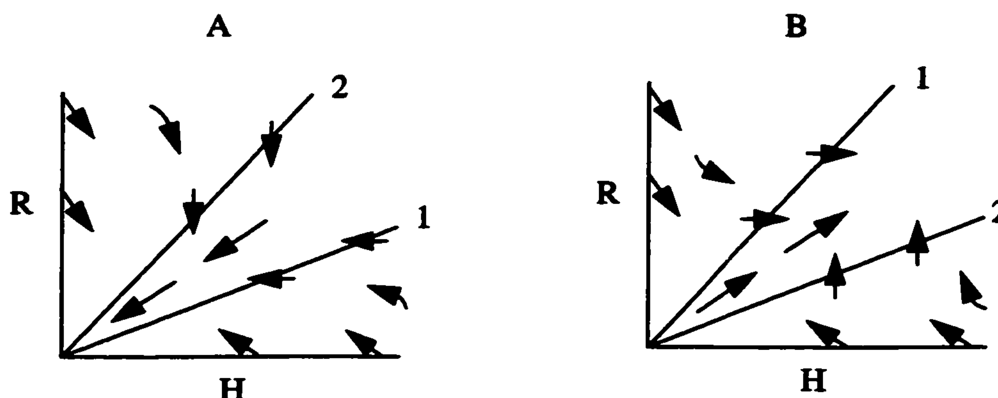

Figure 34 Phase-plane analysis. The arrows represent trajectories as the population numbers change with time.

If $\frac{\gamma}{\alpha_1} < \frac{\gamma_2}{\beta}$ then the steady state at (0,0) is stable (Figure 34 A). If $\frac{\gamma}{\alpha_1} > \frac{\gamma_2}{\beta}$, the steady state is unstable (Figure 34 B), and if $\frac{\gamma}{\alpha_1} = \frac{\gamma_2}{\beta}$ then every point on the coincident line is neutrally stable. This last case is uninteresting as it is so improbable. The same conclusions regarding stability can be arrived at by linearizing about the equilibrium and finding the eigenvalues using the Jacobian.

Translating this result back into our original variables and solving for I, we see that the (0,0) state is stable if $I > \alpha - \mu_1 - \frac{\mu_2\beta}{\gamma + \mu_2}$, whereas without a dormant state, the requirement is $I > \alpha - \mu_1$. Thus assuming no reproduction in the hiding out state reduces the level of immune functioning necessary to clear the disease. If no mortality occurs in the dormant state, the necessary immune functioning is the same with or without a dormant phase, though the rate at which the bacteria are cleared is reduced with dormancy. Note that if immune functioning is dependent on the level of R (the reproducing phase), the

dormant phase may reduce immune functioning enough to ensure an endemic population of bacteria in the host.

In the immune system model, the efficacy of the immune function increases with time and exposure to antigens. So I, and thus α_1, increases with time, and the slope of line 1 in the phase plane decreases, which implies that a host-pathogen system originally in state B in Figure 34 with both bacterial phases increasing, may eventually move into state A with both classes of bacteria decreasing, indicating the beginning of recovery. If I decreases as R decreases, then lines 1 and 2 may continually switch dominance, and periodic or non-periodic bounded solutions are possible. In this case there can be long term persistence of the bacteria at a fairly low (and oscillating) level.

Suppose instead we consider the dynamics of R and I in the absence of a dormant phase (H), assuming simple predator-prey type dynamics:

$$\begin{aligned} \frac{dR}{dt} &= (\alpha - \mu_1 - I)R \\ \frac{dI}{dt} &= \rho RI - \mu_3 I \end{aligned} \tag{69}$$

This is the standard Lotka-Volterra predator prey system In which the trivial (0,0) steady state is stable to the addition of predators (I) but not to prey (R). The other steady state at $(\mu_3/\rho,\alpha_2)$ is neutrally stable, which results in periodic solutions in the phase plane.

For a system with all three components:

$$\begin{aligned} \frac{dR}{dt} &= (\alpha - \beta - \mu_1 - I)R + \gamma H \\ \frac{dH}{dt} &= \beta R - (\gamma + \mu_2)H \\ \frac{dI}{dt} &= \rho RI - \mu_3 I \end{aligned} \tag{70}$$

or more simply:

$$\frac{dR}{dt} = (\alpha_2 - I)R + \gamma H$$
$$\frac{dH}{dt} = \beta R - \gamma_2 H \qquad (71)$$
$$\frac{dI}{dt} = \rho R I - \mu_3 I$$

The Jacobian is:

$$J = \begin{bmatrix} \alpha_2 - I & \gamma & -R \\ \beta & -\gamma_2 & 0 \\ \rho I & 0 & \rho R - \mu_3 \end{bmatrix} \qquad (72)$$

Equilibria occur at (0,0,0) and $(\frac{\mu_3}{\rho}, \frac{\beta\mu_3}{\rho\gamma_2}, \alpha_2 + \frac{\gamma\beta}{\gamma_2})$.

For the (0,0,0) equilibrium:

$J = \begin{bmatrix} \alpha_2 & \gamma & 0 \\ \beta & -\gamma_2 & 0 \\ 0 & 0 & -\mu_3 \end{bmatrix}$, which leads to the following equation to determine stability:

$\lambda^3 + (\mu_3 + \gamma_2 - \alpha_2)\lambda^2 + (\mu_3\gamma_2 - \beta\gamma - \alpha_2\gamma_2 - \alpha_2\mu_3)\lambda - \mu_3(\alpha_2\gamma_2 + \beta\gamma) = 0$, or

$\lambda^3 + a\lambda^2 + b\lambda + c = 0$

If a, b and c are all positive then there are no real positive roots. If ab > c as well, by the Routh-Hurwitz conditions (see Murray 1989) the equilibrium is stable. For this to be the case, α_2 would have to be negative because all other coefficients are assumed to be positive. Note that when α_2 > or = 0, then c < 0. If b and/or c < 0 and a > 0, or if a < 0 then b < 0 and c < 0, then the Routh-Hurwitz conditions for the real parts of all roots being less

than zero are not met, so there exists at least one root with real part > 0. The equilibrium is unstable under the assumption that the birth (reproductive) rate (α) is greater than the combined rate of natural mortality (μ_1) and transfer to the dormant phase (β). This model represents a state in which the immune system is unable to fully remove the bacteria.

For the $(\frac{\mu_3}{\rho}, \frac{\beta\mu_3}{\rho\gamma_2}, \alpha_2 + \frac{\gamma\beta}{\gamma_2})$ equilibrium,

$$J = \begin{bmatrix} -\frac{\gamma\beta}{\gamma_2} & \gamma & -\frac{\mu_3}{\rho} \\ \beta & -\gamma_2 & 0 \\ \rho\left(\alpha_2 + \frac{\gamma\beta}{\gamma_2}\right) & 0 & 0 \end{bmatrix}$$, which gives us

$$\lambda^3 + \left(\gamma_2 + \frac{\gamma\beta}{\gamma_2}\right)\lambda^2 + \left(\gamma\beta + \mu_3\left(\alpha_2 + \frac{\gamma\beta}{\gamma_2}\right)\right)\lambda + \mu_3(\alpha_2\gamma_2 + \beta\gamma) = 0,$$ or

$$\lambda^3 + a\lambda^2 + b\lambda + c = 0$$

Here, a > 0, necessarily. If c > 0 then ab > c, then this equilibrium is stable by Routh-Hurwitz conditions. Otherwise it is unstable. If α_2 is greater than zero, as it is assumed to be, the zero steady state is unstable and the non-zero steady state is stable. This model represents a state in which the immune system is unable to fully remove the bacteria, yet is able to keep the disease in a latent state. This idea of latency is expanded upon below.

The existence of the dormant state results in no change in the R value, $(\frac{\mu_3}{\rho})$, but a smaller I value, $\alpha - \mu_1 - \frac{\mu_2\beta}{\gamma_2}$ versus $\alpha - \mu_1$, unless $\mu_2 = 0$. The time scales however change, which is important when considering the persistence of the carrier state. The dormant state also eliminates the existence of periodic solutions. If the bacteria are allowed to reproduce in the dormant state, then the results are much different. $\gamma 2$ is possibly larger than γ in this case and thus the immune response, I, necessary to fight off the disease is greater than without the existence of the dormant state.

Figure 34 B indicates that a constant immune response may be inadequate to suppress the disease. This represents a fish with reduced immune system functioning which is not able to increase its immune system functioning in response to increasing bacterial load. The analysis of Equations 70-72 shows that a non-zero steady state is possible under simple assumptions, indicating the possibility of latency and long term carrier individuals when the immune system is functioning perfectly. The actual dynamics are a bit more complex with time delays between increases or decreases in the bacterial load and the concomitant increases or decreases in immune system levels. The dormant bacterial phase ensures that some bacterial will likely survive as the immune system ramps down.

7. 4. Models of disease dynamics in a single salmon population

7. 4. 1. Overview

In this section the dynamics of disease are considered using mathematical models. Major aspects of the disease dynamics include transmission of the disease from infected to susceptible individuals, the rate of eruption of the disease in latent individuals, the rate of recovery from the disease and the rate of disease induced mortality.

7. 4. 2. SI model

A simple model of the dynamics of the disease in a single population is presented here. The assumption is made that there is no recovery but only increased mortality in the infected population. "S" represents the number or proportion of susceptible individuals and "I" the infected individuals. This model contains no recovered/immune class.

$$\begin{aligned} \frac{dS}{dt} &= -\lambda SI - \mu_S S \\ \frac{dI}{dt} &= \lambda SI - \mu_I I \end{aligned} \tag{73}$$

where λ represents the rate of transmission of the disease. Assuming that $\mu_I > \mu_S$, the disease increases the overall rate of mortality of the population.

To analyze the steady states and dynamical properties of such a model, continuous births must be assumed. To simplify, vertical transmission is ignored. While this is unrealistic for semelparous salmon species, it allows some insights regarding the ability of infection to persist in a population and the interactions between two populations, which is explored below.

$$\begin{aligned}\frac{dS}{dt} &= \beta(S+I) - \lambda SI - \mu_S S \\ \frac{dI}{dt} &= \lambda SI - \mu_I I\end{aligned} \tag{74}$$

The steady states for this system are (0,0) and $(\frac{\mu_I}{\lambda}, \frac{\mu_I(\beta-\mu_S)}{\lambda(\mu_I-\beta)})$. The Jacobian of this system is:

$$J = \begin{bmatrix} \beta - \lambda I - \mu_S & \beta - \lambda S \\ \lambda I & \lambda S - \mu_I \end{bmatrix} \tag{75}$$

The trivial steady state is stable if $\beta < \mu_S$. The other steady state is stable if $\mu_S < \beta < \mu_I$. If $\beta > \mu_I$, this model predicts exponential growth of both I and S, which will eventually be constrained by some other process, such as density dependence, but which is not included in the mathematical model above. This model with continuous births has no direct application to semelparous salmon species.

7. 4. 3. Model of infection with *R. salmoninarum* from an aggregate source

Based upon Pascho et al.'s (1991) division of the population into those that test negative for BKD and those with low, medium and high level infections, the dynamics of infection levels and mortality among the population might more realistically be described

by a model based upon Figure 35.

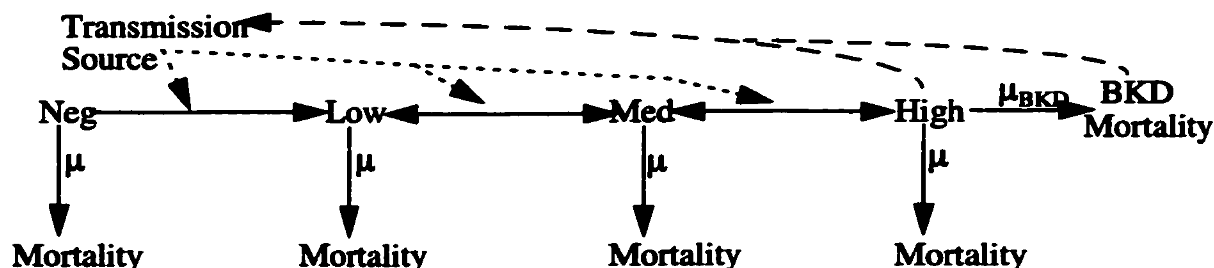

Figure 35 The dynamics of disease transmission and disease severity with four infection states and one source of infection.

This can be described by a Markov chain model with transition matrix T, representing the probabilities p_{ij} of transition from one state to another in one unit time t, with both i and j ranging from 1 to 4, representing those fish with no, low, medium, and high levels of infection.

$$T = \begin{bmatrix} p_{11} & 0 & 0 & 0 \\ p_{21} & p_{22} & p_{23} & 0 \\ 0 & p_{32} & p_{33} & p_{34} \\ 0 & 0 & p_{43} & p_{44} \end{bmatrix} \tag{76}$$

Each of the p values in this model is dependent upon the strength of the source as well as temperature, stress and developmental stage. The source strength depends on the infection levels of the fish in the population, external sources, and water flow rate. While this model is perhaps more realistic than the continuous differential models given in equations 73 and 74, it requires substantial knowledge of the transition parameters. Another approach is to add a reservoir of infection to the SI model above. This looks like:

$$\frac{dS}{dt} = \beta(S+I) - \lambda SW - \mu_S S$$
$$\frac{dI}{dt} = \lambda SW - \mu_I I \qquad (77)$$
$$\frac{dW}{dt} = \rho I - (\sigma(S+I) + \delta)W$$

W, here, represents the density of bacteria in the water, following Boots (1999). The reservoir increases from contributions from the infected class (ρ) and decreases due to both uptake by individuals (σ) and mortality and removal (i.e. due to water flow) (δ).

This model can then be improved by a second addition, that of a single carrier or "latent" class, representing both the low and medium infection levels defined by the ELISA test, symbolized by "L" for latent. At this point "I" represents all infectious rather than all infected individuals, as the latent individuals are infected as well. Also, in this model, ϕ represents the rate of eruption of the disease in latent individuals and ν represents the rate of recovery to latency among infectious individuals.

$$\frac{dS}{dt} = -\lambda SW - \mu_1 S$$
$$\frac{dL}{dt} = \lambda SI - \phi L + \nu I - \mu_1 L$$
$$\frac{dI}{dt} = \phi L - \nu I - \mu_2 I \qquad (78)$$
$$\frac{dW}{dt} = \rho I - (\sigma(S+L+I) + \delta)W$$

This is the most realistic of the above models, but too complex to gain insight without quite good parameter estimation.

7. 4. 4. SLI model

A simplified version of the above model assumes a direct link between the number of infectious individuals and new infections, dropping the bacterial density factor.

$$\frac{dS}{dt} = -\lambda SI - \mu_1 S$$
$$\frac{dL}{dt} = \lambda SI - \phi L + \nu I - \mu_1 L \qquad (79)$$
$$\frac{dI}{dt} = \phi L - \nu I - \mu_2 I$$

While we lose the idea of a reservoir of infection, the more important addition, and the one for which there is some data, the latent class, remains. Only a portion of the latent fish become infectious in the particular life stage modeled, and many of those may recover from the latent class. A slightly modified version of this model is analyzed numerically in section 7. 6.

Another approach is to simplify this even further, simply predicting the infection levels of spawners based upon the infection levels of their parents. Too great a level of life-cycle variability and a lack of data precludes analysis of this model. If a long time series of spawner infection level data were available, this might be attempted.

7. 5. Epidemiological models of two interacting populations

7. 5. 1. SI model

Another goal of modeling the disease dynamics is to analyze the impact of one stock on the maintenance or severity of the disease in another. This is especially of interest for the interactions between wild and hatchery stocks.

Begin with a simple compartmentalized susceptible-infected (S-I) model of two separate populations of the same species which have some contact and disease transmission between them. Assume as well that there is no recovery, and that the mortality rate among infecteds is greater than that of susceptibles ($\mu_1 < \mu_2$). Also assume that the contact and infection rate among a group is greater than that between groups ($\eta < \lambda$).

$$\begin{aligned}
\frac{dS_1}{dt} &= -\lambda S_1 I_1 - \eta S_1 I_2 - \mu_S S_1 \\
\frac{dI_1}{dt} &= \lambda S_1 I_1 + \eta S_1 I_2 - \mu_I I_1 \\
\frac{dS_2}{dt} &= -\lambda S_2 I_2 - \eta S_2 I_1 - \mu_S S_2 \\
\frac{dI_2}{dt} &= \lambda S_2 I_2 + \eta S_2 I_1 - \mu_I I_2
\end{aligned} \tag{80}$$

Basically, the result of the interaction is increased transmission in both populations. However, to accurately describe the dynamics of wild and hatchery salmon, we must include different life stages, varying within and between group infection rates (contact rates) with lifecycle stage, and also considering effects of straying on vertical transmission and immunotolerance induction.

7. 5. 2. Model with birth process

In order to get an analytical solution as was done above with the single population model, a continuous birth process is added. This model was previously analyzed by Holt and Pickering (1985).

$$\begin{aligned}
\frac{dS_1}{dt} &= \beta(S_1 + I_1) - \lambda S_1 I_1 - \eta S_1 I_2 - \mu_S S_1 \\
\frac{dI_1}{dt} &= \lambda S_1 I_1 + \eta S_1 I_2 - \mu_I I_1 \\
\frac{dS_2}{dt} &= \beta(S_2 + I_2) - \lambda S_2 I_2 - \eta S_2 I_1 - \mu_S S_2 \\
\frac{dI_2}{dt} &= \lambda S_2 I_2 + \eta S_2 I_1 - \mu_I I_2
\end{aligned} \tag{81}$$

There are four possible steady states associated with this model:

$$(0,0,0,0),\ (\frac{\mu_I}{\lambda},\frac{\mu_I(\beta-\mu_S)}{\lambda(\mu_I-\beta)},0,0),\ (0,0,\frac{\mu_I}{\lambda},\frac{\mu_I(\beta-\mu_S)}{\lambda(\mu_I-\beta)}),\text{ and}$$

$$(\frac{\mu_I}{\lambda+\eta},\frac{\mu_I}{(\lambda+\eta)}\frac{(\beta-\mu_S)}{(\mu_I-\beta)},\frac{\mu_I}{\lambda+\eta},\frac{\mu_I}{(\lambda+\eta)}\frac{(\beta-\mu_S)}{(\mu_I-\beta)}).$$

The coexistence steady state is stable given the same conditions as for the single population (if $\mu_S < \beta < \mu_I$) and additionally if $\eta < \lambda$. If $\eta > \lambda$, then the coexistence steady state is unstable, and the two steady states with only one population extant are stable, with the larger original population surviving. This, however, assumes disease transmission between the two groups is greater than that within each group, which seems unlikely.

Assuming, on the other hand, that each population experiences unique within-group and between-group disease transmission parameters, a more realistic model can be produced:

$$\begin{aligned}
\frac{dS_1}{dt} &= \beta(S_1+I_1)-\lambda_1 S_1 I_1-\eta_1 S_1 I_2-\mu_S S_1\\
\frac{dI_1}{dt} &= \lambda_1 S_1 I_1+\eta_1 S_1 I_2-\mu_I I_1\\
\frac{dS_2}{dt} &= \beta(S_2+I_2)-\lambda_2 S_2 I_2-\eta_2 S_2 I_1-\mu_S S_2\\
\frac{dI_2}{dt} &= \lambda_2 S_2 I_2+\eta_2 S_2 I_1-\mu_I I_2
\end{aligned} \tag{82}$$

There are four possible steady states associated with this model:

$$(0,0,0,0),\ (\frac{\mu_I}{\lambda_1},\frac{\mu_I(\beta-\mu_S)}{\lambda_1(\mu_I-\beta)},0,0),\ (0,0,\frac{\mu_I}{\lambda_2},\frac{\mu_I(\beta-\mu_S)}{\lambda_2(\mu_I-\beta)}),\text{ and}$$

$$(\frac{(\lambda_2-\eta_1)\mu_I}{(\lambda_1\lambda_2-\eta_1\eta_2)},\frac{(\lambda_2-\eta_1)\mu_I}{(\lambda_1\lambda_2-\eta_1\eta_2)}\frac{(\beta-\mu_S)}{(\mu_I-\beta)},\frac{(\lambda_1-\eta_2)\mu_I}{(\lambda_1\lambda_2-\eta_1\eta_2)},\frac{(\lambda_1-\eta_2)\mu_I}{(\lambda_1\lambda_2-\eta_1\eta_2)}\frac{(\beta-\mu_S)}{(\mu_I-\beta)})$$

Under our assumptions ($\mu_S < \beta < \mu_I$ and $\lambda_1 > \eta_2, \lambda_2 > \eta_1$), the coexistence steady state exists and is stable (Holt and Pickering 1985).What does this mean for the depression of the population numbers? The interaction of the two stocks reduces total equilibrium numbers from single stock situation. The susceptible and infected sub-populations are both reduced by a factor of $\lambda_2(\lambda_1-\eta_2)/(\lambda_2\lambda_1-\eta_1\eta_2)$ for population 2, with the subscripts reversed for population 1.

If, on the other hand $\lambda_1 > \eta_2$, but $\lambda_2 < \eta_1$, (or vice versa) then there is no coexistence steady state, and the second population will dominate, with the first dying out. This is because the second population has a greater propensity to infect individuals within the other population than individuals within itself. This interaction may be realistic for upstream and downstream populations of salmon, with the upstream population infecting the downstream population. However, the addition of a carrying capacity control would add greatly to the realism, allowing for a coexistence steady state.

Consider the upstream-downstream case, with population one being upstream, and population two being downstream, and assume, further, that there is no transference of disease from population two to population one (η_1=0). In this case, the coexistence steady state becomes:

$$\left(\frac{\mu_I}{\lambda_1}, \frac{\mu_I(\beta-\mu_S)}{\lambda_1(\mu_I-\beta)}, \frac{(\lambda_1-\eta_2)\mu_I}{(\lambda_1\lambda_2)}, \frac{(\lambda_1-\eta_2)\mu_I}{(\lambda_1\lambda_2)}\frac{(\beta-\mu_S)}{(\mu_I-\beta)}\right)$$

and the first population reduces that of the second by a factor of $(\lambda_1-\eta_2)/\lambda_1 = 1 - \eta_2/\lambda_1$.

7. 6. Numerical analysis of the SLI model

The model given in Equation 79 is used to explore the data from Pascho et al. (1991). This data, given in Figure 23 and Table 17, gives the distribution of infection levels and cumulative mortality for the low-BKD and high-BKD groups at five time periods after hatch. Here only the last three, the parr, pre-smolt and smolt infection levels, at 8, 12 and 16 months after hatch are considered. Three time periods of transmission and

development of the disease are considered concomitant with these monitoring times. The first 8 months, during which water temperatures were mainly cold (Periods A, B and C in Figure 13), the next four months (Period D in Figure 13), during which temperatures were generally warmer and during which the mortality of the two groups diverged, with the infection levels diverging somewhat, and the following four months (Period E in Figure 13), being the final winter before release, with cold temperatures, and during which a great divergence in infection levels occurs, with greater mortality observed in the high-BKD group as well.

Table 16 Values of parameters used and the division of the population by infection class at the beginning of each time period and at end of the last period. The letters represent time periods, and "high" and "low" represent the offspring of the high-BKD and low-BKD broodstock respectively.

Parameter	A-C low	A-C high	D low	D high	E low	E high	End low	End high
$\mu 1$	.0002	.0002	.0004	.0004	.00001	.00001		
$\mu 2$	.002	.002	.004	.01	.002	.002		
λ	.02	.02	.01	.03	.03	.03		
c	.2	.2	0	0	0	0		
ϕ	.0004	.0004	.0002	.005	.001	.01		
v	.001	.001	.1	.1	.1	.01		
t (days)	240	240	120	120	120	120		
S	1	.98	.348	.329	.330	.285	.324	.143
L	0	.01	.575	.586	.572	.561	.571	.405
I	0	.01	.026	.031	.001	.026	.006	.277
Total Survival to Smoltification							.901	.825

The near matching values of infection levels and total mortality at the parr stage, 8 months after hatch cannot be achieved, using realistic parameter values, without an outside source of infection (c) to modify Equation 79 as follows:

$$\begin{aligned}\frac{dS}{dt} &= -\lambda S(I+c) - \mu_1 S \\ \frac{dL}{dt} &= \lambda S(I+c) - \phi L + \nu I - \mu_1 L \\ \frac{dI}{dt} &= \phi L - \nu I - \mu_2 I\end{aligned} \tag{83}$$

A fourth-order Runge-Kutta numerical approximation was used to analyze the dynamics of the SLI model given in Equation 83. Parameters were chosen to fit the data and to represent reasonable dynamics and are given in Table 16 along with the modeled infection levels and cumulative mortalities at each of the three monitoring periods for the two groups. The results are compared to the data in Table 17.

The initial percent of the population in each of the Latent and Infectious classes in the high-BKD group do not significantly affect the end point for that group (Note that if (So,Lo,Io) = (1,0,0), then after the final stage, (S,L,I) = (0.153, 0.403, 0.274) versus (0.143, 0.405, 0.277)). However, without the external infection source, the end point for the high-BKD group would be very different (0.786 0.074, 0.037).These last values are not unlike those seen by Lee and Evelyn (1989, and see Chapter 3), when vertical infection was followed by rearing for 195 days in treated water. While the conditions were obviously different in this latter experiment, this serves to reinforce the importance of the external source of infection. The difference in the parameters among the two groups after the first time period can be attributed to the effects of antigen inclusion induced immunotolerance, decreasing resistance to the disease and recovery rates from the infectious state. Differences in parameters between time periods are due to differences in both temperature regimes and developmental stages.

To examine how well the model fits the data, it is assumed that only those individuals with high level infections, according to the partitioning done by Pascho et al. (1991) and my analysis (see Chapter 5), are in the Infectious class, with those classified as

having low or medium level infections being in the Latent class. For the parr and pre-smolt stages, there is no distinction made in the data between the negative and low infection classes, but the levels have to be such as to lead up to the levels seen in the smolt stage. Table 17 compares the data with the model fit, and shows the model to fit quite nicely.

Table 17 Comparison of data and model results. Here both low and medium levels of infection by ELISA are considered Latent, and only High levels considered Infectious. Values are the percent of original population in each class or dead.

Stage	Group	Data/Model	S	S+L	L	I	Dead
Parr	Low	Data		.878		.024	.089
		Model	.348	.923	.575	.026	.051
	High	Data		.908		.031	.061
		Model	.329	.915	.586	.031	.054
Pre-Smolt	Low	Data		.906		0	.095
		Model	.330	.902	.572	.001	.097
	High	Data		.844		.013	.143
		Model	.285	.846	.561	.026	.138
Smolt	Low	Data	.339	.897	.558	.004	.099
		Model	.324	.895	.571	.006	.099
	High	Data	.122	.570	.448	.255	.176
		Model	.143	.548	.405	.277	.175

7. 7. Sensitivity analysis

The sensitivity of the model to variation in parameters and initial conditions is considered below. As almost all infection occurs before the onset of downstream migration (or after return), the proportion of uninfected individuals at release may be a critical factor in predicting returns. The change in the value of this variable with the doubling of each parameter (except for c, which is increased by 0.1, and the initial S, L and I proportions, which are increased by 0.01) is examined in Table 18.

Table 18 Final values of S with doubling of parameter values (except c = + 0.1, S, L, I = + 0.01). "The letters represent time periods, and "high" and "low" represent the offspring of the high-BKD and low-BKD broodstock respectively. Compare to base values.

Parameter	A-C low	D low	E low	A-C high	D high	E high
$\mu 1$	.309	.309	.324	.141	.141	.142
$\mu 2$	.326	.324	.324	.146	.149	.150
λ	.110	.323	.318	.039	.126	.066
c	.196	.287	.226	.077	.091	.097
ϕ	.308	.323	.318	.134	.128	.095
ν	.325	.325	.327	.144	.151	.165
So	.327			.143		
Lo	.323			.141		
Io	.312			.135		
Base	.324	.324	.324	.143	.143	.143

The value of c, the external infection source, is important in each stage and both groups, as is not surprising. The outcome appears fairly independent of initial (vertical) infection or of mortality rates. The same is true for rates of entering the infectious stage (ϕ) or recovery (ν) except in the case of the final stage for the high-BKD group. The infection coefficient (λ) is important for both groups in the first time period, and in the high-BKD group for the final time period.

Similar results are seen when the percent of fish in the Infectious class is considered (Table 19), with the only difference being that ϕ and ν are important for the final stage of the low-BKD group as well. However, depending on the conditions experienced during migration and early ocean entry, as well as the timing of the latter, the proportion of the population in the Latent and Infectious classes can be expected to change as individuals pass between these two states. While transmission may be rare during

migration, the proportion of individuals with moderate versus severe infections at ocean entry may be of great importance in determining survivorship. Individuals uninfected at release or the beginning of migration have a greater probability of surviving than those with either latent or active infections at release.

Table 19 Final values of I with doubling of values (except c = + 0.1, S, L, I = + 0.01). The letters represent time periods, and "high" and "low" represent the offspring of the high-BKD and low-BKD broodstock respectively. Compare to base values.

Parameter	A-C low	D low	E low	A-C high	D high	E high
$\mu 1$	.005	.005	.006	.263	.263	.277
$\mu 2$	.006	.006	.006	.274	.265	.247
λ	.008	.006	.006	.326	.285	.303
c	.007	.006	.006	.308	.302	.294
ϕ	.006	.006	.011	.279	.276	.418
v	.006	.006	.003	.277	.278	.199
So	.006			.281		
Lo	.006			.282		
Io	.006			.283		
Base	.006	.006	.006	.277	.277	.277

Halving the values of each parameter gives the same information as seen in Tables 18 and 19 regarding the relative effects of altering the various parameters.

To get a better idea of how much the proportions in the Susceptible, Latent and Infectious classes at the smolt stage change with changing parameter values, these proportions are graphed versus various values of c, λ and ϕ in each time period (Figures 36, 37, and 38).

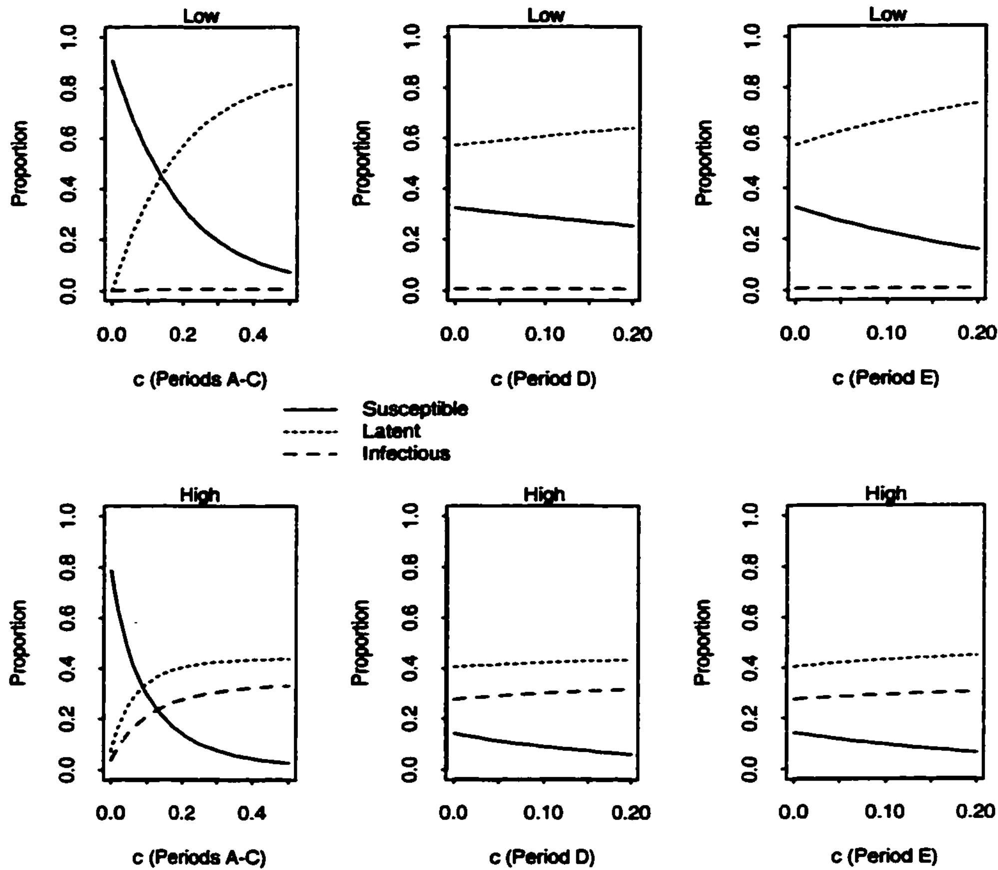

Figure 36 Sensitivity analysis with varying c (external infection source).The graphs represent the proportion of the original population that is predicted by the model to be in the Susceptible, Latent and Infectious classes at smoltification and the beginning of migration.

From these results it can be concluded that the best methods of reducing infection and increasing survivorship of salmon released from hatcheries include, most importantly (1) eliminating outside sources of infection (c), perhaps by treating all incoming water, as well as (2) reducing transmission rates (λ) which can be achieved by decreasing density and/or increasing water flow, and (3) reducing the eruption of the disease in carrier salmon (ϕ) by reducing stress and improving the overall condition of the fish.

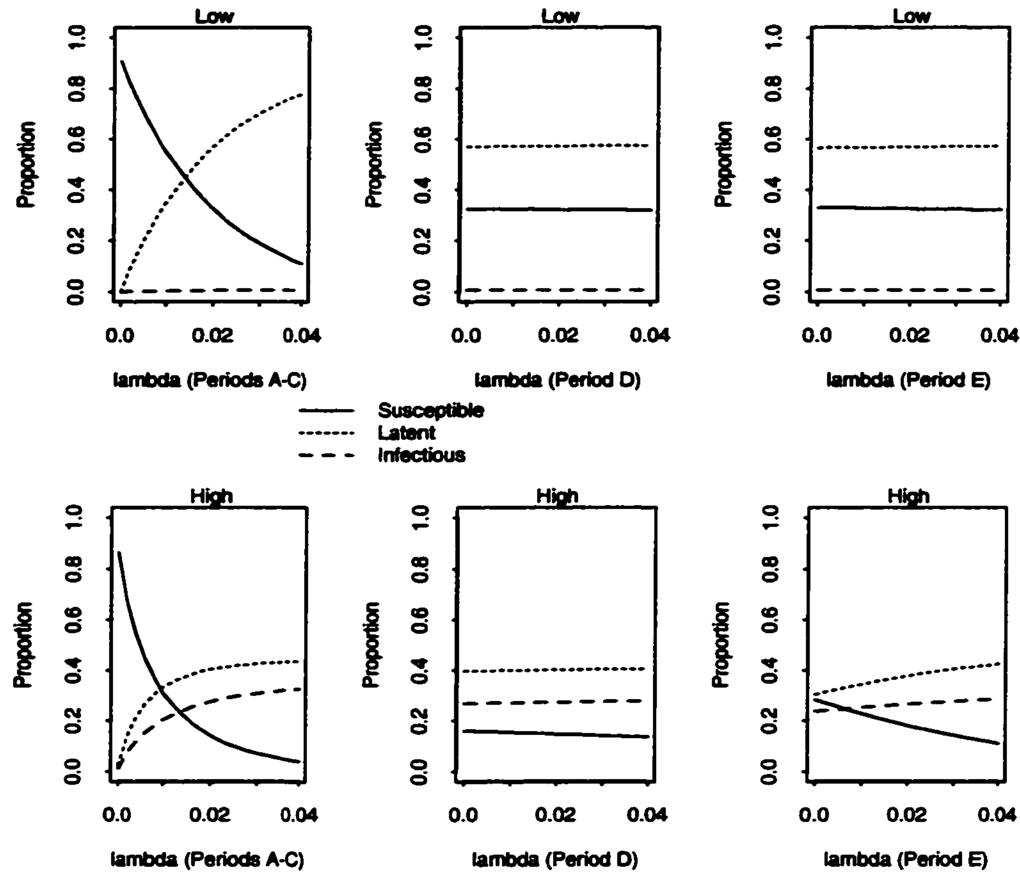

Figure 37 Sensitivity analysis with varying λ (infection rate). The graphs represent the proportion of the original population that is predicted by the model to be in the Susceptible, Latent and Infectious classes at smoltification and the beginning of migration.

Finally, the proportion of individuals in the Susceptible, Latent and Infectious stages at ocean entry is considered. This is accomplished by modeling the migration stage using Equation 78. No transmission is assumed to take place during migration, thus $\lambda = 0$ and $c = 0$, but mortality in all three classes is important. Parameters are assumed to be constant during migration for 30 or 60 days (the former representing transported salmon) under differing conditions represented by varying ν, the rate of recovery to the latent class (Table 20 and Figure 39).

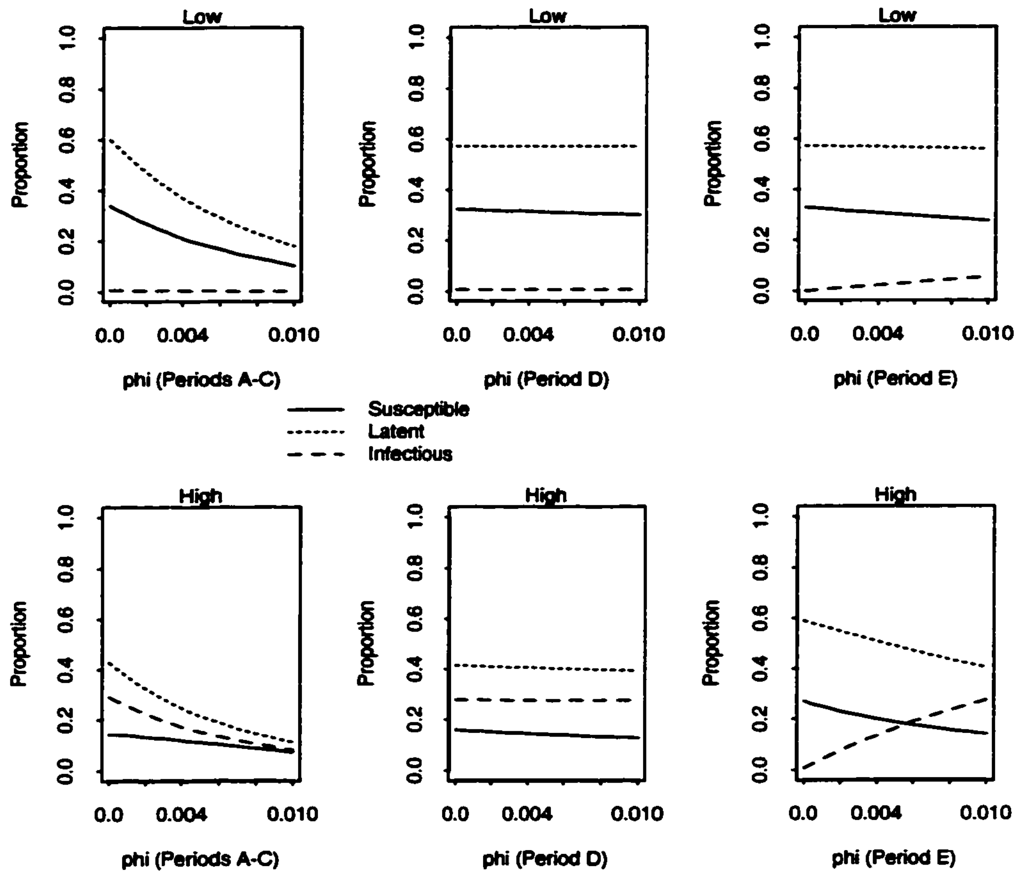

Figure 38 Sensitivity analysis with varying ϕ (rate of eruption of disease from latent state).The graphs represent the proportion of the original population that is predicted by the model to be in the Susceptible, Latent and Infectious classes at smoltification and the beginning of migration.

It is expected that the survivors come from both the Susceptible and Latent classes at ocean entry, with essentially all of the infectious individuals dying soon after entering the ocean. The baseline number of salmon with a potential for survival is somewhere between the "Susceptible" and "Susceptible + Latent" curves in Figure 39. The results show this baseline number at ocean entry may be heavily dependent upon conditions, including temperature, during migration, upon infection levels at the beginning of migration, and upon the length of migration. There may be trade-offs, as mentioned above. If a shortened migration due to transportation results in ocean entry before warm

temperatures are experienced, then the longer migration may be preferable. This can be seen by comparing the 30 day migration with low ν to the 60 day migration with high ν in either of the low-BKD or high-BKD groups.

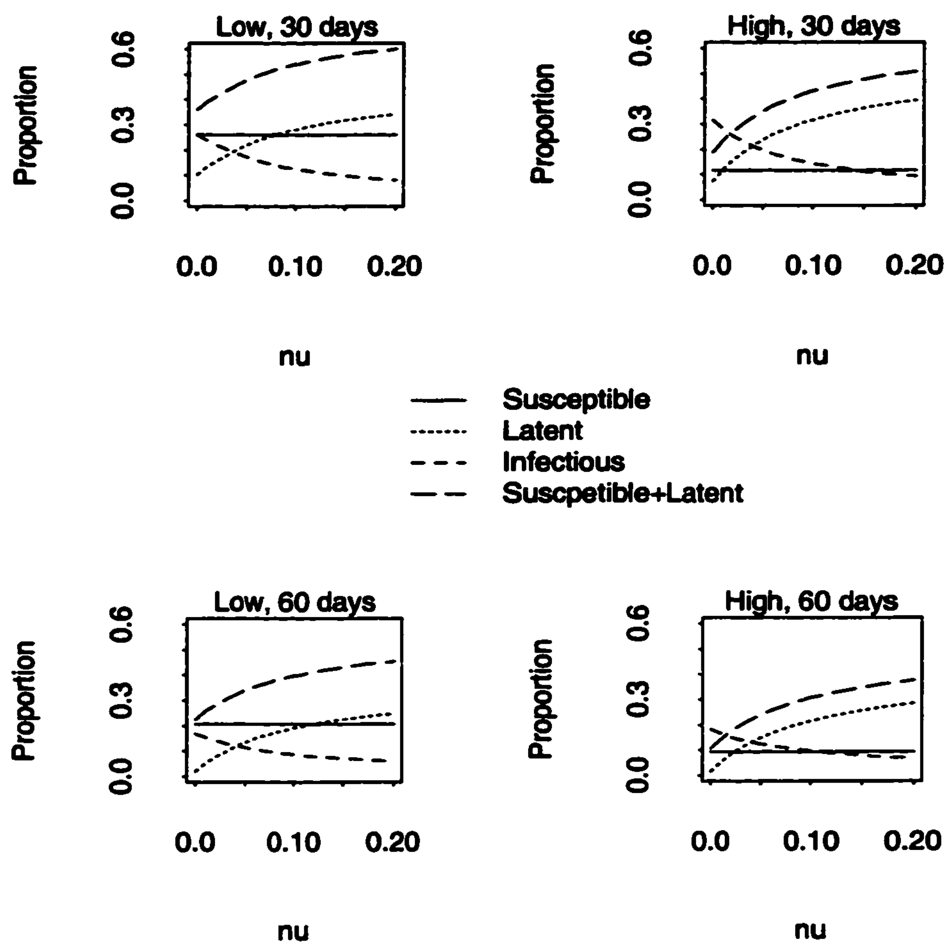

Figure 39 Sensitivity analysis for the low and high-BKD groups under migration for 30 or 60 days to the ocean with better (high ν) or worse (low ν) conditions, perhaps being highly related to water temperature. The graphs show the proportion of the original population that is predicted by the model to be in Susceptible, Latent and Infectious classes at ocean entry.

Table 20 Values of parameters used for the migration model and the distributions of population by degree of infection at the beginning of migration. 30 and 60 refer to the length in day of migration. ν is the only variable parameter.

Parameter	Low 30	High 30	Low 60	High 60
$\mu1$	.0075	.0075	.0075	.0075
$\mu2$	.025	.025	.025	.025
λ	0	0	0	0
c	0	0	0	0
ϕ	.05	.05	.05	.05
ν	0-.2	0-.2	0-.2	0-.2
So	.324	.143	.324	.143
Lo	.571	.405	.571	.405
Io	.006	.277	.006	.277
time (days)	30	30	60	60

7. 8. Discussion

The analysis techniques explored in this chapter provide a basis for detailed mathematical modeling of the dynamics of BKD. The limitations of the data preclude firm conclusions about those dynamics at this time. Despite these limitations, this analysis does support some of the conclusions made earlier in this dissertation. The SLI disease model was able to mimic the dynamics of both the low-BKD and high-BKD groups, but only when different parameters were invoked for each group and for each of three time periods, and only when an external (upstream) source of infection was invoked. The sensitivity analysis suggests that the external source of infection is extremely important, but only early on in the life cycle. Once the disease is established in the population, the state of the population in terms of immunotolerance and overall condition are more important.

8. Management and Research Implications

8. 1. The management problem

Among Columbia River basin salmon stocks, the prevalence of BKD is highest and its effects most severe in spring and summer chinook salmon (Pascho et al. 1991, Bullock and Wolf 1986) in both rivers (Maule et al. 1996, Elliott et al. 1997) and the ocean (Banner et al. 1986). BKD is prevalent in hatcheries on the Columbia and Snake rivers (Maule et al. 1996,VanderKooi and Maule 1999), and returns of spring and summer chinook to these hatcheries have been consistently low.

The research reviewed in the Chapter 5, which followed salmon under hatchery rearing and under subsequent saltwater challenge or recapture during downstream migration, and other large scale studies provide the best knowledge we have concerning the dynamics and effects of BKD. While still limited in time and scope, these studies, along with laboratory studies, do provide a basis for both testable hypotheses and management decisions.

8. 2. Hatchery management

8. 2. 1. Use of antibiotics

Erythromycin is effective at reducing infection level in female spawners before spawning, and in hatchery smolts before release. Antibiotic mediated reduction of infection in female spawners may reduce both vertical infection and antigen inclusion induced immunotolerance if the antigen has not already entered the egg in sufficient quantities before treatment. However, the antigen has been shown to remain in tissues for at least three months (Pascho et al. 1998). The effect of treating spawners with erythromycin on immunotolerance in their offspring is therefore not clear.

The short term reduction in mortality for smolts treated with erythromycin during hatchery rearing may reduce overall mortality levels, though the effect of the treatment is

short lived after release, and thus the overall effect is likely small.

Future research should address both the effect of the use of erythromycin in spawners, if any, on reducing immunotolerance in offspring and the effect of erythromycin in hatchery fish on both in hatchery survival and SAR (smolt to adult returns). These could both be accomplished using segregation and tagging techniques.

8. 2. 2. Segregation or culling of eggs of highly infected female spawners

Some hatcheries have begun culling or segregating eggs from highly infected broodstock and reducing stocking densities in raceways (Pascho et al. 1991). Whether either practice will translate into reduced mortality and increased returns in the long term remains unclear. The situation is complicated by a number of factors. Very low level infections in female spawners may lead to vertical infection in a very small proportion of offspring (Lee and Evelyn 1989), so that unless all females with any evidence of infection are eliminated, the disease will not be completely eliminated from hatchery raceways, even if dechlorinated city drinking water or similarly sterilized water is used.

Vertical transmission does not appear to be the principal effect of high bacterial loads in female spawners. Only 14% of eggs from a very highly infected female spawner contained detectable numbers of *R. salmoninarum* cells (Evelyn et al. 1984), and less than 10% of eggs exposed to extremely highly infected ovarian fluid for 12 or 24 hours were infected (see Chapter 3). Antigen inclusion induced immunotolerance may be the factor driving the reduced in-hatchery survivorship and SARs of offspring of highly infected female spawners (see Chapter 5).

Segregation may not be useful given that, at least in the 1988 Dworshak segregation study, the principal cause of infection is neither vertical infection nor subsequent horizontal infection from vertically infected fish (see Chapter 5). Segregation may reduce the bacterial load in the water in the low-BKD raceways, but at the same time results in increases in the bacterial load in the water for those fish in the high-BKD

raceways, many or even most of which may have not been directly affected by the infections in female spawners.

Culling is not necessarily a good solution, at least not under the parameters used in the 1988 Dworshak study. While culling may reduce horizontal infection to the low-BKD stock in hatchery and to other populations during migration and transportation, it also removes many fish that would survive to return and spawn. If there is an overabundance of female spawners in a particular year or at a particular hatchery, than culling is a reasonable approach. If not, then more information is necessary to decide if to cull and which spawners to cull. If indeed, it is only the very highly infected spawners with high levels of antigen in the ovarian fluid which cause immunotolerance in offspring that is the problem, as suggested in Chapter 5, then those female spawners alone should be culled. If, on the other hand, immunotolerance is spread more evenly across the offspring of female spawners with different levels of infection, then the benefits of reduced horizontal transmission during migration and transportation versus the loss of returning spawners to the hatchery in the next generation must be considered.

Another consideration is genetic effects. While no strong genetic differences in resistance to BKD have been found within salmonid species, that does not mean that they do not exist. If, in fact, those offspring or a portion of those offspring that survive despite having *R. salmoninarum* cells or the p57 antigen present in the egg stage, survive due to some genetic advantage, culling highly infected spawners may remove an important route of adaptation.

8. 2. 3. Hatchery rearing strategies: density, temperature and feeding

Banks (1994) found that increased rearing densities resulted in decreased smolt to adult returns (SAR). Whether or not the effects of density are disease related, it is clear that there are optimal densities for hatchery rearing, above which no per-raceway gain in returns is made.

In Chapter 4 the effects of high temperatures (with as low as 10 °C being considered high for naturally infected populations) on the progression and outcome of BKD were explored. High temperatures for days, weeks or months before release may have a increase the SAR of released smolts by reducing bacterial loads in these smolts before migration.

Winter feeding and temperature regimes that mimic those in natural systems result in higher quality smolt populations with fewer and less severe *R. salmoninarum* infections. High growth rates before release, as would be seen in natural systems with cold winters and higher food availability in the spring, correlate strongly with high SARs (Beckman et al. 1999).

8. 3. Effects of diversion, holding and transportation during migration

8. 3. 1. Temperature effects

Low temperatures in river may cause immunosuppression and prevent significant reduction in bacterial loads before salt water entry. Warm water during migration, as in the case of warmer water before hatchery release, may result in lower bacterial loads in smolts before salt water entry. Early season transport before the river temperatures have heated up may move smolts to the ocean before warmer temperatures have helped reduce bacterial loads, thus increasing the BKD related mortality upon ocean entry.

8. 3. 2. Stress effects

The stress produced by diversion, and holding at dams, and transportation may cause short term immunosuppression. The high levels of cortisol produced during smoltification are similar to amounts produced under stress. Mesa et al. (1999) found that while infection with BKD did not appear to affect the process of smoltification, smoltification appeared to induce outbreaks of BKD, probably due to reduced immune function during smoltification.

8. 3. 3. Transmission

Transportation increases the proximity of fish for short periods (up to 16 hours from Lower Granite Dam) and may increase the incidence of horizontal transmission, though the levels observed in barge water are too low to cause significant horizontal transmission. Bacteria laden feces may provide a direct route of transmission during transportation.

8. 4. Proposed research

8. 4. 1. Infection and antigen inclusion in eggs, measurement via PCR, ELISA

Lee and Evelyn (1989) saw conflicting results when eggs from spawning females with very low bacterial loads (28-113 cells ml^{-1}) were examined for *R. salmoninarum* cells and when 195 day old smolts were tested for BKD (see Chapter 3). PCR (polymerase chain reaction) techniques are more sensitive that the FAT (fluorescent antibody technique), gram stain or culture in detecting *R. salmoninarum* in individual salmonid eggs (Brown et al. 1994). A more exact measure of the relationship of ovarian fluid infection levels to vertical transmission can be made by PCR screening eggs of individual female spawners with different levels of infection.

8. 4. 2. Vertical infection and immunotolerance in offspring of individual female spawners.

An experiment in which offspring of individual female spawners were reared separately from the offspring of other female spawners with different ovarian fluid infection levels would give insight into the vertical transmission and antigen inclusion induced immunotolerance effects of varying natural infection levels in female spawners. Infection levels should be tested in eggs, and frequently, at least weekly, during rearing.

8. 4. 3. Effect of temperature changes on survival of hatchery juveniles with moderate incidences of BKD

An experiment should be undertaken in which rearing raceways were subjected to various temperature regimes and samples tested frequently (weekly) to determine the prevalence and levels of R. salmoninarum infection. This would allow for testing the effect of temperature changes at different life stages on the infection levels and mortality rates experienced by the juvenile salmonids. In particular, by sampling frequently the changes in infection levels could be better tracked and linked to life stages and temperature.

8. 4. 4. Level of infection in returning salmon from high and low BKD groups

Along with tagging hatchery releases of smolts from females with high and low levels of infection to compare SARs, infection levels of returning fish should be measured to determine if and to what extent spawners from one group or the other are more likely to have high levels of infection upon return.

8. 4. 5. Effect of temperature and intermittent or chronic stress on survivorship of bypassed or transported downstream migrants held in fresh or salt water.

Park et al. (1986) held yearling chinook salmon smolts for 21 weeks in seawater at Lower Granite Dam. Delayed mortality ranged from 60 to 76%. Over 99% of all mortalities were found to be associated with BKD, with high levels of *R. salmoninarum* infection in most dead smolts.

Gilbreath et al. (2000) proposed research to compare the physiology and delayed mortality of transported, single bypass and multiple bypassed Snake river hatchery spring chinook held in artificial seawater tanks at Bonneville dam. This study should be extended to include the effects of temperature and intermittent and chronic stress on BKD induced mortality. In particular, while they propose holding fish in saltwater at 10 °C, subsets of these fish should be held in fresh water for a week to ten days with each subset subjected to a different temperature, within the range of 4 to 20 °C, before being transferred to the

saltwater tanks.

Subsets of these fish should also be subjected to intermittent or chronic stress. Moles (1997) found that naturally infected fish with clinical *R. salmoninarum* infections could not withstand saltwater (30 ppt) challenge after a 12 hour swimming exercise (3x body length/s) (no survival), whereas non-infected smolts subjected to the same conditions did much better (95% survival). Mesa et al. (1999) found that rare intermittent stress did not cause higher mortalities in BKD infected smolts over non-infected smolts. The effects of intermediate amounts of stress, perhaps more representative of the experience of migrating salmon, should be explored.

Another experiment should artificially infect spring chinook smolts and then hold subsets of them for one to two weeks at temperatures ranging from 4 to 20°C, before transferring them to saltwater tanks. This would allow for a more direct measure of the effect of different temperatures in river on survival of infected spring chinook smolts upon salt water entry. As Banner et al. (1983) found that BKD related mortality continued for 200 days after salt water entry, these experiments should continue for at least that length of time.

Glossary

Affinity
Univalent bonding strength of an antibody for a particular antigen.

Anadromous
Describes fish such as salmon which spawn in fresh water but spend the majority of their adult life in the ocean.

Antibody
A protein produced by B cells in the body in response to the presence of an antigen. Antibodies are a primary form of specific immune response and act by attaching themselves to a foreign antigen and weakening or destroying it.

Antigen
A substance, usually a protein, on the surface of a cell or bacterium that stimulates the production of an antibody.

Avidity
Multivalent bonding strength of an antibody for a particular antigen.

Avidity maturation
The process, which occurs in germinal centers, by which successive lines of B cells are selected for and adapt to a particular antigen, with the final selected lines of B cells producing antibodies with high avidities for the antigen selected for.

B cell
A type of lymphocyte, formed in bone marrow in mammals and in the kidney of teleosts that creates antibodies in response to a specific antigen.

Immunocompetence
The ability of the body to develop an immune response in the presence of a pathogen or antigen.

Immunosuppression
The inhibition of the immune response, also referred to as immunodepression.

Immunotolerance
The state in which the immune system is tolerant of a pathogen in that it does not develop a complete immune response to the pathogen.

Lymphocyte
A class of cells in the immune system that produces antibodies and is responsible for rejecting foreign tissue. A kind of white blood cell.

Obligate pathogen
A pathogen which depends upon the tissues of its particular host for survival and reproduction, as apposed to an opportunistic pathogen.

Opsonization
Process by which foreign bodies such as bacteria are made more susceptible to destruction by phagocytic cells by coating them with a protein such as an antibody or a component of the complement system.

T cell
A type of lymphocyte that matures in the thymus and is essential for various aspects of immunity.

Teleost
Bony fish. Fish in the suborder Teleostei, including most extant species of fish but excluding cartilaginous fish, such as sharks, sturgeon, etc.

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Appendix A: Overview of the Human Immune System

A.1. Basic components

The human immune system is composed of a great variety of cellular and humoral factors which act independently or, more often, in concert to rid the body of pathogens. The major components of the human immune system are as follows:

A. 1. 1. Phagocytes

Phagocytes are cells which engulf and destroy exotic organisms and particles by ingesting them into a phagosome and then releasing cytotoxic agents (including free radicals, lysozyme and other enzymes) into the phagosome and digesting its contents. Phagocytic cells include polymorphonuclear neutrophils which are circulating phagocytes, meaning they occur almost exclusively in the blood. Neutrophils only live for about 24 hours. They comprise the vast majority of leucocytes (white blood cells) in our blood, as every one of us with a healthy immune system produce some 50-100 million of them every day. Neutrophils are the expendable foot soldiers of the immune system.

The other major type of phagocytic cells are mononuclear phagocytes which are know as monocytes when found in the blood and macrophages elsewhere. These cells also engulf and destroy pathogens, but they survive for longer periods of time and occur in strategic locations for finding invading pathogens, including in the liver, kidneys, lungs, brain, spleen and lymph nodes. Macrophages and monocytes are more effective individually but occur in lower numbers than neutrophils.

A. 1. 2. Complement

The complement system is a complex of serum proteins which are involved in triggering inflammation, phagocyte activation, direct lytic attack on cell membranes, and the tagging (or *opsonization*) of pathogens for phagocytosis. The complement system is activated either via the classical pathway, which is antibody mediated, or via the

alternative pathway which is a direct result of contact with the pathogen.

Tagging of pathogens for phagocytosis is known as opsonization. The molecules which perform the tagging are known as opsonins, which include antibodies and certain elements of the complement system, most notably C3b.

A. 1. 3. Mediators of inflammation

Inflammation is a response to cellular injury or infection which results in an increase in blood flow to, capillary permeability in and migration of phagocytes and other leukocytes to the affected area. The aggregation of phagocytes helps prevent the infection from spreading. Mast cells and basophils mediate inflammation and release cytokines (chemical mediators of communication between cells) that affect other immune system cells.

A. 1. 4. Cytotoxic T cells

Cytotoxic T cells are lymphocytes that mature in the thymus (thus the T) and kill virally infected cells and certain tumors through releasing lytic substances into those cells. They recognize infected cells and tumors by the presence of non-self peptides (short strings of amino acids which are breakdown products of proteins) on major histamine complex (MHC) type I, which occurs on all cells in the human body. Cytotoxic T cells are specific to particular peptides, and are selected for subsequent to activation by antigen presenting cells (APCs). T cells carry on their surface T cell receptor (TCR) which is specific to particular peptides presented on MHC.

A. 1. 5. Natural killer cells

Natural killer cells are lymphocytes, and thus in the same class of cells as B cells and T cells. In particular they are large granular lymphocytes which like cytotoxic T cells kill virally infected cells and certain tumor cells through cell lysis. Unlike cytotoxic T cells they are not specific to particular peptide sequences.

A. 1. 6. Antibodies

Antibodies (or immunoglobulins) are molecules specific to epitopes (small sites) on antigens (protein or other surface components or secretions of pathogens). They are produced by B cells, are have 5 known structural types in humans - IgA, IgM, IgG, IgD, and IgE, with IgG being the most potent circulatory type. IgM is multivalent and is often the first humoral antibody response, with IgG being produced after avidity maturation has begun. IgA is mostly found in seromucous secretions rather than in the blood or lymph. Much less is known about the role of IgD and IgE, both of which are scarce in serum. There are two measurements of an antibody's effectiveness in attaching to a particular antigen. Affinity refers to the binding strength of a single antibody effector site to an epitope, whereas avidity refers to the total binding strength of multivalent antibody to an antigen, including the possibility of multiple binding sites.

A. 1. 7. B cells

B cells (B from "bursa Fabricii", the specialized organ where B cells mature in birds) are lymphocytes that develop in the bone marrow, and carry on their surface many copies of a single antibody morphology. There are millions of such cells with tens of thousands of different antibody forms in our circulatory system at any time. Thus there are, on average, a few tens of each extant type present. When activated by antigen, B-cells become involved in a complicated series of event that results in creation of short lived antibody producing plasma cells, avidity maturation (the increase in the avidity of antibody for the particular antigen) and eventually the creation of long-lived plasma cells and memory cells. The first plasma cells that differentiate mostly produce IgM, whereas later, most plasma cells produce IgG. The whole process generally takes on the order of a week or two.

A. 1. 8. Helper T cells

Helper T cells recognize peptides presented on MHC type II which, unlike MHC type I, occurs only on particular types of cells, termed antigen presenting cells (APCs).

Like B-cells and cytotoxic T cells, helper T cells are specific to parts of antigens, and like cytotoxic T cells, helper T cells respond to particular peptides presented to them. Once activated, helper T cells release cytokines which help activate other immune cells. There are two types of helper T cells, one which helps mediate the humoral immune system (B-cells) and one which helps mediate the cell-mediated immune system (cytotoxic T and phagocytic cells).

A. 1. 9. Antigen presenting cells (APCs)

Antigen presenting cells are particular cell types most of which have MHC type II on their surface and which process antigens taken in by the cell and present resulting peptides on their surface. Macrophages, B cells and dendritic cells (DCs) and in particular follicular dendritic cells (FDCs) are all APCs. FDCs lack MHC type II and are especially important in the avidity maturation of B cells, as they take up antigen into their folds and, without processing it, slowly present it to B cells over time. This takes place in special locations called germinal centers where avidity maturation takes place. The continuous presentation of antigen is important so that avidity maturation can continue even after the pathogen is wiped out, and more optimally avid memory cells can be produced, thus ensuring a powerful secondary response and conferring subsequent immunity against the pathogen.

APCs are also present in the thymus where they take up self antigens and present it to developing T cells. This provides a means of identifying and deleting self-reacting T cells within the thymus, and thus preventing auto-immunity.

A. 1. 10. Cytokines

Cytokines are chemical mediators of communication between cells. Major groups of cytokines include interleukins (ILs) and interferons (IFNs). Most ILs, the majority of which are produced by helper T cells, direct particular cell types to divide and differentiate. IL1 is produced by activated macrophages and directs helper T cells to

proliferate and produce IL2 which stimulates both T cells and B cells to proliferate, differentiate and produce cytokines or antibodies. IFNs are produced by virally infected cells and by T cells, and induce resistance to viruses in receptor cells. IFNs can also activate macrophages.

A.2. Specific versus non-specific immunity

The immune system is often considered to consist of two parts, the specific and the non-specific responses. The former is that which is antibody or T-cell mediated. The latter can take place without recognition of a particular antigen, and includes the action of phagocytes and the alternative complement pathway.

The specific immune system, which takes longer to respond effectively to an initial infection, but which is also generally more effective and usually confers subsequent immunity, includes T cells, B cells, APCs and antibodies. The two systems interact, with antibodies tagging pathogens and infected cells for phagocytosis (opsonization), some helper T cells stimulating phagocytes through secreted cytokines, and some phagocytes acting as APCs. In fact, the entire repertoire of cells, chemicals, and interactions is somewhat more complicated than I have outlined here, nor is it fully elucidated to date.

A.3. The self/non-self differentiation problem

While the immune system is wonderfully complex and capable, all its protective benefits would be more than negated if it could not distinguish self from non-self and thus attacked components of the organism it protects (this does in fact occur at times and is known as autoimmune disease). How does the immune system distinguish between self and non-self? During development in the thymus or bone marrow, T cells and B cells which react with too high affinity or avidity to self antigens present in those places are deleted. The immune system also has checks and balances so that helper T interaction is necessary for B cell response to most antigens. Thus if there is T cell tolerance, the avidity of B cells for the antigen is usually irrelevant in inducing an immune response. Other

mechanisms include hiding self antigen from humoral lymphocytes, or shutting down through over-stimulation of self reacting T cells which manage to exit the thymus intact.

A.4. Interactions among parts of the immune system

The immune system, especially the specific immune system, has many checks and balances built into it. This helps avoid autoimmunity and prevents runaway proliferation of immune cells and secretion of cytokines and antibodies. For example, B cell proliferation and differentiation resulting in antibody producing plasma cells and latent memory cells is dependent not only on activation by antigen, but also on activation by helper T cell secreted cytokines such as Interleukin 2.

A.5. Avidity maturation

Avidity maturation is the process in which selection and mutation of B cells in germinal centers gives rise to more effective antibody. This happens with the assistance of follicular dendritic cells (FDCs) presenting antigen, and helper T cells releasing cytokines. It is partially responsible for the increased power of the secondary response. While T cells also have a stronger secondary response, this is apparently due only to selection and proliferation of T cells during primary response, and not any intrinsic increase in individual T cell affinity for the antigen.

A.6. Immunotolerance

Immunotolerance occurs when those B or T cells which are specific to an antigen are inactivated. This happens naturally during early development and continuously in the organs where the lymphocytes develop, to prevent auto-immunity. Inactivation also occurs in serum due to overstimulation by exogenous or self-antigen. Thus immunotolerance can be initiated in early developmental stages due to presence of antigen, or may be induced after completion of development due to extremely large doses of antigen.

A.7. Endocrine-immune system interactions

The immune and endocrine systems interact in a number of complex ways (Weyts et al. 1999). Some of this interaction appears to ensure homeostasis. Others, such as the stress response, are more complex. Under physiological stress, elevated levels of glucocorticosteroids such as cortisol are seen. Glucocorticosteroids can induce apoptosis (cell suicide) in lymphocytes, reduction of numbers of lymphocytes (and increases in neutrophils) in the blood through redistribution to lymphatic organs, and inhibition of cytokine production. These effects can have significant negative impacts on the ability of the immune system to fight off disease agents already established in the organism, though they may increase the ability of the organism to prevent initial infection through redistribution of immune strength to the periphery.

A.8. Down-regulation of the immune system

The immune system includes a number of negative feedback mechanisms to avoid runaway immune response. Some processes stop once stimulation by antigen ends, while suppressor T cells may directly reduce the immune response and antibodies may be produced against other antibodies, thus creating a cascading or network effect (Perelson and Weisbuch, 1997).

Appendix B: Epidemiological Modeling

B.1. Overview

Mathematical models of disease dynamics can help us predict and control epidemics and endemic diseases in humans and in nature. Understanding and quantifying the routes and rates of transmission, the heterogeneity of hosts (including effects of age, gender and activity), the course and effects of the disease (including death or immunity), external factors which effect the transmission and course of the disease (such as seasonality), and other particularities of a disease-host system allow us to predict what actions will be most effective at controlling the disease or its effects.

Daniel Bernoulli (1760) is often credited with the first use of mathematics in evaluating the effects of a disease. He did no more, however, than to use simple probability theory to predict the increase in survivorship to age 25 that would occur with the elimination of smallpox (that more virulent cousin of chicken pox, and which, although currently eradicated outside the laboratory, is again raising fears in these days of bio-terrorism). The result is somewhat depressing by today's standards, for while successful inoculation of all infants against (with) smallpox would increase by 6 the percentage of newborns expected to reach the age of 25, the total predicted survivorship to 25 even with this windfall was still only 48%. Thus the problem of disease was quite a serious one in 18th century Europe.

Dynamical models of disease first appeared in the early 20th century with publications by Hamer (1906), who modeled the periodic return of measles epidemics, and Ross (1908), who considered the relationship of malaria outbreaks to mosquito numbers. It was Hamer who suggested the enduring idea that the contact rate between infected and susceptible individuals is a key factor in determining the course of an epidemic. Kermack and McKendrick (1927, 1932, 1933) formalized the mathematical theory of epidemics, including the basic SIR (susceptible-infected-recovered) model and extensions thereof.

B.2. Characteristics of diseases and their dynamics

The factors affecting the dynamics of a disease in a population can be grouped into six classes: The routes and rates of transmission, infectiousness and latency, recovery and immunity, mortality and reduction in fecundity, host heterogeneity and environmental heterogeneity in space and time.

B. 2. 1. Routes and rates of transmission

For an epidemic to occur or for a disease to remain endemic in a population, there must be adequate transmission. From the modelers point of view, the important factor is the probability of transmission from each individual to each other individual at any point in time. In the simplest cases it is enough to assume homogeneity, e.g. that all individuals have equal probability of infecting all other individuals at every point in time. In more complicated cases, the population must be broken down into classes based upon location, age, stage, gender, activity, health and/or genotype. There may also be temporal variation in transmission rates, often due to seasonal variation in contact rates. The route of transmission often determines the temporal variation as related to seasonal changes in activity.

Vertical transmission (from parent to offspring) can ensure entrance of the disease into the next generation, especially in species with non-overlapping generations. Vectors, intermediate host species which transfer the disease from one individual to another, can act as reservoirs of infection, and can significantly increase the complexity of disease dynamics.

B. 2. 2. Infectiousness and latency

The length of the infectious period is important in predicting the probability of passing the disease on to one or more other individuals. Increases in the total time of latency and infectiousness together heighten the probability of transmission between sub-populations.

B. 2. 3. Recovery and immunity

The long term effects of the disease, including mortality, infertility or reduced fecundity, and immunity may affect future dynamics of both host and pathogen. Where immunity is conferred, or death results, a disease is not likely to persist in a population unless combined latent and infectious periods are long and transmission rates are low relative to birth and immigration rates.

Maternal antibodies can confer temporary immunity to offspring, reducing the incidence of disease in the first few months of life. Immunity may be temporary after recovery from infection, so that the susceptible class may be continuously augmented by previously immune individuals.

B. 2. 4. Mortality and reduction in fecundity

Mortality and reduction in fecundity decrease the number susceptible hosts at a particular point in time. This also reduces the number of new susceptible individuals entering the population through births.

B. 2. 5. Host heterogeneity

Host heterogeneity in susceptibility to and effects of a disease affects the course of an epidemic in a population, especially if some individuals are completely immune. Vaccination can confer immunity, and can cause an endemic or periodically epidemic disease to be driven from a population. Heterogeneity in overall health due to nutrition and stress can effect both susceptibility to a disease and its effects, such as the likelihood of mortality from the disease. Host heterogeneity can result from differences in gender, age, stage and activity. There can be intrinsic genetic differences in susceptibility and reaction to a disease. Diseases can have a selective effect on populations as well.

B. 2. 6. Environmental heterogeneity in space and time

Periodic outbreaks of disease are caused not only by boom-bust cycles inherent in the dynamics of disease in a population, but also by changes in the environment or changes in contact rate over time, and by reintroduction of disease from another population. Seasonal changes such as in humidity and temperature may increase the survival of the pathogen outside the host, thus increasing the likelihood of successful transmission. Increases in density and contact rate of hosts, as occurs in humans every fall with the start of school, and every winter with increased time spent indoors, can increase the transmission rate of a disease above the critical value for an epidemic to occur.

B.3. The SIR model

B. 3. 1. The basic model

The SIR model is the standard basic epidemiological model. We assume a closed population of size N which is divided into three classes: susceptible (S), infected, which are also infectious (I), and recovered and immune (R). Susceptible individuals are infected by interaction with infected individuals assuming the law of mass action, while infected individuals recover at a constant rate (ν) (Figure 4.1).

$$\frac{dS}{dt} = -\alpha IS \qquad \text{(B.1)}$$

$$\frac{dI}{dt} = \alpha IS - \nu I \qquad \text{(B.2)}$$

$$\frac{dR}{dt} = \nu I \qquad \text{(B.3)}$$

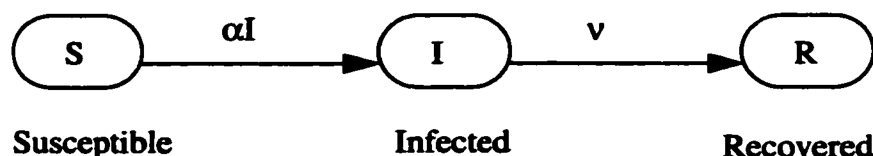

Figure 40 The basic S-I-R model.

For analysis of epidemics, we typically assume a population of susceptible individuals to which is introduced one or a few infected individuals. From this we can come to the following conclusions. The peak of infection occurs when $S = \rho = \nu/\alpha$, and the epidemic runs its course without infecting all individuals. This can be shown by dividing equation (2) by equation (1) and then solving (see Murray, 1989):

$$\frac{dI}{dS} = -1 + \frac{\rho}{S} \tag{B.4}$$

$$I + S - \rho \ln S = C = I_0 + S_0 - \rho \ln S_0 = N - \rho \ln S_0 \cong N - \rho \ln N \tag{B.5}$$

So that S is always greater than zero. N/ρ is known as R_0, the basic transmission rate of the infection, which is the number of secondary infections caused by a single infected individual entering a population of N susceptible individuals. The initial growth of the epidemic is approximately exponential, with exponent $\alpha-\nu$. If ν is greater than α, no epidemic occurs. Note that the mathematical definition of epidemic is simply that the total number of infected (rather than susceptible or recovered) individuals is increasing at some point. This is different than the dictionary definition which requires "sudden rapid spread", or at least "excessive prevalence".

The basic SIR model predicts the time course of an epidemic in a closed

population, with no birth, death, immigration or emigration. The end result is always the elimination of the disease, though as it is a continuous approximation to a discrete process, the elimination is only asymptotic. Extensions of the model can expand the range of diseases and situations that can be modeled. Also note that assuming the disease confers lasting immunity, it cannot successfully re-enter the population subsequently.

B. 3. 2. The model with births and deaths and/or immigration and emigration

With births and deaths, or immigration and emigration a number of different results can occur. Even assuming a constant population size, with births equaling deaths and all newborns being susceptible, the disease may become endemic, reaching a non-trivial steady state. This endemic state may be approached in an oscillatory manner, or infection may occur in a periodic or chaotic manner. Large scale fluctuations in the number of infected individuals can result in the extinction of the pathogen in the population due to stochastic events when the infected population is small ("endemic fade-out"; Anderson and May, 1991). Diseases which die out in a population may re-infect that population after the susceptible population has been replenished with new births or immigration.

Assuming a non-constant population size leads to other possible solutions. Epidemic disease may severely reduce the population size or fecundity of a population, if only temporarily. Endemic disease may control the size of a population as well, though some populations may continue to grow despite endemic infection, with the size of both the susceptible and infected classes growing with time.

B. 3. 3. Age structured models

Including the age of individuals creates another complication. If we assume susceptibility to the disease is age dependent, and also consider chance of infection between individuals is dependent on their ages.

B. 3. 4. Latent classes

When the infected individuals are not initially infectious, this adds a delay into the system. These infected, non-infectious individuals can be represented by another class.

B. 3. 5. Structured models

Few host populations are truly homogenous. For those cases where heterogeneity significantly effects the disease dynamics, structured models are often useful. We are typically concerned with heterogeneity in susceptibility to the disease, in the course and effects of infection and in infectiousness.

The effects of the disease may be such that carriers of the disease are unaware of their infection, and thus not seeking treatment nor avoiding contact that may result in infection in others as in the case of in women with sub-clinical gonorrhea infections (Anderson and May, 1991).

B. 3. 6. Sexually transmitted diseases

One obvious structured model is that of a sexually transmitted disease in a heterosexual population. We assume that females are infected only by males, and males are infected only by females. Thus we have two host sub-populations which interact in a fairly simple fashion. The simplest version assumes homogenous mixing between the two groups (realizing this is unrealistic, but suspending our disbelief for the moment). Another approach is to also divide males in females into different activity classes with each male class having a different probability of contact with each female class, and including transmission through homosexual contact as well.

B. 3. 7. Sub-populations and spatially structured models

Models containing sub-populations with different contact rates are useful for analyzing disease in populations divided by space or activity. For village type models, there is a much lower probability of contact among individuals from different villages than

among individuals within a village. For activity based models, one group might have high levels of contact with the group with another having much lower internal contact rates, with contact between groups at an intermediate or low level.

B. 3. 8. Non-structured models of heterogeneity in susceptibility

Heterogeneity in susceptibility can lead to the appearance of lower contact rates as an epidemic progresses. This is due to more susceptible individuals more likely being infected early on, leaving the remaining susceptible population with lower likelihood of infection. If immunity is not a result of recovery, endemism can result even when the average susceptibility is too low for the disease to persist in a population, due to a sub-population with high susceptibility which is continually re-infected. Variation in susceptibility can be the result of genetic differences, or of differences in activity, as in sexually transmitted diseases. While a structured model would work in this case, it is also sometimes possible to simply include non-linearity in the transmission dynamics. One way to accomplish this is to replace Eqn. (B.2) in the basic SIR model with (Dwyer et al. 1997):

$$\frac{dI}{dt} = \alpha I^a S^b - \nu I \tag{B.6}$$

B.4. Ecological examples

In the field of ecology, the subject of disease dynamics and effects has gained much attention in recent years, though not in aquatic systems. Endemic disease may regulate the size of a population, though it is often difficult to discern to what extent disease induced death is compensatory rather than additive. Epidemic diseases can have large and important effects on population sizes, and both epidemics and endemic diseases can affect the genetic makeup of a population. Studies of disease in ecology have included the question of what maintains a disease in host populations, how to best control disease in wildlife populations, what risks are posed by disease to endangered species, and what are the dynamics of multi-species host-parasite models (including single parasite-several host, single host-several parasite, and vector transmission models) (Grenfell and Dobson, 1995).

One published model of disease in salmonids (des Clers 1993) is more akin to the work done by Bernoulli in 1760 than contemporary dynamics disease modeling, though having a more complex data set than that of Bernoulli. This models explores the impact of disease-induced mortality at different points in the lifecycle on total population numbers using an age structured model. While this is useful for predicting where in the lifecycle particular decreases in disease induced mortality would have the most impact, it does not address the actual dynamics of any disease.

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