IMMUNOTOXICOLOGY OF PACIFIC HERRING: DETERMINATION OF REFERENCE RANGES AND THEIR APPLICATION TO ASSESSING EXPOSURE TO THE WATER-SOLUBLE FRACTION OF CRUDE OIL

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Abstract

Declines in Pacific herring returning to spawn in Prince William Sound four years after the 1989 *Exxon Valdez* oil spill, and isolation of pathogens, raised concern that crude oil exposure might alter immunocompetence and increase disease susceptibility. While adverse effects of sublethal crude oil exposure on eggs, embryos and larvae of Pacific herring and median lethal concentrations for juveniles and adults were known, little information existed on immunological consequences of sublethal exposure in juvenile and adult herring. Therefore, a suite of assays from the 3-tiered immunotoxicological approach examined if sublethal water soluble fraction of oil (WSFO) exposure of juvenile and adult herring affects their hematology, plasma biochemistry and immunological status.

Wide intraspecific variance of several variables was identified in control fish, which was largely attributable to age, size, and holding time prior to experiments, as well as duration of experiments. Additionally, skin lesions were associated with elevation of plasma lysozyme and hematocrit in juveniles, while hematocrit, leucocrit, spleen-somatic index (SSI) and plasma cortisol varied with gender in adults. A significant captivity effect was observed in control and WSF fish alike.

In a series of experiments using a pulse 16 to 28 day exposure to WSFO (26 to 321 ppb total polyaromatic hydrocarbons range), few statistically significant ($p<0.05$) changes occurred consistently in Tier 1 and 2 variables in either age class. Comparison with the 95^{th} percentile estimation and inter-quartile reference ranges (derived from baseline and control herring) and the literature aided interpretation of statistical changes. Only plasma lysozyme, lactate and phagocytosis varied in association with WSFO exposure in both age classes, but changes were not concentration-related. Despite transitory changes in plasma biochemistry, lysozyme and phagocytosis, no single variable was a consistent predictor of WSFO exposure. Viral Hemorrhagic Septicemia Virus (VHSV) challenges, in conjunction with WSFO exposures, could not induce clinical disease. Mortality was not altered in adults and overall they were resilient to WSFO exposure, under the experimental conditions described. Juveniles exposed to WSFO and VHSV showed increased cumulative mortality. It is recommended that age, size and captivity time be considered important modifying factors in future studies of this nature.

I dedicate my thesis to my parents, Margaret and Gordon Sanders. The strength, courage and love they demonstrated in the face of great adversity motivated me to persevere throughout the completion of my research and writing.

"*O, the turbulent waters of your imagination,*
the calm of happy memories"

(Cirque du Soleil)

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α-MSH	α-Melanocyte stimulating hormone
A/G	Albumin/globulin ratio
ACTH	Adrenocorticotropic hormone
Ah	Aryl hyrocarbon
AK	Alaska
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
B(a)P	Benzo(a)pyrene
BC	British Columbia province
BMSC	Bamfield Marine Sciences Centre
BS	Barkley Sound
Ca^{2+}	Calcium
CF	Condition factor
Cfu	Colony forming units
Cl^-	Chloride
CMR	Crude mortality rate
CV	Coefficient of variation
DFO	Department of Fisheries and Oceans
DMBA	Dimethylbenz(a)anthracene
EDTA	Ethylenediamine tetra-acetic acid
EPC	Epithelioma *papulosum cyprini*
EVO	*Exxon Valdez* oil
EVOS	*Exxon Valdez* oil spill
FBS	Fetal bovine serum
GIT	Gastro-intestinal tract
GLM	General linear model
GSI	Gonad-somatic index
HBSS	Hank's Balanced Salt Solution
Hct	Hematocrit
HEWL	Hen Egg White Lysozyme
HPA	Hypothalamus-pituitary-adrenal
I. hoferi	Ichthyophonus hoferi
i.p.	Intraperitoneal
IHNV	Infectious hematopoeitic necrosis virus

IQR	Inter-quartile range
L-15	Leibovitz's -15 medium
LC_{50}	Median lethal concentration
Lct	Leucocrit
LR	Likelihood Ratio
LSI	Liver-somatic index
MEM	Minimum Essential Medium
MFS	Marrowstone Field Station
mø	Macrophage
MS-222	Tricaine methanesulfonate
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
NOAA	National Oceanic and Atmospheric Administration
NRDA	Natural Resource Damage Assessment
OD	Optical density
OWD	Oil water dispersion
p.o.	*per os*
PAH	Polyaromatic hydrocarbon
PAHs	Polyaromatic hydrocarbons
PBCO	Prudhoe Bay crude oil
PBS	Phosphate Buffered Saline
PBS	Pacific Biological Station
PCBs	Polychlorinated biphenyls
PE	Percentile estimation
Pfu	Plaque forming units
P_i	Inorganic phosphorus
PNW	Pacific Northwest
ppb	Parts per billion
ppm	Parts per million
PS	Puget Sound
PVC	Polyvinyl chloride
PWS	Prince William Sound
QI	Quadra Island
RBCs	Red blood cells
ROIs	Reactive oxygen intermediates
SD	Standard deviation
SE	Standard error of the mean

SIN	Sample identification number
SMR	Standardized mortality rate
SPF	Specific pathogen free
SPMD	Semi-permeable membrane device
S-R value	Spearman's Rho correlation coefficient
SSI	Spleen-somatic index
T_0	Time zero (baseline)
T_E	Experimental sample time
T_H	Holding time
TP	Total protein
T_P	Pooled times
TPAH	Total polyaromatic hydrocarbons
TSA	Trypsin soy agar
TSB	Trypsin soy broth
UV	Ultra-violet
V. anguillarum	*Vibrio anguillarum*
VA	Vancouver Aquarium
VHS	Viral hemorrhagic septicemia
VHSV	Viral Hemorrhagic Septicemia Virus
WA	Washington
WAF	Water-soluble fraction
WBCs	White blood cells
WSF	Water soluble fraction
WSFO	Water-soluble fraction of crude oil

Chapter 1 – Outline

"Life is either a daring adventure or nothing at all. Security is mostly a superstition. It does not exist in nature."

(Helen Keller, 1880 - 1968)

1.1. OVERVIEW

The goal of this study was to assess the potential impact of exposure to the water soluble fraction (WSF) of Prudhoe Bay crude oil from the Alaska North Slope on Pacific herring (*Clupea pallasi*) following sublethal exposure in terms of the physiological consequences and the potential for an increase in disease susceptibility. The test species, Pacific herring, are not cultured; therefore, it was necessary to use wild-caught herring from several sources within the Pacific Northwest (PNW), as they became available. Herring were obtained from the Barkley Sound (BS) region and the Georgia Strait area in British Columbia (BC) and from the Puget Sound (PS) region of Washington (WA) State, USA.

Herring are maintained at a few public aquaria in North America but are not widely used as a research animal. Consequently, there is very little published information with respect to their physiological and immunological status. A baseline range for a given variable is a necessary benchmark against which to judge the impact of exposure to any specific stressor on that variable (Barham *et al.*, 1980; Schreck, 1990). However, baseline ranges for immunological and hematological variables in Pacific herring are sparse. As such, it was evident that characterizing a reference range of normal values for measured variables in captive herring was essential prior to assessing the impact of exposure to the water soluble fraction of oil (WSFO) and the key research questions.

This thesis established reference ranges for the variables being measured for the first time for BC herring. Age, size, and time in captivity (both holding and experimental) were found to be significant determinants of reference range, while geographical origin and gender were of lesser importance.

The suite of indicators used in this study was selected based on their prior use in other fish immunotoxicological studies and on the analytical instruments available for the processing of blood and tissue samples. Due to the general fragile nature of herring, it was not feasible to blood-sample each fish repeatedly or *via* cannulation. Instead, blood and tissue samples were of a terminal, acute nature and were collected after anaesthesia and euthanasia respectively. Comparisons were made to other fish species in the published literature to establish if herring were similar or different in their hematological, plasma biochemical and immunological responses when held in captivity.

Using the reference ranges for the suite of indicators, the impact of WSFO exposure on the selected variables in juvenile (0+year) and adult herring was evaluated. The suite of indicators were divided into three tiers: Tier 1 – general hematology, condition indices and plasma biochemical responses; Tier 2 -- macrophage cell function assays; and Tier 3 – response to pathogen challenge. Viral hemorrhagic septicemia virus (VHSV) was used as a challenge pathogen for both juvenile and adult herring either in conjunction with, or directly following, WSFO exposure. In addition, *Vibrio anguillarum* (*V. anguillarum* (*Listonella*)) was used as a challenge pathogen following WSFO exposure of juvenile herring. Preliminary pathogen challenges, without WSFO exposure, were also conducted using VHSV and *Ichthyophonus hoferi* (*I. hoferi*). The association of increased prevalence of VHSV and *I. hoferi* in Prince William Sound (PWS) herring populations in 1994 with declines in the herring returning to spawn was the basis for using these pathogens in this study. In addition, they are also pathogens with known population regulating effects in herring.

The term "normal" is problematic. Fish that have not been reared in a laboratory under controlled conditions inherently come with unknown exposure histories. Therefore, the potential for herring to be incubating disease or to have recently encountered xenobiotics prior to capture always exists. As such, they should not be considered "normal" in terms of fish that are pathogen-free, unstressed and unexposed to xenobiotics. Rather, they are "representative" of the status of wild caught herring from the PNW when held in specific captive conditions. Additional handling and physical damage associated with capture and captivity may influence measurement variables. Potentially, they may also affect the conclusions generated if unknown stressors are acting in a differential manner between treatment groups, which is possible despite randomization of treatment groups. Until cultured herring are available, these problems will remain. However, cultured fish may not be representative of wild fish.

It has been strongly recommended that reference ranges be considered in the context of the species, the age of fish, gender, and the source population (Blaxhall, 1972; Love, 1980; Campbell, 1988; Fänge, 1994) because these factors can influence the expected range (McIntyre and Pearce 1980; Hille, 1982). Distinct regional and interannual variations in mass, length, and energy content of groups of herring in PWS have been observed (Paul and Paul, 1999). Baseline values have not previously been established

for Pacific herring; therefore, they should be valuable for future studies beyond those performed in this thesis.

1.2. THESIS OBJECTIVES

My research examined the effect of sub-lethal exposure to WSFO on a selected suite of immunotoxicological indicators (Table 2.2). The rationale was to determine if crude oil exposure results in detectable changes in markers of immune function in juvenile and adult Pacific herring.

The questions that this research strives to answer are:

1) What constitutes the "representative" Pacific herring in terms of the central tendency and variation of key hematological, biochemical and immunological variables in wild caught herring held in captivity?
2) Does sub-lethal experimental exposure to WSFO lead to changes in immune variables and disease susceptibility in Pacific herring?
3) Does the immunotoxicological approach lend itself to the assessment of the impact of xenobiotic exposure on the health of Pacific herring?
4) Can the results from captive PNW herring be extrapolated to explain population declines in PWS herring?

To answer these questions, the following sets of objectives were addressed:

1) To establish reference ranges for each of the hematological, biochemical and immunological variables (Chapter 2). To evaluate the degree of variance due to intra-specific biological variation by determining if the variability in a specific measurement variable is associated with age, size, gender, population source, lesions, anemia, holding time (T_H), or experimental sampling time (T_E) are factors that significantly affect the measurement variables. To make recommendations regarding how statistical significance can be interpreted biologically.
2) To evaluate changes in hematological, biochemical, and immunological variables of herring following sub-lethal exposure to WSFO (Chapter 3).
3) To evaluate changes in hematological, biochemical, and immunological variables of herring following a combined WSFO and pathogen challenge (Chapter 3).
4) To determine the usefulness of the immunotoxicological approach for assessing xenobiotic exposure of Pacific herring. To comment on the applicability of these results to the PWS herring decline. To make recommendations for adapting the immunotoxicological approach for wild fish populations (Chapter 4).

Chapter 2

Characterization of factors that affect variation in the hematological, biochemical and immunological status of Pacific herring (*Clupea pallasi*)

"One step closer to knowing"
(U2, 2004)

2.1. INTRODUCTION

2.1.1. Life History of the Pacific herring

Pacific herring belong to the family Clupeidae. This species can be found from northern Baja, California to Cape Bathurst in the Beaufort Sea (Hourston and Haegele, 1980; Middaugh *et al.*, 1998). Coastal British Columbia is the centre of abundance for herring in North America.

Herring are an extremely important species within the marine Pacific Northwest (PNW) ecosystem because they are key components in the diets of a wide range of marine organisms (Haegele 1993). Eggs are preyed upon by invertebrates, marine birds, fish species including adult herring, and marine mammals (Bishop and Green, 2001). Juveniles are the principal diet of young salmonids, while adults are a key food source of adult salmon, marine mammals and birds (Mackinson, 1999; Cooney *et al.*, 2001). Depletion in herring stocks has the potential to significantly impact a wide range of species because of their ecological importance in many food webs. They are also important economically as a commercial and subsistence fishery (Hart, 1973).

Herring in southern BC generally spawn in late February to early March. Herring spawn on either intertidal or subtidal vegetation and substrate (Blaxter, 1985). The eggs hatch synchronously in 10 to 21 days and the larvae live on their yolk sacs for about 6 days before feeding on plankton at the surface. At 25 mm in length, larvae metamorphose into juveniles (approximately 70 days post-hatch), and begin to develop scales and exhibit schooling behaviour. During the summer, juveniles form large schools in protected waters (nurseries) near spawning areas. They move offshore to feeding grounds by September and can be found at a depth of 150 to 200 m (DFO, 1984).

Mature adults leave offshore feeding grounds during October to December and migrate to inshore spawning areas. They remain inshore in deep channels and bays near spawning areas to complete gonadal maturation. The age of onset of spawning varies with geographical location (Trumble and Humphreys, 1985). In the southernmost extent of the range in California, spawning may begin in 2-year olds, but begins in 4-year olds in Alaska (Norcross *et al.*, 2001). Herring begin spawning at 2+ years of age in BC (Hay *et al.*, 2001). The exact timing of spawning also varies on a north-south gradient, with the San Francisco herring spawning as early as November and those in Alaska

spawning mid-April (Norcross *et al.*, 2001). On the west coast of Vancouver Island in BC, spawning peaks in late February to early March, while on the North Coast of Vancouver Island and the Queen Charlotte islands spawning peaks in late March to mid-April. After spawning, adult Pacific herring move offshore to return to feeding grounds at a depth of 100 to 150 m (DFO, 1984).

2.1.2. Immunotoxicology

Historically, the field of environmental toxicology has concentrated on observing the effect of xenobiotics upon organisms at concentrations near the median lethal concentration LC_{50} (Cairns, 1992). In recent years, the effects of sub-lethal concentrations of toxic substances have been recognized to be of biological importance (MacCubbin *et al.*, 1990; Niimi, 1990; Stein *et al.*, 1992; Zelikoff, 1993). The exposure of fish to sub-lethal levels of xenobiotics can be immunosuppressive (Iwama, 1977; Knittel, 1981; Sindermann, 1993), as well as decrease growth, impair reproduction, decrease swimming performance and decrease ability to appropriately respond to additional stressors (Kennedy *et al.*, 1995).

This trend towards evaluating lower concentrations has driven the suite of toxicological assessment endpoints away from death and towards sub-lethal indicators. These include impairment of physiology, reproduction, and performance, which are more subtle measures but would ultimately represent impaired fitness (Adams *et al.*, 1989; Hinton, 1989; Anderson, 1990). It has also been recognized that xenobiotic exposure can exacerbate disease states by decreasing immune function (Sharma, 1981; Khan, 1990; Zelikoff, 1994). This has lead to the development of the field of immunotoxicology, which focuses on exposure to low concentrations of xenobiotics and the evaluation of sub-lethal effects on the immune system and its function. Chemicals in the marine environment that have been shown to have immunosuppressive potential include copper (Donaldson and Dye, 1975), cadmium (Schreck and Lorz, 1978), dioxins (Spitsbergen *et al.*, 1986), tributyltin (Wishkovsky *et al.*, 1989) and PAHs (Arkoosh *et al.*, 1991).

Immunotoxicology specifically evaluates the impact of chemical exposures on an organism's mechanisms to ward off pathogens and disease. However, no single endpoint change (hematological, biochemical or immunological) in fish can currently be considered pathognomic (Weeks *et al.*, 1992). Adams *et al.* (1989) recommends the use of immunotoxicological biomarkers as part of a suite of indicators that examine

different levels of biological organization. Establishing reliable biomarkers in fish immunotoxicology has been the focus of researchers because some fish diseases are often related to environmental quality (i.e., hypoxia and organic wastes) and because many environmental pollutants can be potentially immunotoxic (Zeeman and Brindley, 1981; Luster *et al.*, 1988; Adams *et al.*, 1989; Wester *et al.*, 1994; Karrow *et al.*, 2003).

2.1.3. Immuntoxicology and the Teleost Immune System

The hematological and immunological systems of fish are thought to be as comprehensive as those of mammals and birds (Zeeman and Brindley, 1981). The major lymphoid tissues in fish are the kidney, thymus and spleen, as well as mucosal lymphoid tissue in the skin and gills (Zelikoff, 1993; Press and Evensen, 1999). While the teleost immune system bears many similarities in immune structures and responses to that of higher vertebrates, it has some significant structural and functional differences (Zeeman and Brindley, 1981; Zelikoff, 1993).

The fish immune system is comprised of non-specific and specific components, somewhat analogous to mammals. Non-specific defenses are considered more important in fish *vs.* their mammalian counterparts because the specific component is less developed (Ingram, 1980; Grinde *et al.*, 1988; Pilström and Bengtén, 1996). Non-specific defense mechanisms are of key importance as a first line of defense (Dalmo *et al.*, 1997; Ainsworth and Dexiang, 1990). External barriers such as skin, gills, and mucus comprise part of the non-specific defenses (Ellis, 1981; Sakai, 1992). Other non-specific mechanisms include inflammation and phagocytosis by macrophages and neutrophils (Secombes and Fletcher, 1992; Lamas *et al.*, 1994; Rice and Schlenk, 1995; Boonstra *et al.*, 1996; Ellis, 1999). Impairment of the non-specific system has the potential to be particularly harmful for fish (Low and Sin, 1996). Specific immunity includes both humoral mediated immunity (HMI) that produces antibodies specific to antigens and cell-mediated immunity (CMI) from cytotoxic T cells and natural killer cells, both of which confer resistance to infection (Weeks *et al.*, 1992).

Fish do not possess bone marrow; instead, the anterior (head) kidney and spleen serve as the main hemopoietic organs (Corbel, 1975; Fänge, 1986, Zelikoff, 1994). Fish also do not have discrete lymph nodes. They do appear to possess the equivalent of a lymphatic system (Corbel, 1975; Zeeman and Brindley, 1981; Zelikoff, 1994) but in some teleosts, a secondary vascular system has been described rather than a true lymphatic

system (Steffensen and Lomholt, 1992; Olson, 1996). The third and greatest difference is the strong association between environmental temperature and immunological status (Bly and Clem, 1992; Anderson, 1993; Hardie *et al.*, 1994). Fish are ectothermic and therefore immune responses are temperature sensitive.

Water temperature governs the activity of the non-specific and specific immune responses in fish. For example, the immune function of sockeye salmon (*Oncorhynchus nerka*) showed differential reliance on the two components of the immune system with varying water temperature. At 8°C, non-specific immune responses were activated, whereas specific responses were activated at 12°C (Alcorn *et al.*, 2002). Tench (*Tinca tinca*) had higher numbers of active phagocytes and enhanced phagocytosis at 12°C compared to 22°C (Collazos *et al.*, 1994). In carp (*Cyprinus carpio*), exposure to low temperature enhanced phagocytosis activity (Le Morvan *et al.*, 1997) and in channel catfish (*Ictalurus punctatus*) phagocytosis was an important compensatory mechanism at lower temperatures (Dexiang and Ainsworth, 1991). While the non-specific responses are improved at lower water temperatures, B and T cell functions (specific responses) were suppressed for 3 to 5 weeks in channel catfish after reducing the temperature from 23°C to 11°C (Bly and Clem, 1991). Therefore, if any generalization can be made, non-specific responses predominate at lower water temperatures and specific responses predominate at higher water temperatures.

2.1.4. Assessment of immunocompetence

Immunotoxicology attempts to identify measures of the immune system defenses that are altered in individuals by exposure to a xenobiotic beyond the boundaries of normal variation, or baseline range, for a given population. Evaluation of the immune system when examining the effects of xenobiotic exposure on disease susceptibility is important because immunocompetent cells are necessary for disease resistance (Anderson and Zeeman, 1995).

A generalized tiered approach has been widely accepted as a method of evaluating the immunotoxicological potential of a given compound, or mixture of compounds (Vos & van Loveren, 1987; Luster *et al.*, 1988; Adams, 1990; Weeks *et al.*, 1992; Wester *et al.*, 1994; Deschaux and Khan, 1997). This approach places the wide variety of bio-indicators used to examine responses to chemical exposure into three tiers, which reflect the complexity of the immune system (Speirs *et al.*, 1978; Vos, 1980; Luster *et al.*,

1982; Kerkvliet, 1986; Luster *et al.*, 1988; Vos *et al.*, 1994). Tier 1 screens for changes in general immune function, including condition indices, hematology, and blood biochemistry (Luster *et al.*, 1988; Anderson 1990; Weeks *et al.*, 1992). Tier 1 assays are generally inexpensive, relatively easy to perform and require little specific equipment, making them ideal for field conditions (Weeks *et al.*, 1992). Tier 2 assesses for changes in function of humoral and cell-mediated responses and includes antibody titers and macrophage assays (Weeks *et al.*, 1992; Vos *et al.*, 1994). Tier 3 assays examine the integrated functioning of the immune system *via* pathogen challenges and an examination of differences in mortality (Weeks *et al.*, 1992; Cooper, 1997). This is the most integrative test of an organism's ability to defend itself against disease (Luster *et al.*, 1988).

Assays of immune function in fish have shown large variation due to species differences, specific reactions to captivity and handling, and differences in laboratory facilities and procedures (Stolen *et al.*, 1990). When performing disease challenges there is especially a need to standardize the challenge prior to its use as a biomarker (Weeks *et al.*, 1992). Fish exposed to various environmental stressors can exhibit either an increase (Peters *et al.*, 1991) or a decrease (Weeks and Warinner, 1984; Walczak *et al.*, 1987) in phagocytotic activity. More consistent findings are increases in leucocrit (Lct) (McLeay and Gordon, 1977; Peters and Schwarzer, 1985); decreases in lysozyme activity (Peters *et al.*, 1991) and increased susceptibility to bacterial infection (Angelidis *et al.*, 1987; Kennedy *et al.*, 1995).

In order to isolate the effects of oil or pathogens, it was important to determine which factors need to be accounted for in experimental design and analysis. The goal of this chapter was to determine the influence of demographic factors (age, size, gender, and population), pathology indices (lesions and anemia) and captivity (T_H and T_E) on the variability of a suite of hematological, biochemical, and immunological variables in wild caught captive-held Pacific herring. Age, gender, and population were examined here, as they are known to influence immune system variables in other fish species (Niimi, 1990). T_H and T_E differences have also been shown to be potential confounders of reference ranges (Torres *et al.*, 1986; Marty *et al.*, 1998). Wide ranges in plasma biochemistry values have been reported for wild PWS herring that were caught and sampled at sea (Marty *et al.*, 1998), but a profile of reference ranges of hematological

and immunological measures in captive Pacific herring has not been previously established. Therefore, reference range profiles of hematological, plasma biochemistry and immunological measures for captive, non-manipulated Pacific herring were generated to fill this knowledge gap.

The subsequent data chapter examines the effects of exposures (WSFO and pathogens) on immunological variables by comparing exposed herring to unexposed control groups of herring, therefore, reference ranges for variables were not needed *per se*. Nevertheless, biological variability will exist among control groups and it is important to express the response to a stressor in the context of normal biological variability (Heath, 1990; Schreck, 1990). The results of this chapter could also be useful if herring were sampled under field conditions without controls as it is critical to have a reference range that reflects the representative captive condition.

2.2. METHODS

Methods common to all experiments are described in this chapter. Details that vary between experiments, details of WSFO exposure, and pathogen challenges are discussed in the subsequent chapter. Experiments were conducted over a 4-year period from the fall of 1995 to the spring of 1999. Herring used for experiments ranged from 0+ year (young of the year) to adults (2 + years) and were obtained from three geographic locations: Barkley Sound (BS), BC; Quadra Island (QI), BC; and Puget Sound (PS), WA (Table 2.1, Figure 2.1). Herring used for experiments were also compared with fish sampled from PWS herring and Vancouver Aquarium (VA) herring (originally obtained from QI). Age was determined by the time of year when capture occurred and by the size of fish. 0+year juveniles were obtained in eelgrass beds during summer months. This habitat is generally accepted as the nursery habitat for herring, thereby placing them as young of the year, i.e., born in the year of capture (Hay *et al.*, 2001) and > 3 months but < 6 months old at the time of capture. Adults (2 years and older) were collected just prior to spawning.

Different source populations were used for two reasons. Adult BS herring could only be obtained once a year, just prior to spawning. The QI population were available year round and allowed experiments to continue at other times of the year. PS herring were

used in this research due to the collaborative nature of some experiments with researchers from the University of Washington.

Table 2.1. Description of the herring populations used during the experiments.

Date of Experiment	Population Source	Age
September - November 1996	Barkley Sound, BC	0+year
November 1998 - January 1999	Barkley Sound, BC	0+year
Nov 1995 - Jan 1996	Puget Sound, WA	0+year
July 1996	Barkley Sound, BC	Adult
July - August 1998	Barkley Sound, BC	Adult
Jan - June 1997	Puget Sound, WA	Adult
October 1997	Quadra Island, BC	Adult
November 1997 - January 1998	Quadra Island, BC	Adult
October 1997	Vancouver Aquarium (QI), BC	Adult
Spring 2001	Prince William Sound	Adult

A summary table of specific experimental conditions, sample times, exposure concentrations, pathogen challenge doses and variables measured is shown at the end of the methods section of the exposure chapter (Table 3.2). It was not possible to perform all assays or observations on all fish sampled within each experiment. This was due to limitations of whole blood and plasma volume, assay limitations (i.e., minimum macrophage cell count required), loss of some samples during centrifugation processes, limitations on sample size for processing costly assays, and constraints on performing post mortems on all fish. Consequently, the sample size reported for measurement variables and for observations regarding gender and the presence of lesions reflect the sub-samples within each experiment that could be tested and examined for each assay and observation. As such, it was not possible to maintain a constant sample size for all assays within or between experiments.

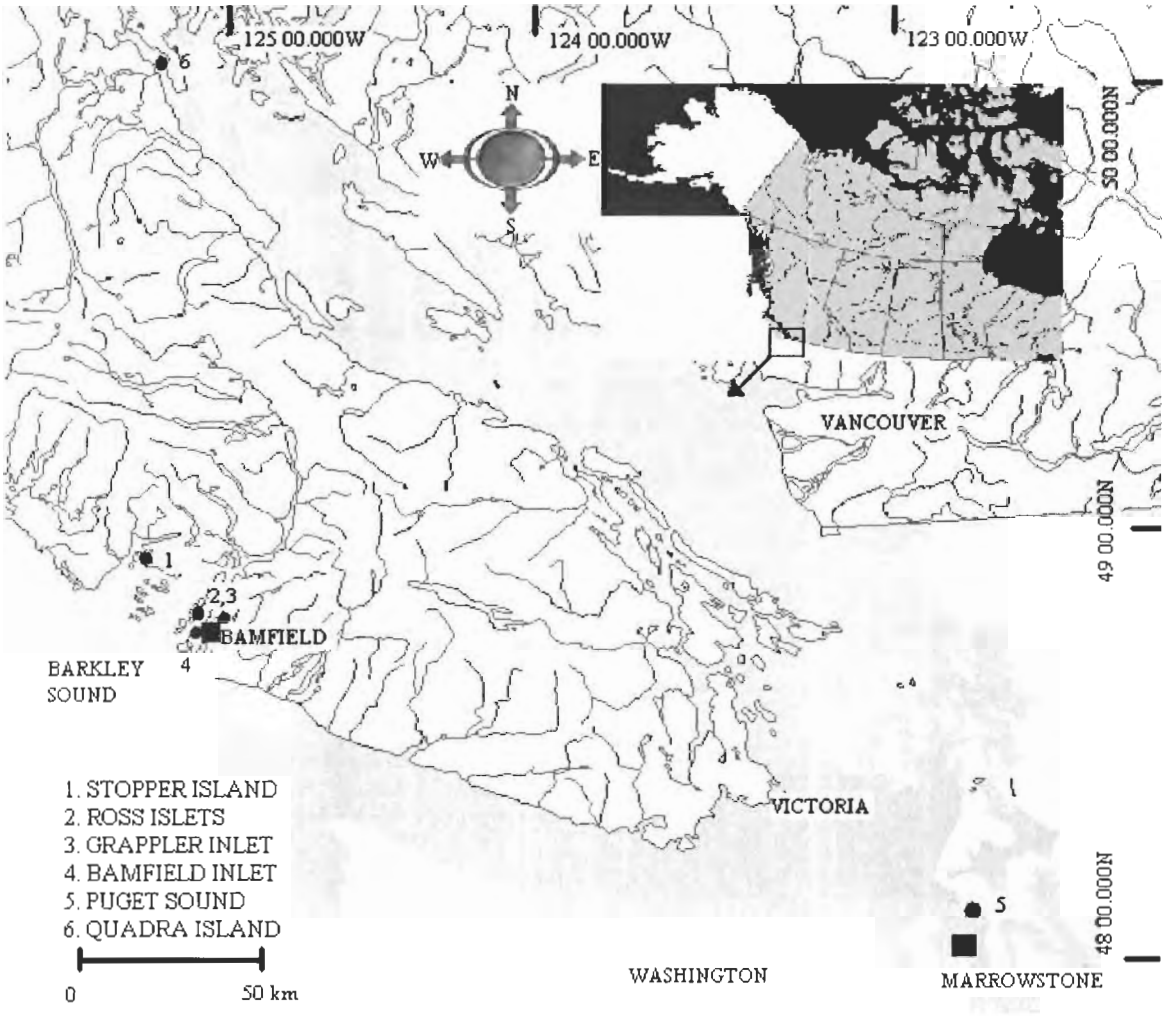

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Figure 2.1 Location of field stations and herring collection areas. Squares represent the two field stations: Bamfield Marine Sciences Centre located in Bamfield, BC and the Marrowstone Field Station located in Marrowstone, Washington. Circles represent the areas from which herring were collected. Reproduced with the permission of the Community Mapping Network.

2.2.1. Fish capture methods

Herring from BC were collected under a research permit granted to the Bamfield Marine Sciences Centre (BMSC) and those from PS were collected under a similar Washington Department of Fish and Wildlife research permit. Adult BS and PS herring were captured *via* commercial purse seine nets. The BS herring were transferred from the seine net (while still in the water) to totes (1600 L) onboard research vessels using large dip nets. Approximately 20 fish were transferred in each dip net load, and 400 fish were placed in each tote. Juvenile BS herring were beach-seined at several locations close to Bamfield (Figure 2.1) using a small research beach seine or a small purse seine. Fish were transferred from nets into plastic bins (70 L for 100 fish) onboard small outboard boats using dip nets. Juvenile PS herring were obtained by dip net from bait balls and transported in an oxygenated tank back to the laboratory (Elder, pers. comm., 2005). Adult and juvenile herring were also obtained from a live bait holding facility on QI, BC. Fish were initially caught by seine boats in the Georgia Strait and were maintained in large sea storage pens within Quathiaski Cove, QI.

Pacific herring are particularly susceptible to scale loss following handling and contact with nets. Although measures were taken to minimize handling to avoid descaling injuries, it was necessary to use dip nets at least once for each batch of fish and to initially collect fish with either a beach or commercial purse seine.

2.2.2. Fish transport conditions

The totes onboard the research vessels were supplied with flowing saltwater and were supplemented with oxygen *via* bubble diffusion sets. The transportation time for BS adults back to the BMSC was 1.5 to 2.5 h. Fish from PS were transported within 30 to 60 min to the Marrowstone Field Station (MFS) in a similar manner. Juvenile herring were transported *via* boat to BMSC within 15 to 20 min of capture.

QI herring were transported *via* truck in oxygenated totes (1600 L and 400 L per 100 adults and juveniles, respectively) with saltwater ice to maintain the water temperature and salinity at the same level as that of the sea pen for the duration of the return trip to the BMSC (8 to 10 h). Oxygen, salinity, and temperature were measured at intervals during the journey and adjustments were made to oxygen flow as needed using 100% oxygen (dissolved oxygen levels were keep at or near saturation at all times).

2.2.3. Holding facilities

On arrival at BMSC, juvenile BS and adult QI herring were assigned by lots directly to either sanitized experimental tanks (450 L and 265 L) or larger holding tanks (800 L) to begin acclimation. Adult BS herring were initially held in a large holding tank (138,300 L). Fish were exposed to an ambient photoperiod since the tanks were outdoors. Saltwater at BMSC was obtained at 30 m in depth from Bamfield Inlet. This entered a header tank that was 25 m above sea level. The header tank was accessible only by maintenance staff and was plumbed off to various flow-through saltwater tank locations and to individual tanks. BMSC research staff monitored water quality parameters at the facility level. All tanks were covered with lids and were located within outdoor enclosures to prevent access by predators and people. All effluent was treated with a Canadian Department of Fisheries and Oceans (DFO) approved chlorination/dechlorination system.

After 48 hours in captivity, herring were offered a commercial frozen krill diet (Murex Aquafoods, Langley, BC), which they readily accepted, and then were fed once daily *ad libitum* thereafter. Fish were generally held for at least two weeks prior to the initiation of experiments and some were held for up to 124 d. During the initial 2-weeks of the acclimation period, 10 to 30 fish were euthanized and submitted to the Fish Health Laboratory at the Pacific Biological Station (PBS) for bacteriology and virology screening. This involved culture for bacteria (*Vibrio* spp., *Renibacterium salmoninarum*, and *Pseudomonas spp.*) and virology testing for VHSV, infectious hematopoeitic necrosis virus (IHNV), infectious pancreatic necrosis virus, and *Oncorhynchus masu* virus using cell culture. A record of mortalities during the period of acclimation to experimental tanks was maintained and dead fish were removed daily. Following acclimation, fish were transferred by dip net and bucket to experimental tanks.

Variables such as water temperature, noise, O_2 saturation, and toxic algal blooms varied between years and experiments and may have contributed to mortality variation during holding and acclimation periods. For example:

- Water temperature during 1997/98 was unusually high (14 to 16°C) compared with other years (8 to 12°C). This was most likely attributable to El Niño. High losses of acclimating juvenile herring occurred in 1997 (50 to 73%).

- The location of experimental tanks at BMSC was in an area where pedestrian traffic was high, relative to the isolation of BMSC and other holding areas. Despite measures to minimize the noise to which acclimating and experimental fish were exposed, the tanks located closest to pedestrian walkways would have experienced higher noise levels than those further away.
- On several occasions, a 100% tank mortality occurred on the day following a red-tide notice from the DFO. Although inflow pipes for the water supplying the BMSC were located at a depth of 30 m, it was still feasible that some dinoflagellates and toxic algae may have entered the system. Regardless, the surviving tanks of fish were not subsequently used for experiments or to establish reference ranges.
- Gas super-saturation occurred during the fall of 1997 (up to 106% saturation). This caused gas emboli and death among herring. Subsequently, individual header tanks were installed to reduce gas partial pressures in the water.

2.2.4. Fish sampling

Plasma chemistry may show diel cycles that can confound the impact of environmental factors (Carillo *et al.*, 1986; Thorpe *et al.*, 1987; Laidley and Leatherland, 1988). Therefore, fish were sampled at the same time of day (08:00 to 12:00 h). All fish were sampled by dip net and immediately anesthetized in 100 mg/L tricaine methanesulfonate (MS222, Sigma) until opercular motion ceased. Blood was collected by either caudal venipuncture (adults) or severing of the tail (juveniles) once fish were completely anesthetized. Cervical dislocation was performed prior to dissection.

2.3. MEASUREMENT VARIABLES

Measurement variables were selected as potential biological indicators for Pacific herring based on a review of the fish immunotoxicological literature (Anderson, 1990; Secombes *et al.*, 1992; Wester *et al.*, 1994; Zelikoff, 1994; Anderson and Zeeman, 1995). Each variable is introduced and discussed below with respect to its usefulness as an indicator of immune toxicity. Measurement variables are then summarized at the end of this section (Table 2.2). Additional details and the specific assay protocols are provided in Appendix 1. A comprehensive listing of average values and ranges for each measurement variable in other fish species, and of previously reported biochemical values in herring, is provided in Tables 2.3 and 2.4.

2.3.1. Tier 1 responses

2.3.1.1. Condition indices

Condition indices are simple and rapidly performed indicators of fish health at the organismal level (Bolger and Connolly, 1989; Goede and Barton, 1990). They represent a means of comparing general body condition among individuals based on length, weight, and organ weight data. Condition factor (CF) is thought to be a measure of energy storage (van den Heuvel *et al.*, 1999) and it was determined using Fulton's formula based on length and weight measurements (Rakitin *et al.*, 1999). This was calculated as body mass/length^3 x 100, and assumes isometric growth with unchanged body proportions and specific gravity (Rakitin *et al.*, 1999). Liver somatic index (LSI), gonad somatic index (GSI), and spleen somatic index (SSI) provide evidence of changes in organ weight that may have a pathological origin. These indices were calculated for most adult fish. Only LSI was determined in juvenile fish. Organ mass/body mass x 100 was used to calculate these indices (van den Heuvel *et al.*, 1999).

2.3.1.2. Hematology

Hematology is the study of the various cellular components that comprise blood, including red blood cells (RBCs), white blood cells (WBCs), and thrombocytes. The measurement of RBC and WBC parameters have long been used in both human and veterinary medicine in both diagnosis and prognosis (Campbell, 1988; van Vuren *et al.*, 1994; Houston, 1997; Tierney *et al.*, 2004). Fish hematology is commonly used in research to identify abnormal physiological functioning in fish (van Vuren *et al.*, 1994). The hematological measures made in this study included hematocrit (Hct), leucocrit (Lct), and white blood cell (WBC) differential counts.

Hct is the percentage of blood by volume that is composed of erythrocytes (RBCs) and has been called a primary tool for assessing fish health (Allen, 1993, Anderson and Zeeman, 1995). Hct varies with fish species but 41.0 ±14.3% (standard deviation) has been reported for teleosts as a group (Wilhelm-Filho *et al.*, 1992). Anemia (low Hct) can result from blood loss, increased red cell destruction, or from reduced red cell production. Polycythemia (elevated Hct) is rare, although the effects of stress can cause splenic contraction and an increase in Hct that varies in magnitude among fish species (Gallaugher and Farrell, 1998). In addition, dehydration can elevate Hct due to hypovolemia.

Lct is the percentage of blood by volume that is composed of WBCs and is an estimator of total WBC count and is considered an indicator of resistance to disease in fish (Wedemeyer 1996). It is less comprehensive than a total white blood cell count but the relative simplicity involved in obtaining the measurement lends itself well to fieldwork and intense sampling. The absence of detectable Lct or an increase above an individual's normal level is suggested to be more important than minor changes in the absolute Lct value (Anderson and Zeeman, 1995). Generally, increased Lct indicates stimulation of WBC production whereas a decrease indicates either suppression or WBC loss.

Hct and Lct were measured by collecting blood into 100 µL heparinized microcapillary tubes (in duplicate whenever possible). The tubes were spun at 13,460 xg for 3 min using a microcentrifuge (IEC Micro-MB), which separated red and white cells from the plasma. Averages for Hct and Lct were calculated from measurements of the percentage of packed red and white blood cells using digital calipers (VWRbrand®, Edmonton, AB) and a dissecting microscope. The plasma remaining after centrifugation was preserved and frozen at -20°C for subsequent analysis of plasma biochemistry and lysozyme activity analysis.

The WBC differential count is a means of determining cell percentages of the cell types that comprise the WBCs. Although there is some controversy surrounding the significance of WBC differentials in interpreting the WBC status of fish, the method has been adopted by some researchers (McLeay and Gordon, 1977; Wedemeyer *et al.*, 1983) in a manner comparable to mammalian hematology. The controversy exists because many of the descriptions of fish hematology and the indices used have been built upon the classification scheme of mammalian blood cells (Ellis, 1977; Tierney *et al.*, 2004). Fish blood cells possess many similarities with higher vertebrates but also have unique features, including nucleated red blood cells and a more poorly defined association between the classes of WBCs and their function. Because of these differences, methods adopted from human and mammalian studies need to be used with caution (Houston, 1997). Due to morphometric differences in piscine WBCs, the differential count is generally divided into four categories: granulocytes (neutrophils and eosinophils), lymphocytes, thrombocytes, and monocytes (Campbell, 1988).

One drop of herring blood produced two blood smears. These were allowed to air dry and were then stained with a Diff-Quik staining set (Difco™, BBL™, BO Biosciences, Mississauga, ON). The slides were examined at 1000x power with a Nikon Alpha-Phot 2 YS-2 microscope. 100 WBCs were counted on each of 2 slides per fish, providing a resolution of 0.5%, with each cell assigned a classification of either a neutrophil, eosinophil, lymphocyte, monocyte, or thrombocyte (Fänge, 1992; Ainsworth, 1992). As circulating macrophages are extremely rare (<1%) in fish (Ellis, 1977), any such cells were classified as monocytes. Small lymphocytes and thrombocytes were found to be quite similar in their appearance. However, it was possible to differentiate between them with careful examination and any potential bias due to the subjective nature of the WBC differential count was minimized by having the same person perform all counts.

2.3.1.3. Plasma biochemistry

Biochemical measures have been shown to be sensitive to sublethal stressors and the degree of change can be associated with the severity of the stressor (Livingstone, 1985; Thomas *et al.*, 1987; Thomas, 1990; Jeney *et al.*, 1996). Biochemical assays (glucose, lactate, albumin, total protein, calcium, phosphorus, chloride, and cortisol) were performed on plasma using Sigma brand diagnostic kits (Sigma, St. Louis, MO). In general, these assays were based on enzymatic reactions of various dyes with the plasma constituents. The subsequent degree of colour development was read by measuring the optical density at specific wavelengths using a spectrophotometer.

All plasma samples were processed within 6 months of collection and assays were conducted at room temperature. Plasma, rather than serum, was used in this study because comparisons of paired plasma and serum samples from rainbow trout (*Oncorhynchus mykiss*), channel catfish, hybrid tilapias (*Oreochromis* spp.) and hybrid striped bass (*Morone* spp.) showed that levels determined from serum may not accurately reflect blood chemistry levels in the circulation (Hrubec and Smith, 1999). Rapid changes in fish serum can occur even when samples are handled properly (Warner *et al.*, 1979; Hrubec and Smith, 1999). In this study, fish were caught from the tank, anesthetized, and then sampled. While this sampling method can alter some aspects of blood chemistry more than the chronic methods using cannulation, (e.g. gain of electrolytes, McDonald and Milligan, 1992; and a bias towards elevated Hct, Gallaugher and Farrell, 1998) only a terminal sample was taken and all fish were sampled the same, allowing for within study comparison.

Electrolyte disturbances occur in fish plasma during both acute and chronic exposures to petroleum hydrocarbons (Payne *et al.*, 1978; McKeown & March, 1978). Plasma chloride, calcium and inorganic phosphorus assays all measured the concentration of each ion within the plasma colorimetrically. Plasma chloride (Cl^-) is sensitive to many environmental and internal factors. Alterations in the plasma concentration of this electrolyte can occur following gill damage and skin damage after exposure of the gills to external pollutants (McDonald and Milligan, 1992). Osmoregulation can be compromised by catecholamine-induced gill and cardiac changes that are seen in stressed fish (Pickering and Pottinger, 1995). For example, marine fishes such as herring will gain ions if gills and skin are damaged and therefore elevations in the concentration of major ions within the plasma are to be anticipated. Plasma Cl^- concentration is considered useful as a tool in assessing whether toxicant exposure is stressful for fish (Heath, 1995). Plasma Cl^- levels of 141 to 197 mEq/L have previously been reported in spawning herring (Marty *et al.*, 1998).

Blood calcium (Ca^{2+}) levels in marine fish tend to range from 3.2 to 3.4 mmol/L in teleosts to 5.6 mmol/L in elasmobranchs (McDonald and Milligan, 1992). Stressors such as hypoxia and sustained exercise have shown no effect on plasma Ca^{2+} concentration in rainbow trout (Andreason, 1985), however, little is known about the effects of WSFO exposure on this plasma ion. In marine fish, 22% of the total Ca^{2+} is protein-bound. Therefore, any changes in plasma protein may impact the total plasma Ca^{2+}, however free Ca^{2+} remains constant and this is what our assay measured (McDonald and Milligan, 1992). Inorganic phosphorus (P_i) is also partially protein-bound. Therefore, it may also change with alterations in plasma protein levels. The concentration of free phosphorus has been shown to generally remain the same (Björnsson and Haux, 1985), however, the effects of stressors on phosphorus are unknown. Plasma P_i concentrations of 5.5 to 38 mg/dL have been measured in spawning herring (Marty *et al.*, 1998).

Total plasma protein concentrations in fish can range from 2 to 8 g/dL (Fletcher, 1975; Sandnes *et al.*, 1988) and in teleosts, albumin concentrations range from 1.0 to 2.4 g/dL and make up 25 to 50% of total plasma protein (Sandnes *et al.*, 1988; Miller *et al.*, 1983). Changes in plasma protein concentration can occur due to changes in plasma water volume. An increase can be due to movement of fluid from plasma to the intracellular

compartment and a decrease can be due to hydration of the plasma. Osmotic imbalances and any stressor that disrupts osmoregulation can bring about these shifts and therefore, alter total plasma protein levels (McDonald and Milligan, 1992).

The general response of fish to acute stressors is a well-defined set of primary and secondary effects. The primary stress effects include increased secretion of catecholamines and corticosteroids (i.e., cortisol). Increases in these compounds can then result in a range of secondary stress effects on various biochemical (i.e., glucose and lactate), immunological and physiological parameters (Anderson, 1993). Stressors such as handling, pollutant exposure, temperature, starvation and disease can all induce the primary stress response. This response is a complicated pattern of changes, not only in physiology but also in behaviour, which has evolved to allow for increased survival (Pickering and Pottinger, 1995).

The circulating concentrations of plasma glucose and lactate were measured colorimetrically. An elevation in blood glucose due to mobilization of liver glycogen in fish under stress is one of the characteristics of the general stress response (Love, 1980) and is thought to be a sensitive reliable indicator of environmental stress (Sibergeld, 1974; Mazeaud *et al.*, 1977; Love, 1980; Martin and Black, 1996; Lumanlan-Mayo, 2000). A decrease in plasma glucose has been reported previously in herring in association with T_H and in spawning herring glucose ranges from 0.94 to 22.8 mmol/L (Marty *et al.*, 1998).

Lactate is the major end product of anaerobic metabolism in vertebrates and is usually <1 mM in fish blood (McDonald and Milligan, 1992). An increase in plasma lactate usually indicates an oxygen limitation and therefore, increases are common under conditions of either environmental hypoxia, internal hypoxemia, or exhaustion due to excessive activity (McDonald and Milligan, 1992). Plasma lactate increased in Atlantic salmon and in rainbow trout following stress and exercise regimes (Thomas *et al.*, 1999) and netting (Love, 1980).

Cortisol was measured using an ELISA kit (Neogen™ Corporation, Lexington, KY). Cortisol is the main (≥80%) circulating corticosteroid of teleosts and originates from interrenal gland cells (Olsen *et al.*, 1992; Weyts *et al.*, 1999). It acts as both a

glucocorticoid and a mineralocorticoid in teleosts (Balment and Henderson, 1987). It is generally accepted that acute stress leads to increases in plasma cortisol levels (Olsen *et al.*, 1992; Weyts *et al.*, 1999). Plasma cortisol levels have often been used to examine primary endocrine responses to xenobiotics (Donadson *et al.*, 1984). However, plasma cortisol does not always change with exposure to adverse environmental conditions (Schreck, 1981) or with disease (Laidley *et al.*, 1988). Stress from handling increases cortisol concentrations, but it takes approximately 1 hour for cortisol to reach a peak in the plasma, so fish capture and sampling effects should not confound any elevation due to treatment stress (Milligan, 1996).

2.3.2. Tier 2 responses

2.3.2.1. Plasma lysozyme

Lysozyme (mucopeptide N-acetylmuramylhydrolase) is a stable hydrolytic antibacterial enzyme of fish and is part of the first line of non-specific defenses against pathogens and other tissue insults. It originates primarily from the lysosomes of neutrophils, monocytes and macrophages, and is secreted into the peripheral blood stream by these classes of cells (Murray & Fletcher, 1976; Kokoshis and DiLuzio, 1979). This enzyme attacks the peptidoglycan in the cell walls of mostly Gram-positive bacteria, but also Gram-negative bacteria to some degree (Grinde *et al.*, 1988; Alexander and Ingram, 1992; Holloway *et al.*, 1993; Hutchinson & Manning, 1996). In the current study, determination of plasma lysozyme activity was performed using the lysoagar plate technique (Ellis, 1990).

2.3.2.2. Macrophage assays

The fish macrophage is essential for phagocytosis, inflammation and antigen processing (Zeeman and Brindley, 1981; Adams and Hamilton, 1984), and it also secretes lysozyme. The phagocytosis of foreign particles and damaged self-cells is a key function of the macrophage. Head kidney macrophages were obtained *via* the sterile dissection of head kidney tissue that was placed immediately into tissue culture medium (see Appendix 1 for details). The protocol for the isolation of head kidney macrophage cells for each experiment was from Stolen *et al.*, (1990). Tissue macrophages were evaluated for their phagocytic ability and their capacity to produce reactive oxygen species (a process termed respiratory burst activity).

Phagocytosis is an important component of the immune defense of fish and its effectiveness is considered an indicator of the competency of the cell-meditated arm of

the immune system (Neumann *et al.*, 2001). An elevation in phagocytosis compared with controls implies immunostimulation, whereas a decrease suggests immunosuppression. Phagocytosis is the ability of phagocytic cells (blood monocytes, most neutrophils and kidney macrophages) to engulf and destroy foreign particles. Phagocytic capability was assessed by incubating macrophages with Congo Red-stained yeast particles (Kaminiski *et al.*, 1985; Seeley *et al.*, 1990). Phagocytosis was quantified spectrophotometrically and the result interpreted by comparing to a standard curve made from serial dilutions of Congo Red-stained yeast stock solution (Seeley *et al.*, 1990).

After isolation, head kidney macrophage cells were incubated with a solution of nitroblue tetrazolium dye (NBT) to evaluate the ability of these cells to produce reactive oxygen intermediates (ROIs) that have bactericidal activity (Stasiak and Baumann, 1996). ROIs are used by tissue macrophages as cytotoxic agents to destroy engulfed microorganisms and to damage extracellular targets (Pick, 1986). This suite of oxidizing agents includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), myeloperoxidase, and the hydroxyl radical (OH^-), (Baboir, 1978). The respiratory burst consists of a sequence of reactions that not only result in the production of ROIs, but also leads to a marked increase in oxygen consumption and increased use of glucose. The amount of respiratory burst activity was measured spectrophotometrically.

2.3.2.3. Antibody titre

Antibodies are produced by the fish immune system in response to exposure to foreign protein antigens, primarily by lymphocytes originating in the spleen and head kidney in teleosts (Ellis, 1977). IgM is the main class of immunoglobulin reported in teleosts (Watts, *et al.*, 2001) and has been identified specifically in herring plasma in the range of 0.13 to 5.32 mg/ml (Marty *et al.*, 1998; Davis *et al.*, 1999). In general, the antibody titre is the accepted measure of the ability of the humoral arm of the immune system to respond to a challenge. Antibody titre is a measure of the B-cell mediated response of the immune system whose function it is to neutralize antigen with antibodies and to activate the complement cascade system (Rijkers *et al.*, 1980; Dorson, 1981). The antibody assay that was used in the current study is based on the slide agglutination technique (Roberson, 1990). This is a rapid, screening test for fish antibodies, especially under field conditions (Toranzo *et al.*, 1987). Commercially prepared latex beads coated with anti-*Vibrio anguillarum* antibodies were obtained from Microtek®

International Ltd. (Saanichton, BC). Both a negative control, using 0.85% sodium chloride (NaCl), and a positive control, using commercial antisera, were run simultaneously.

Table 2.2 Hematological, plasma biochemical and immunological indicators used to assess toxicant exposure in fish.

Variable	Tier	Definition, interpretation and application	References
Hematocrit	1	Volume of packed red blood cells/unit volume of whole blood; indicator of hemopoietic activity, hydration status, and blood loss. Used to define anemia and polycythemic states. Nonspecific, easy, low sensitivity, non- specific immune impairment.	(Anderson, 1990; Ellsaesser and Clem, 1986; Anderson and Zeeman, 1995)
Leucocrit	1	Volume of leucocytes (white blood cells)/unit volume of whole blood. Indicator of the quantity of white blood cells. Elevation can indicate immunostimulus, reduction can indicate immuno-suppression. Non-specific, easy, low sensitivity. Does not identify a specific immune impairment.	(Anderson, 1990; Pickering and Pottinger, 1987; McLeay and Gordon, 1977)
White blood cell (WBC) differential count	1	Proportional contribution of different types of WBC to the total WBC population. Low sensitivity, no specific immune impairment, labour intensive, wide variation. Provides clues regarding how the body is responding.	(Anderson, 1990; Blaxhall, 1972; Anderson and Zeeman, 1995)
Condition factor	1	Body weight/length^3 x 100, an energy storage or use parameter. Easy to obtain.	(Van den Heuvel *et al*, 1999)
Liver-somatic index	1	Liver weight/body weight x 100, energy storage parameter Easy to obtain, can indicate abnormally small or large liver.	(Van den Heuvel *et al*, 1999)
Spleen-somatic index	1	Spleen weigh/body weight x 100. Easy to obtain, can indicate splenic contraction.	(Van den Heuvel *et al*, 1999)
Gonad-somatic index	1	Gonad weight/body weight x 100, energy utilization measure. Easily obtained, varies with reproductive stage and season.	(Van den Heuvel *et al*, 1999)
Plasma glucose	1	Identifies an alteration of glucose metabolism. Sensitive, reliable indicator of environmental stress in fish, measure of secondary stress effect.	(Silbergeld, 1974; Martin and Black, 1996: Lumanlan-Mayo, 2000)
Plasma lactate	1	Indicator of anaerobic metabolism. Can reflect stress or exertion.	(Thomas *et al.*, 1999; Driedzic and Kiceniuk, 1976)
Plasma albumin	1	Component of blood protein level. Decrease can indicate intestinal inflammation or hemorrhage, or reduced production by the liver.	(Pfeifer and Weber, 1979; Turner, 1983)

Table 2.2 Continued

Variable	Tier	Definition, interpretation and application	References
Plasma total protein	1	Measure of blood protein level. Can indicate dehydration or reduced protein production. Affected by season, age, metabolism, temperature, and increases with increased globulin production.	(Denton and Yousef,1975; Pfeifer and Weber, 1979)
Plasma chloride	1	Plasma electrolyte involved with osmoregulation. Changes can reflect stress (increases in salt water).	(Randall, 1972; Wedemeyer and Nelson *et al*, 1975)
Free plasma calcium	1	Plasma electrolyte, important in membrane function and muscular activity. Generally unaffected by stress in other fish species.	(Andreason, 1985)
Phagocytosis	2	Evaluates phagocytosis capability of macrophages. Labour intensive, good sensitivity, wide statistical variation, reflects changes early in immune kinetics.	(Anderson, 1990; Olivier *et al*, 1986; McLeay and Gordon, 1977)
Respiratory burst, NBT	2	Neutrophil oxidative radical NBT (nitroblue tetrazolium) activity Evaluates ability to produce bactericidal reactive oxygen species. Subjective, labour intensive.	(McLeay and Gordon, 1977: Lamas and Ellis, 1994)
Trypan blue	2	Macrophage cell viability assessment permits determination of the percentage of live macrophage cells. Provides clear distinction between viable and non-viable cells.	(McLeay and Gordon, 1977)
Plasma lysozyme activity	2	Antibacterial enzyme present in serum, mucus, plasma, and head kidney. Relatively inexpensive, fast assay. Indirect measure of macrophage function.	(Fletcher and Grant, 1968; Grinde *et al.*, 1988)
Virus neutralization	2	Evaluates circulating antibody and tissue viral load. Moderate degree of preparation, time lag of one week for results.	(McLeay and Gordon, 1977)
Antibody agglutination	2	Measures circulating antibody titre. Easy, good sensitivity, late in immune kinetics, requires prepared antigen.	(Anderson, 1990; Robohm, 1986)
Pathogen challenge	3	Evaluates protection against infection. Mortality rate comparisons, relative % survival. Difficult, mid-point in immune kinetics, requires live pathogens.	(Anderson, 1990; McLeay & Gordon, 1977; Hetrick *et al.*, 1979; Knittel, 1981; MacFarlane *et al.*, 1986)

Table 2.3 Literature values from other adult fish species for the variables measured in the current study with adult Pacific herring. Values are ranges or mean ± standard error (SE) or standard deviation (SD) where indicated with an asterisk. Sample size is shown where known. All values are from control fish only. Resp.=respiratory.

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Hematocrit (%)		41.0 ±14.3*	Teleosts	52	Wilhelm-Filho *et al.*, 1992
	20 - 43	32 ±5	*Salmo trutta*	73	Blaxhall and Daisley, 1973
	32 - 53		*Salmo salar*	16	Olsen *et al.*, 1992
		42 ± 1	*Salmo salar*	40	Knoph and Thorud, 1996
		47 ±1	*Salmo salar*	140	Sandnes *et al.*, 1988
	32 - 45	38.4 ±3.8*	*Oncorhynchus mykiss*	23	Barham *et al.*, 1980
	26 - 44	34.9 ±1.2	*Oncorhynchus mykiss*	16	Barnhart 1969
		22.8 ±1.5	*Oncorhynchus mykiss*	18	Wood *et al.*, 1979
		26.3 ±1.4	*Oncorhynchus mykiss* (summer)	9	Gallaugher *et al.*, 1995
		32.5 ±1.0	*Oncorhynchus mykiss* (winter)	13	Gallaugher *et al.*, 1995
	21.5 - 43.9	32.0 ±5.5*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
	24.0 - 43.0	34.1 ±4.6*	*Oncorhynchus mykiss*	200	Wedemeyer and Nelson, 1975
		27.2 ±4.8*	*Ictalurus punctatus*	5	Ellsaesser and Clem, 1986
		35.4 ±5.5*	*Ictalurus punctatus*	5	Martin and Black, 1998
	20 - 44	34.6 ±2.8	*Ictiobus cyprinellus*	10	Chlebeck and Phillips, 1969
		29.7 ± 0.5	*Carassius auratus* (L.) (female)	65	Summerfelt *et al.*, 1967
	20.0 - 43.5	33.9	*Esox lucius* (L.) (male)	12	Mulcahy 1970
	20 - 33	27.9	*Esox lucius* (L.) (female)	9	Mulcahy 1970
		42.2 ±6.8*	*Dicentrarchus labrax*	20	Alvarez-Pellitero and Pintó, 1987
		14.9 ±0.6	*Platichthys flesus* (L.) (female)	6	Larsson *et al.*, 1984
		32 ±1	*Perca fluviatilis* (female)	16	Sjöbeck *et al.*, 1984
		14.5 ± 1.3	*Platichthys stellatus*	10	Wood *et al.*, 1979
		22.8 ± 1.2	*Pleuronectes americanus* (male)	10	Payne *et al.*, 1995
		39.3 ± 2.2*	*Hoplosternum littorale*	5	Brauner *et al.*, 1999
	34.4 - 39.3	35.4 (median)	*Oreochromis aureus*	6	Allen, 1993
		23.5 ± 1.0	*Ameiurus nebulosus*	37	Gilmour and MacNeill, 2003
	7.0 - 28.0	21.4 ±2.6	*Limanda limanda* (L.)	29	Tahir *et al.*, 1993

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Hematocrit (%)		32.9 ±0.4	*Carassius auratus* (L.) (male)	75	Summerfelt *et al.*, 1967
	22.5 - 27.8	25.5 ±2.9	*Tilapia nilotica*	20	Badawi and Said, 1971
		18.7 ±0.6	*Scyliorhinus canicula*	21	Torres *et al.*, 1986
		33.6 ±3.2	*Cyprinus carpio*	6	Harikrishnan *et al.*, 2003
		30.3 ±3.9*	*Rutilus rutilus* (L.)	9	Jeney *et al.*, 1996
		36.4 ±6.8*	*Lepomis sp.*	5	Lohner *et al.*, 2001
		18.7 ±0.6	*Scyliorhinus canicula*	21	Torres *et al.*, 1986
Leucocrit (%)		0.68 ±0.05	*Salmo salar*	140	Sandnes *et al.*, 1988
		2.48 ±0.19	*Scyliorhinus canicula*	21	Torres *et al.*, 1986
		0.75 ±0.27*	*Rutilus rutilus* (L.)	7	Jeney *et al.*, 1996
Lymphocytes (%)	63 - 93	79	*Esox lucius* (L.)	8	Mulcahy, 1970
	56 - 100	90 ±9	*Salmo trutta*	73	Blaxhall and Daisley, 1973
		37.5 ±21.4*	*Dicentrarchus labrax*	20	Alvarez-Pellitero and Pintó, 1987
		67.3 ±10.9*	*Limanda limanda*	16	Secombes *et al.*, 1991
	64.2 - 97.0	51.6 ±30.0	*Limanda limanda* (L.)	23	Tahir *et al.*, 1993
		80.0 ±28.8*	*Lepomis* sp.	6	Lohner *et al.*, 2001
		76.1 ±5.3	*Oncorhynchus mykiss*	20	Lamas *et al.*, 1994
Neutrophils (%)	0 - 25	6.6 ±6.5	*Salmo trutta*	73	Blaxhall and Daisley, 1973
		6.5 ±2.2	*Oncorhynchus mykiss*	20	Lamas *et al.*, 1994
		62.5 ±21.9*	*Dicentrarchus labrax*	20	Alvarez-Pellitero and Pintó, 1987
		16.8 ±5.0*	*Limanda limanda*	16	Secombes *et al.*, 1991
		4.7 ±8.1*	*Lepomis* sp.	6	Lohner *et al.*, 2001
Thrombocytes (%)		10.6 ±8.6*	*Limanda limanda*	16	Secombes *et al.*, 1991
		15.7 ±3.9	*Oncorhynchus mykiss*	20	Lamas *et al.*, 1994
Monocytes (%)		0.0 ±0.0	*Dicentrarchus labrax*	20	Alvarez-Pellitero and Pintó, 1987
		5.3 ±3.6*	*Limanda limanda*	16	Secombes *et al.*, 1991
		18.4 ± 22.7*	*Lepomis* sp.	5	Lohner *et al.*, 2001
	0 - 1		*Oncorhynchus mykiss*	20	Lamas *et al.*, 1994
Condition factor		1.31 ±0.04	*Limanda limanda* (at sea)	10	Hutchinson and Manning, 1996
		1.15 ±0.03	*Limanda limanda* (on shore)	10	Hutchinson and Manning, 1996
	1.07 -1.45		*Limanda limanda* (female)	62	Hutchinson and Manning, 1996

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Condition factor	0.69 - 1.12	0.96 ± 0.09	*Limanda limanda* (L.)	29	Tahir *et al.*, 1993
		0.71 ± 0.02	*Glyptocephalus cynoglossus*	5	Khan, 2003
		0.66 ± 0.02	*Limanda ferruginea*	10	Khan, 2003
		1.21 ± 0.06	*Pleuronectes americanus*	18	Khan, 2003
		1.41 ± 0.04	*Coregonus clupeaformis*	24	Cooley *et al.*, 2002
	0.81 - 1.11	0.94 ± 0.09*	*Clupea pallasi*	44	Elston *et al.*, 1997
		1.06 ± 0.02	*Ictalurus punctatus*	8	Rice *et al.*, 1995
Gonad-somatic index	0.45 - 8.48		*Limanda limanda* (female)	62	Hutchinson and Manning, 1996
	0.05 -1.09		*Limanda limanda* (male)	58	Hutchinson and Manning, 1996
		8.88 ± 0.43	*Pleuronectes americanus* (male)	10	Payne *et al.*, 1995
	1.4 - 25.4	15.2 ± 5.5*	*Clupea pallasi*	44	Elston *et al.*, 1997
Liver-somatic index	1.76 - 3.26		*Limanda limanda* (female)	62	Hutchinson and Manning, 1996
	1.72 - 3.65		*Limanda limanda* (male)	58	Hutchinson and Manning, 1996
		0.89 ± 0.04	*Perca fluviatilis* (female)	18	Sjöbeck *et al.*, 1984
		0.77 ± 0.03	*Pleuronectes americanus* (male)	10	Payne *et al.*, 1995
	0.78 - 4.00	1.69 ± 0.44	*Limanda limanda* (L.)	29	Tahir *et al.*, 1993
		1.16 ± 0.06	*Glyptocephalus cynoglossus*	5	Khan, 2003
		0.92 ± 0.04	*Limanda ferruginea*	10	Khan, 2003
		1.01 ± 0.05	*Pleuronectes americanus*	18	Khan, 2003
		0.79 ± 0.02	*Coregonus clupeaformis*	24	Cooley *et al.*, 2002
		1.14 ± 0.06	*Ictalurus punctatus*	8	Rice *et al.*, 1995
Spleen-somatic index		0.15 ± 0.02	*Pleuronectes americanus* (male)	10	Payne *et al.*, 1995
	0.04 - 0.21		*Ictalurus punctatus*	30	Martin and Black, 1998
Glucose (mmol/L)		2.78 ± 0.15	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		4.77 ± 0.16	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		3.89 ± 0.14	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		2.72 ± 0.12	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
		2.78 ± 0.11	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		2.08	*Etheostoma nigrum*	54	Silbergeld, 1974
		4.21 ± 0.14	*Clupea pallasi* (female)	117	Marty *et al.*, 1998
		5.00 ± 0.24	*Clupea pallasi* (male)	116	Marty *et al.*, 1998

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Glucose (mmol/L)	0.9 - 22.8	4.6 ± 2.2*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	0.17 - 9.10		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998
	0.94 - 22.8	4.16 ± 0.40	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		5.72 ± 0.34	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
	0.87-4.78	2.19 ± 1.22*	*Oncorhynchus mykiss*	23	Barham *et al.*, 1980
		5.6 ± 0.19	*Salmo salar* L.	20	Knoph and Thorud, 1996
		4.05 ± 0.7	*Oncorhynchus mykiss*	5	Milligan, 1996
	4.0 - 12.1	7.99 ± 2.26*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
	1.23 - 30.83	11.2 ± 2.3	*Oncorhynchus mykiss*	16	Barnhart, 1969
		6.88 ± 0.20*	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		6.6 ± 0.94*	*Cyprinus carpio*	6	Harikrishnan *et al.*, 2003
		2.24 ± 0.12	*Perca fluviatilis* (female)	16	Sjöbeck *et al.*, 1984
		5.54 ± 0.84	*Cyprinus carpio* (L.)	5	Price *et al.*, 1997
		3.17 ± 0.67*	*Rutilus rutilus* (L.)	6	Jeney *et al.*, 1996
Lactate (mmol/L)		2.25 ± 0.75	*Perca fluviatilis* (female)	10	Sjöbeck *et al.*, 1984
		4.8	*Platichthys flesus* (L.)	9	Larsson *et al.*, 1980
	0.3 - 2.5		*Salmo salar*	16	Olsen *et al.*, 1992
Total protein (g/dL)	4.16 - 5.66	4.2 ± 0.4*	*Salmo salar*	20	Sandnes *et al.*, 1988
		3.1 ± 0.09	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		3.0 ± 0.09	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		2.64 ± 0.18	*Limanda limanda* (at sea)	10	Hutchinson and Manning, 1996
		2.68 ± 0.13	*Limanda limanda* (on shore)	10	Hutchinson and Manning, 1996
		3.8 ± 0.07	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		3.8 ± 0.05	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		3.1 ± 0.09	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
		3.1 ± 0.10	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		2.14 ± 0.06	*Clupea pallasi* (female)	117	Marty *et al.*, 1998
		2.30 ± 0.05	*Clupea pallasi* (male)	116	Marty *et al.*, 1998
	0.2 - 3.8	2.2 ± 0.6*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	1.0 - 3.1		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Total protein (g/dL)	3.96-8.38		*Esox lucius* (L.)	32	Mulcahy 1970
		3.0 ± 0.2	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		2.7 ± 0.1	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
	0.38 - 5.51	4.32 ± 0.58*	*Oncorhynchus mykiss*	23	Barham *et al.*, 1980
	3.96 - 8.38	6.34	*Esox lucius* (L.)	32	Mulcahy, 1970
		2.79	*Oncorhynchus mykiss*	12	Pfeifer and Weber, 1979
	1.6 - 3.5	2.25 ± 0.5*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
	4.8 - 12.0	8.0 ± 0.48	*Oncorhynchus mykiss*	16	Barnhart, 1969
		2.0	*Carassius auratus* (L.) (female)	65	Summerfelt, 1967
		1.9	*Carassius auratus* (L.) (male)	75	Summerfelt, 1967
		3.42 ± 0.17	*Pleuronectes americanus* (male)	9	Payne *et al.*, 1995
		3.2 ± 0.52	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		3.34 ± 1.32	*Cyprinus carpio*	6	Harikrishnan *et al.*, 2003
		4.0 ± 0.85*	*Limanda limanda*	16	Secombes *et al.*, 1991
	1.57 - 5.99	3.26 ± 0.79	*Limanda limanda* (L.)	24	Tahir *et al.*, 1993
		2.96 ± 0.25	*Cyprinus carpio* (L.)	5	Price *et al.*, 1997
		2.72 ± 0.09	*Limanda limanda* (L.)	24	Secombes *et al.*, 1992
		3.49 ± 0.35*	*Rutilus rutilus* (L.)	7	Jeney *et al.*, 1996
		6.83 ± 0.41	*Oncorhynchus mykiss*	5	Tahir and Secombes, 1995
		6.8 ± 0.8*	*Lepomis* sp.	5	Lohner *et al.*, 2001
Albumin (g/dL)	1.83 -2.43	1.76 ± 0.2*	*Salmo salar*	20	Sandnes *et al.*, 1988
		1.2 ± 0.04	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		1.2 ± 0.03	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		1.5 ± 0.02	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		1.5 ± 0.03	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		1.3 ± 0.03	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
		1.3 ± 0.04	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		0.47 ± 0.02	*Clupea pallasi* (female)	117	Marty *et al.*, 1998
		0.56 ± 0.02	*Clupea pallasi* (male)	116	Marty *et al.*, 1998
	0.0 - 1.1	0.5 ± 0.2*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	0.1 - 0.8		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998
		1.3 ± 0.06	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Albumin (g/dL)		1.2 ± 0.05	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
	1.1 - 2.7	1.7 ± 0.4*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
		1.37	*Oncorhynchus mykiss*	12	Pfeifer and Weber, 1979
		1.1 ± 0.20	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
Globulin (g/dL)		1.9 ± 0.06	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		1.8 ± 0.07	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		2.3 ± 0.05	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		2.3 ± 0.04	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		1.8 ± 0.05	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
		1.8 ± 0.06	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		1.6 ± 0.09	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		1.5 ± 0.10	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
Sodium (mEq/L)		118 ± 1	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		109 ± 1	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		149 ± 1	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		146 ± 1	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		141 ± 1	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		141 ± 1	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
	141 -197	163 ± 12*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	139 -184		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998
	110 -147	129 ± 9*	*Oncorhynchus mykiss*	22	Barham *et al.*, 1980
		166 ± 1	*Clupea pallasi* (female)	117	Marty *et al.*, 1998
		166 ± 1	*Clupea pallasi* (male)	116	Marty *et al.*, 1998
Chloride (mEq/L)		127	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		137 ± 1	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
		131 ± 1	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		167 ± 1	*Pleuronectes americanus* (male)	10	Payne *et al.*, 1995
		151 ± 1	*Salmo salar* (L.)	20	Knoph and Thorud, 1996
		151 ± 1	*Pleuronectes flesus*	16	Alkindi *et al.*, 1996
		127 ± 2	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		95 ± 20*	*Rutilus rutilus* (L.)	7	Jeney *et al.*, 1996

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Calcium (mg/dL)		10.3 ± 0.41	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		10.3 ± 0.64	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		11.6 ± 0.21	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		10.1 ± 0.29	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		13.0 ± 0.74	*Oreochromis* spp. (plasma)	10	Hrubec and Smith 1999
		13.3 ± 0.73	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
	6.7 - 21	11.6 ± 1.9*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	7.9 - 14.8		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998
		11.3	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		9.23 ± 0.18	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
		9.92 ± 0.26	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		10.8 ± 0.15	*Salmo salar* (L.)	20	Knoph and Thorud, 1996
		0.7 ± 0.03	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		19.5 ± 1.36	*Cyprinus carpio*	6	Harikrishnan *et al.*, 2003
	6.7 - 10.6	9.05 ± 0.86*	*Oncorhynchus mykiss*	200	Wedemeyer and Nelson, 1975
		7.96 ± 0.40	*Perca fluviatilis* (female)	16	Sjöbeck *et al.*, 1984
		34.7 ± 6.4*	*Rutilus rutilus* (L.)	7	Jeney *et al.*, 1996
Phosphorus (mg/dL)		7.3 ± 0.42	*Ictalurus punctatus* (plasma)	10	Hrubec and Smith 1999
		9.9 ± 0.59	*Ictalurus punctatus* (serum)	10	Hrubec and Smith 1999
		9.2 ± 0.19	*Morone* spp. (plasma)	10	Hrubec and Smith 1999
		11.8 ± 0.35	*Morone* spp. (serum)	10	Hrubec and Smith 1999
		4.3 ± 0.18	*Oreochromis* spp., (plasma)	10	Hrubec and Smith 1999
		6.5 ± 0.44	*Oreochromis* spp. (serum)	10	Hrubec and Smith 1999
	5.5 - 38	12.7 ± 4.3*	*Clupea pallasi* (spawning)	233	Marty *et al.*, 1998
	3.7 - 21.5		*Clupea pallasi* (VHSV/ITP neg.)	140	Marty *et al.*, 1998
		10.5 ± 0.43	*Oncorhynchus mykiss* (plasma)	10	Hrubec and Smith 1999
		17.5 ± 1.09	*Oncorhynchus mykiss* (serum)	10	Hrubec and Smith 1999
		20.3 ± 6.8	*Perca fluviatilis* (female)	10	Sjöbeck *et al.*, 1984
	5.1 - 12.0	8.68 ± 1.5*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
		13.0 ± 1.3	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
	8.4 - 12.7	10.4 ± 1.10*	*Oncorhynchus mykiss*	200	Wedemeyer and Nelson, 1975

Table 2.3 Continued

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Alkaline phosphatase (U/L)	19 - 91	50 ± 23*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
	0.4 - 4.0	2.2 ± 0.9	*Oncorhynchus mykiss*	16	Barnhart, 1969
		172 ± 87.2	*Oncorhynchus mykiss*	10	Warner *et al.*, 1979
		59.3 ± 2.1	*Clupea pallasi* (female)	117	Marty *et al.*, 1998
		51.1 ± 1.6	*Clupea pallasi* (male)	116	Marty *et al.*, 1998
Albumin/ Globulin	0.54 - 0.92	0.73 ± 0.08*	*Oncorhynchus mykiss*	122	Miller *et al.*, 1983
		0.41	*Salmo salar*	140	Sandnes *et al.*, 1988
Cortisol (ng/ml)		22.4 ± 4.8	*Oncorhynchus mykiss*	5	Milligan, 1996
	0.80 - 11.1		*Salmo salar*	16	Olsen *et al.*, 1992
		<2.0	*Salmo trutta*	24	Pickering and Pottinger, 1987
		13.5 ± 3.1	*Platichthys stellatus*	5	Hogstrand *et al.*, 1999
		0.71 ± 0.10	*Cyprinus carpio* (L.)	100	Siwicki and Studnicka, 1987
		31.6 ± 1.93	*Limanda limanda* (at sea)	10	Hutchinson and Manning, 1996
		23.4 ± 1.21	*Limanda limanda* (on shore)	10	Hutchinson and Manning, 1996
Lysozyme (µg/ml)	3.45 - 19.7	10.9 ± 3.4	*Limanda limanda* (L.)	29	Tahir *et al.*, 1993
		2.0 ± 0.2	*Oncorhynchus mykiss*	30	Karrow *et al.*, 1999
		23.4 ± 2.3	*Oncorhynchus mykiss*	5	Tahir and Secombes, 1995
		4.8	*Clupea pallasi*		Fänge *et al.*, 1976
		3.5	*Clupea pallasi* (RBCs)		Fänge *et al.*, 1976
	3.13 - 6.55		*Oncorhynchus mykiss*	25	Demers and Bayne, 1997
	3.12 - 7.69		*Oncorhynchus mykiss* (handling)	25	Demers and Bayne, 1997
		10.8 ± 9.0*	*Limanda limanda*	16	Secombes *et al.*, 1991
Ig (mg/mL)		14.7 ± 8.9*	*Limanda limanda*	12	Secombes *et al.*, 1991
		14.0 ± 1.0	*Limanda limanda* (L.)	24	Secombes *et al.*, 1992
	13 - 532		*Clupea pallasi*	602	Davis *et al.*, 1999
	173 - 650	398	*Ictalurus punctatus* (10°C)	30	Klesius, 1990
	202 - 638	367	*Ictalurus punctatus* (30°C)	15	Klesius, 1990
Phagocytosis (%)		48.2 ± 1.8	*Limanda limanda* (L.)	24	Secombes *et al.*, 1992
Resp. burst nmol/10^6 cells or optical density (OD)	0.82 - 14.31	9.65 ± 2.70	*Limanda limanda* (L.)	25	Tahir *et al.*, 1993
		0.322 ± 0.026 (OD)	*Heteropneustes fossilis*	5	Fatima *et al.*, 2000

Table 2.4 Literature values from other juvenile fish species for the variables measured in the current study with juvenile Pacific herring. Values are ranges or means ± standard error (SE) (standard deviation (SD) where indicated with an asterisk. Sample size is shown where known. All values are for control fish only.

Variable	Range	Mean ± SE (*=SD)	Species and conditions	N	Reference
Hematocrit (%)	26-54	40	*Pagrus auratus*	300	Canfield *et al.*, 1994
		40.6 ± 4.4*	*Oncorhynchus mykiss*	84	Wlasow, 1984
		45.8 ± 1.73	*Salvelinus fontinalis*	12	Goede and Barton, 1990
		43.4 ± 2.2	*Oncorhynchus mykiss*	10	Farrell *et al.*, 1998
		25.4 ± 1.1	*Platichthys stellatus*	9	Farrell *et al.*, 1998
Leucocrit (%)	0.2-2.4	0.9	*Pagrus auratus*	300	Canfield *et al.*, 1994
		1.54 ± 0.34*	*Oncorhynchus kisutch* (18°C)	98	Wedemeyer *et al.*, 1983
		1.28 ± 0.27*	*Oncorhynchus kisutch* (10°C)	75	Wedemeyer *et al.*, 1983
		0.83 ± 0.21	*Salvelinus fontinalis*	12	Goede and Barton, 1990
		0.84 ± 0.08	*Oncorhynchus mykiss*	10	Farrell *et al.*, 1998
		0.99 ± 0.15	*Platichthys stellatus*	10	Farrell *et al.*, 1998
		0.28 ± 0.10	*Oncorhynchus kisutch* (8°C)	9	Tierney *et al.*, 2004
		0.57 ± 0.10	*Oncorhynchus kisutch* (18°C)	10	Tierney *et al.*, 2004
		0.48 ± 0.06	*Culaea inconstans*	20	Tierney *et al.*, 2004
		0.53 ± 0.10	*Pimephales promelas*	15	Tierney *et al.*, 2004
Condition factor		1.12 ± 0.03	*Salvelinus fontinalis*	20	Goede and Barton, 1990
Glucose (mmol/L)	4.82 - 10.35		*Oncorhynchus mykiss*, exercise	600	Nielsen *et al.*, 1994
	3.5 - 24.6		*Pagrus auratus*	300	Canfield *et al.*, 1994
		5.61 ± 0.21	*Salvelinus fontinalis*	12	Goede and Barton, 1990
		6.11 ± 0.39	*Oncorhynchus mykiss*	9	Farrell *et al.*, 1998
		3.28 ± 0.47	*Platichthys stellatus*	4	Farrell *et al.*, 1998
Lactate (mmol/L)	1.78 – 2.78		*Oncorhynchus mykiss*	600	Nielsen *et al.*, 1994
		1.31 ± 0.24	*Oncorhynchus mykiss*	10	Farrell *et al.*, 1998
		1.42 ± 1.47	*Platichthys stellatus*	10	Farrell *et al.*, 1998
Total protein (mg/dL)	3.6 - 6.9		*Pagrus auratus*	300	Canfield *et al.*, 1994
		3.16 ± 0.27	*Hippoglossus hippoglossus L.*	20	Langston *et al.*, 2002
		6.22 ± 0.57	*Salvelinus fontinalis*	12	Goede and Barton, 1990
Sodium (mEq/L)	187 - 221	204	*Pagrus auratus*	300	Canfield *et al.*, 1994
Chloride (mEq/L)	163 - 194	178	*Pagrus auratus*	300	Canfield *et al.*, 1994

Table 2.4 continued

Variable	Range	Mean ±SE (*=SD)	Species and conditions	N	Reference
Chloride (mEq/L)		111 ± 1.76	*Salvelinus fontinalis*	12	Goede and Barton, 1990
Calcium (mg/dL)	10 – 14.8	3.1	*Pagrus auratus*	300	Canfield *et al.*, 1994
Phosphorus (mg/dL)	7.1 – 13.0	3.2	*Pagrus auratus*	300	Canfield *et al.*, 1994
Cortisol (ng/ml)		12.1 ± 2.04	*Salvelinus fontinalis*	12	Goede and Barton, 1990

2.3.3. Calculations and statistics

All statistical tests were performed using JMP-IN® version 4.0 statistical software using $p<0.05$ as statistically significant (Sall and Lehman, 1996). The Shapiro-Wilk W test for normality of samples with $n<2000$ was used to evaluate the frequency distributions of all variables measured. The assumption of normality of the distribution was only met when sample size was low, i.e., < n=50. Lognormal transformations did not normalize the distributions consistently. Therefore, non-parametric techniques were used to describe distributions and to test for differences between treatment groups to be conservative when generating conclusions. Consequently, the median was generally used to describe the central tendency of data and the 95^{th} percentile estimation (PE) performed on rank ordered data was used to report reference ranges (Wedemeyer and Nelson, 1975; Miller *et al.,* 1983; Green and Knutzen, 2003). As such, distributions were described using the 2.5^{th} and 97.5^{th} percentiles (Reed *et al.,* 1971) to determine the minimum and maximum of the range. Modes were compared when distributions were bimodal.

The Wilcoxon-Kruskal/Wallis test was used, in conjunction with Dunnett's test when necessary, to compare the variables between groups for all continuous data. The Likelihood Ratio test was used in conjunction with contingency tables to make categorical comparisons between treatment groups (Zar, 1984; Sall and Lehman, 1996). Testing for multivariate correlation between variables was performed with the Spearman Rho correlation, a non-parametric association test. This was also used to evaluate time effects in experiments with only two sampling points and to evaluate the effects of differences in holding time (T_H) and duration within an experiment (T_E) (Sall and Lehman, 1996).

It was not possible to perform repeated measures analysis on each fish as each fish was terminally sampled. Therefore, each set of fish sampled represented separate points during the exposure. Limitations on the number of tanks available for experiments meant that most experiments only had duplicates of each treatment. Analysis of the control fish (used to determine reference ranges) for tank effect showed no significant between tank variability ($p<0.05$, Kruskal-Wallis) for juveniles and adults (Tables A2.10 and A2.11). Consequently, most analyses treated the individual fish as the unit of treatment not the tank. However, the effect of time in the time series experiments was

evaluated for control and exposed fish separately, using the tank as the experimental unit rather than the individual (due to the larger number of replicates). In this case, the average of tank medians were tested for time effects using the standard least squares platform of the general linear model (GLM) (Sall and Lehman, 1996). In addition, the effects of holding time (T_H, from capture to initiation of an experiment) and experimental time (T_E) on measurement variables were assessed for control fish using multivariate correlation and the Spearman Rho association at $p<0.05$ as significant with time as a continuous variable. The effects of T_H and T_E on nominal variables were assessed using Nominal Logistic regression and the Effect Wald test at the 95% confidence limit, which determines the significance of the contribution of specific effects (i.e., T_H) to the whole model (Sall and Lehman, 1996).

All comparisons among age classes, gender, and populations were made using the Wilcoxon (ranked sums)-Kruskal/Wallis test at the 95% confidence level due to the non-normal distribution of data. Correlation amongst variables was evaluated with Spearman's non-parametric measures of association test at the 95% confidence level. Bimodal distributions were described using modes rather than the mean or median.

The crude mortality rate (CMR) was used to compare the pre-experimental mortality between experiments as it took into consideration the decrease in the size of the group with time due to mortalities associated with holding, as well as sampling. CMR provides a standardized rate that can allow for comparison across differing time periods such that each group has the same risk period (Jenicek, 1995).

CMR = ((Number of dead fish) / (N at risk))/ unit time

Where: N at risk = $\frac{(\text{N at start of period} + \text{N at end of period})}{2}$

i.e. If 1/100 fish died, the population at risk would be 99.5 = (100 + 99)/2

2.3.4. Mortality

The mortality of herring in captivity prior to experimentation was assessed to determine if there were differences between age-classes and populations (Appendix 2, Table 1). The CMR for each group of herring held at BMSC was calculated for several periods. These included mortality during the initial holding period (from capture to assignment to

experimental tank), mortality during the acclimation period (from experimental tank assignment to start of experiment) and overall pre-experimental mortality (holding plus acclimation). Some groups of herring were ultimately not used in experiments due to unacceptable, ongoing mortality during the acclimation period, i.e., ≥50%. The cumulative experimental mortality rate included only fish that died during the experimental period. Analysis of variance (ANOVA) was used to compare mortality rate between groups of herring.

2.3.5. Age and size effects

The effect of age was evaluated by comparing the median value for juvenile control herring (n=208) with that for adult herring (n=374). The variables common to both age classes are shown in Table 2.5. Juvenile fish were further subdivided into those <11 months old (n=158) and those 11+ months old (n= 50) due to distinct size differences. This age classification assumed that all 0+year herring were born at approximately the same time, (late February to early March), based on knowledge of when adults spawned during the study years. Neither otoliths nor annuli were used to definitively age the herring. The effect of size (mass and length) on variables was also evaluated within each age class.

Table 2.5. Variables common to juvenile and adult herring.

Category	Variables
Size and condition	Body mass, fork length, CF, LSI
Hematology	Hct, Lct, % lymphocytes, % neutrophils, % thrombocytes
Biochemistry	Glucose, lactate, total protein, albumin, globulin, A/G
Immunology	Macrophage cell count, plasma lysozyme, yeast phagocytosed per macrophage

2.3.6. Gender effects

Gender was evaluated as a potential influencing factor in the determination of reference ranges for adult herring. This was done by comparing the median value of the variables measured among females (n=119) and males (n=112). Gender was determined by visualization of gonads post-mortem for a subset (231/374) of the adults. Gender was unknown in the remaining 143 adults because no post mortem was done. Interaction between gender and source population was evaluated for all variables that differed with population.

2.3.7. Population and region effects

The purpose of this analysis was to determine if measured hematological, biochemical and immunological variables were different between the source populations for either juvenile or adult herring. An additional objective of this thesis was to assess if results obtained from the exposure experiments using Pacific Northwest (PNW) herring could be applied directly to the Prince William Sound (PWS) herring as a potential explanation for the population declines seen in the years following the *Exxon Valdez* Oil Spill (*EV*OS). Therefore, it was necessary to know if the source region affected measurement variables. Juvenile herring obtained from BS (n=123) and PS (n=85) were compared. Adults obtained from three sources were compared: BS (n=139), PS (n=160), and QI (n=75). Within each age class, the median values for all variables common to all source populations were compared. In addition, the plasma biochemistry of control adults (n=117) used in this study (collectively identified as PNW) was compared with adults from PWS (n=299). Condition indices and hematological values of adults (n=374) were compared with adult herring held at the Vancouver Aquarium (VA) (n=32) as an additional captive, reference population.

The PWS data set that was used for comparison to the PNW herring in the current study was collected in the spring of 2001 (n=300) by G. Marty (U.C. Davis; Kennedy, pers. comm. 2003). PWS herring were sampled within 4 hours of capture by caudal vein puncture and the plasma was frozen until analysis (Marty *et al.*, 1998). Although differences existed in capture methods between the PNW and PWS herring, and stress due to transportation and captivity was not an issue for the PWS fish, the samples were analyzed in the same manner using kits for the biochemical measurements (Kennedy, pers. comm. 2003). Hematological and condition indices were obtained from adult Pacific herring at the VA to compare with the herring held captive at BMSC. Adult herring obtained from QI were from the same body of water as those obtained by the VA. Therefore, a comparison between this subset and the VA herring was also conducted.

2.3.8. Gross pathology (lesions) effect

An evaluation of the influence of lesion presence on measurement variables was performed by comparing herring with lesions to those without within each age class using 68.0% (396/582) of the total herring. This group corresponded to 51.9% of juveniles (108/208) and 77.0% of adults (288/374). In this study, a lesion was defined as

any grossly detectable alteration of the external or internal anatomy. This was subjective and was based on the experience of the author. In the absence of histopathology, it was not possible to comment on the severity of gross lesions. The remaining 32.0% did not have a post mortem and were not included in the lesions effect analysis as their internal lesions status was unknown. Lesions were first categorized based on their location (external, internal or both), then upon the organ system(s) involved and then sub-categorized with regard to the characteristic of the lesion (Table 2.6). Herring with lesions were categorized into one of three lesion sub-groups (external only, internal only, external plus internal) for comparison with herring without lesions to determine if lesions affected variables. Further classification of the lesion types beyond the level of internal *vs.* external was used to determine if a specific lesion type was associated with significant changes in the variables. This led to the following sub-categories: skin, musculoskeletal, gills, eye, liver, kidney, heart, gallbladder, spleen, peritoneal cavity, liver in combination with any other organ system. Lesions were further divided with respect to other features (Table 2.6).

Table 2.6. Classification of gross pathology observed in Pacific herring based on external, internal or external plus internal occurrence and subdivided based on organ system(s) involved.

EXTERNAL	INTERNAL	EXTERNAL & INTERNAL
Skin	**Liver**	**Skin and liver**
Hemorrhagic	Focal white nodules	Pinpoint breaks among scales and liver green
Pinpoint breaks among scales	Green	Pinpoint breaks among scales and focal white nodules in the liver
Erosions/ulcers	Tan	Hemorrhagic skin, liver green
	Hyperemic	Hemorrhagic skin, focal white nodules in the liver
	Pale	
Musculoskeletal	**Kidneys**	**Gills and liver**
Scoliosis	Pale	Pale gills and green liver
Gills	**Heart**	
Pale	Focal white nodules	
Nodule	**Gall bladder**	
Erosions	Enlarged	
Eye	**Peritoneal cavity**	
Corneal lesions	Ascites	
Missing	**Spleen**	
Petechiae	Mass	

2.3.9. Covariance with anemia

An investigation into the potential confounding effects of low Hct on the other variables was undertaken because anemia (low Hct) in other fish species is known to have detrimental effects (Wood *et al.*, 1979; Alkindi *et al.*, 1996; Gallaugher and Farrell, 1998). To do this, herring with a Hct of <13% were compared to those ≥13%, within each age class. The conservative value of 13% was used as a threshold to identify potentially anemic herring. This was based on the literature values of Hct for other teleost species (Table 2.3 and 2.4, Gallaugher and Farrell, 1998) and the presence of a bimodal distribution of Hct in juveniles that had a distinct lower mode with a peak of 12.8%. For completeness, adults were similarly assessed despite having a unimodal distribution centred at 35.5%. An evaluation for covariance of variables with Hct was performed using the non-parametric Spearman Rho measures of association test (Zar, 1984; Daniel, 1995).

2.3.10. Captivity effects (holding time (T_H), experimental sampling time (T_E))

Captivity is known to impact several of the measurement variables used in this study in other fish (Torres *et al.*, 1986). Therefore, prior to pooling all control values to develop reference range profiles, it was necessary to determine if measurement variables in controls were affected by differences in T_H (from capture to initiation of experiments) or T_E. Comparisons were made between a subset of time-zero (T_0) only controls (fish sampled just prior to the start of an experiment) and the entire pool of controls (T_P) (excluding the time-zero fish) within each age class to evaluate any differences due to experimental time. T_H differences were evaluated for both the T_0 subset and the T_P subsets using Spearman's non-parametric measures of association test to determine the proportion of variation in each variable attributable to a difference in T_H. Interaction between T_H and T_E was also examined using the GLM with T_E nested within T_H.

2.3.11. Reference range profiles

The previous analyses identified which measurement variables could be pooled among all herring that had been either experimental controls or that had been acclimated to holding tanks but not used in experiments and which variables were affected by demographic factors or time and could not be pooled. Reference ranges for each variable were established by sub-dividing for the factors found to be influencing their distribution (age, size, gender, time) in herring. These are sets of ranges for hematological, biochemical and immunological variables defined by the 95th PE values. In addition, inter-quartile reference ranges (IQRs) were produced, using the 25th, and

75th percentiles of the distribution for each variable within each age class (Harms *et al.*, 2002). These provide an alternate set of less conservative ranges and permits comparison with other species that use the IQR to describe distributions (Appendix 2).

Statistically significant responses of control and exposed/challenged fish in experiments over time were evaluated against only the T_0 reference profile (subdivided for T_H when appropriate) to assess captivity effects and differences in captivity effect between control fish and treated fish. The response of exposed/challenged fish in experiments to treatment were evaluated using the T_P reference profile for variables that did not change with T_E (subdivided for T_H where appropriate). However, variables for which differences in T_E account for a significant proportion of the variation were assessed only against the time-matched control ranges within the specific experiment.

2.4. RESULTS

2.4.1. Mortality

Standardized mortality rates for the holding period, acclimation period, and total pre-experimental periods between juveniles and adults were not significantly different ($p>0.05$, ANOVA) even though mortality rate varied by up to a 10-fold difference within age and population groupings, and ranged from 0.1 to 5.7%/day across all comparisons. In juvenile, 0+-year herring, the holding period and acclimation period mortality rate ranges were 0.5 to 4.8%/day and 1.0 to 5.7%/day, respectively. Overall, the combined pre-experimental mortality rate in juveniles (holding and acclimation periods) ranged from 0.5 to 4.3%/day (Table 2.6). The acclimation and total pre-experimental mortality rate ranges were narrower in adults and were 0.4 to 2.2 and 0.3 to 1.8%/day, respectively. The holding period mortality rate range in adults (0.1 to 4.9%/day) was not statistically significantly higher (Table 2.7). The mortality rates for the first group of BS 0+year fish were high and these fish were not subsequently used in experiments or reference range determination. The same was true of the holding mortality rate for the first group of QI adults. However, mortality decreased substantially during the acclimation period, and thus this group of fish was used for experiments.

2.4.2. Age and size effects

Age was a significant factor for CF, LSI, lymphocytes (%), neutrophils (%), Lct (%), Hct (%), plasma glucose, total protein, globulin and the albumin/globulin ratio (A/G), macrophage cell count, phagocytosis, and lysozyme but not for plasma lactate, albumin, or thrombocytes (Table 2.8). Frequency distributions were typically non-normal and

uni-modal (Figures 2.2 to 2.5). However, lymphocytes and Hct in juveniles, and thrombocytes in adults had bimodal distribution. The juvenile lymphocyte modes (25% and 55%) fell on either side of the main mode for adults (35%). The median Hct for juveniles was significantly lower than adults and there were two distinct modes at 12.8% and 35.0%. The second juvenile mode was similar to the median of the unimodal adult distribution (39.1%).

Table 2.7. Mortality rates of herring from each source population during the holding period, acclimation period, and overall pre-experimental period standardized for a population of 100 fish (#deaths/day/100 fish or %/day). (*These herring were originally captured for use by a film crew and mortality was not recorded until the author arrived at BMSC to acclimate the fish).

Population	Holding Mortality rate	Acclimation Mortality rate	Total pre-experimental mortality rate
Barkley Sound, 0+yr, 1996	1.9	n/a	1.9
	1.6	1.0	1.5
Barkley Sound, 0+yr, 1998	4.8	5.7	4.3
	1.4	1.1	1.2
	0.5	n/a	0.5
JUVENILE MEAN	**2.04**	**2.6**	**1.88**
Barkley Sound, Adults, 1996	Unknown*	2.2	0.3
Barkley Sound, Adults, 1997	0.1	1.1	0.5
Quadra Island, Adults, 1997-1	4.9	0.9	1.8
Quadra Island, Adults, 1997-2	1.1	0.4	1.1
Barkley Sound, Adults, 1998	1.4	1.0	1.1
ADULT MEAN	**1.88**	**1.12**	**0.96**
OVERALL MEAN	**1.97**	**1.68**	**1.42**

A substantial proportion of all fish had an undetectable Lct reading, which was counted as zero. The proportion of fish with Lct equal to zero was statistically greater in juveniles compared with adults, and effectively lowered the median Lct value for juveniles. The occurrence of zero Lct ranged from 22 to 29%, depending on the age sub-category and whether or not fish with lesions were included (29% (19/65) < 11 months of age and 25% (3/12) of 11 + months of age with no visible lesions; 22% (34/155) of < 11 months old and 28% (14/50) of the 11 + month group when including fish with lesions). Lct was undetectable in 8% (17/188) of adult herring with no visible lesions and was undetectable in 12% (43/374) of adults when fish with lesions were included.

Comparisons of plasma biochemistry were limited to glucose, lactate, total protein, albumin, estimated globulin, and A/G. Median glucose was three times higher in adults. Total protein, and consequently the estimated globulin (total protein minus albumin),

were four times higher in adults. The A/G ratio was lower by the same degree in adults. The concentration of lysozyme was lower in adult herring. Lactate and albumin levels did not vary with age. The prevalence of lesions was not significantly different between 0+year (n=108) and adult (n=288) herring.

Table 2.8. Comparison of variables among age class using the Wilcoxon/Kruskal-Wallis test at the 95% confidence level. Values are medians. Modes are shown in parentheses below the median. CF=condition factor, LSI=liver-somatic index, Hct=hematocrit, Lct=leucocrit, mø=macrophage • bimodal distribution.

Measure	Juvenile	N	Adult	N	p-value	Relative change
CF	0.79	195	0.84	373	<0.0001	+ 6.3%
LSI	1.13	133	0.89	179	<0.0001	- 21.2%
Hct (%) (modes)	27.8* (12.5/35.0)	205	39.1	367	<0.0001	+ 40.6%
Lct (%)	0.85	205	1.00	367	0.0207	+ 17.6%
Undetectable Lct	23%	48/205	12%	43/467	0.0003	- 47.8%
Lymphocytes (%) (modes)	54.0* (25.0/55.0)	60	34.2	87	<0.0001	- 36.7%
Monocytes (%)	0.00	60	0.00	60	0.0222	+ 0
Neutrophils (%)	6.0	60	24.2	87	<0.0001	+ 303.3%
Thrombocytes (%) (modes)	31.5	60	38.6* (15/ 45)	87	0.6804	+ 22.5%
Macrophage (mø) cell counts	6.63×10^6	108	1.53×10^7	37	<0.0001	+ 130.8%
Glucose (mmol/L)	3.33	72	9.22	117	<0.0001	+ 176.9%
Lactate (mmol/L)	4.31	61	3.16	99	0.1424	- 26.7%
Albumin (g/dL)	0.85	11	0.72	55	0.3618	- 15.3%
Total protein (g/dL)	1.49	53	4.65	55	<0.0001	+ 212.1%
Globulin (g/dL)	0.72	9	3.85	55	<0.0001	+ 434.7%
Albumin/globulin	1.13	9	0.20	55	0.0004	- 82.3%
Yeast per mø	6.00	40	2.69	163	<0.0001	- 55.2%
Lysozyme (µg/ml)	69.86	(165)	1.16	(307)	<0.0001	- 98.3%
Lesions	27%	29/108	29%	83/288	0.6977	+ 7.4%

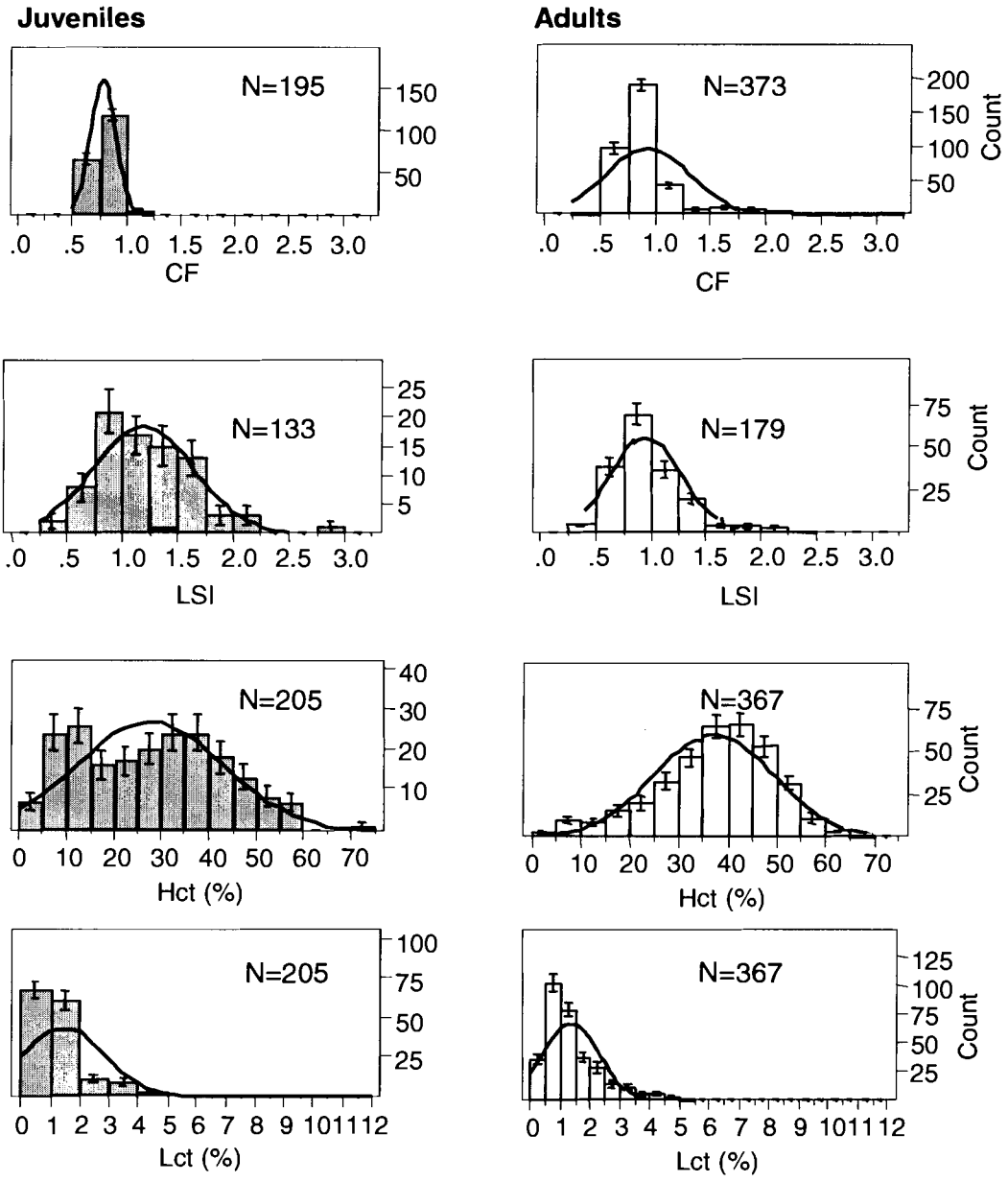


Figure 2.2. The distribution of CF, LSI, Hct, and Lct for juvenile and adult herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

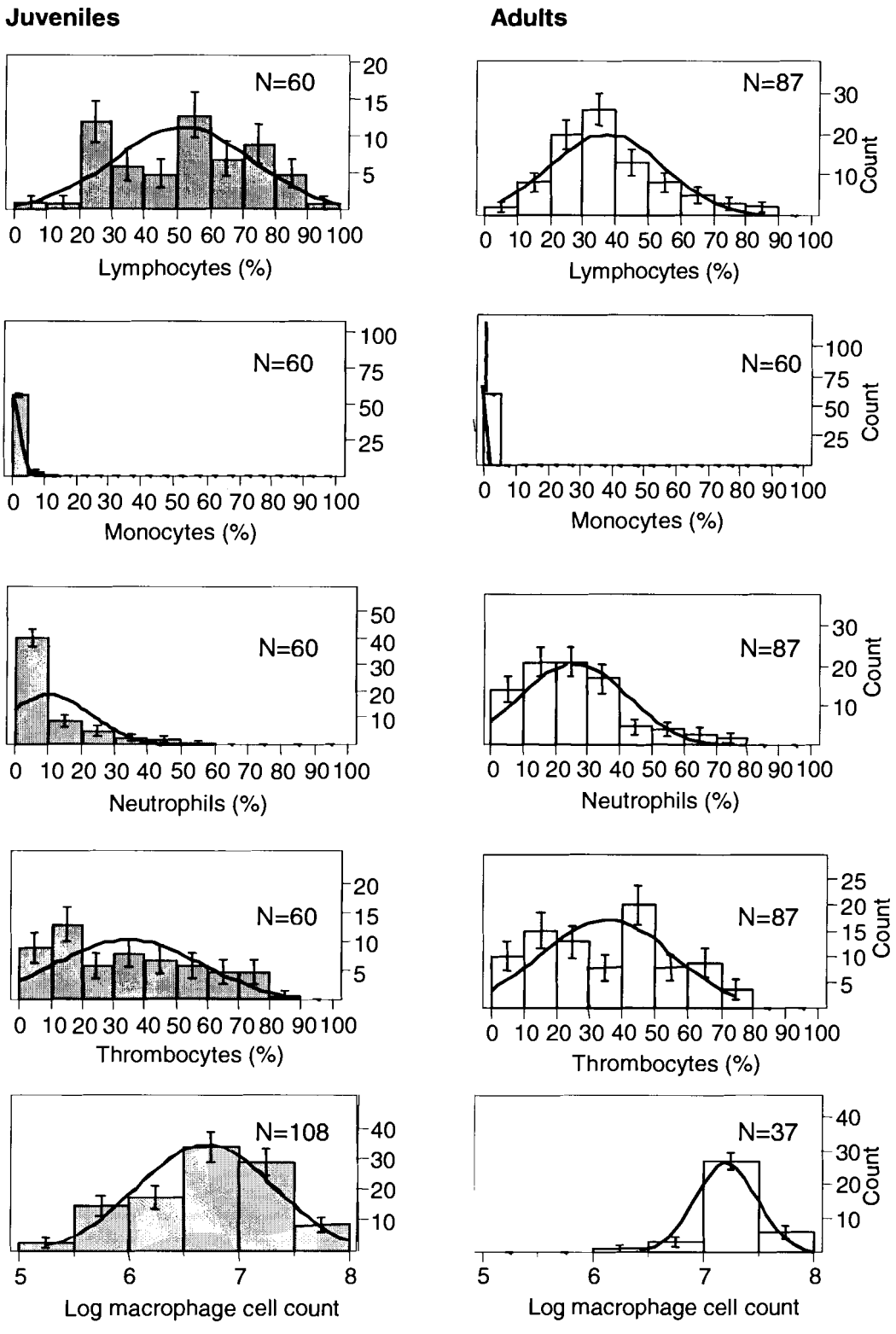


Figure 2.3. The distribution of the percentage of lymphocytes, monocytes, neutrophils, thrombocytes, and the log macrophage cell count for juvenile and adult Pacific herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

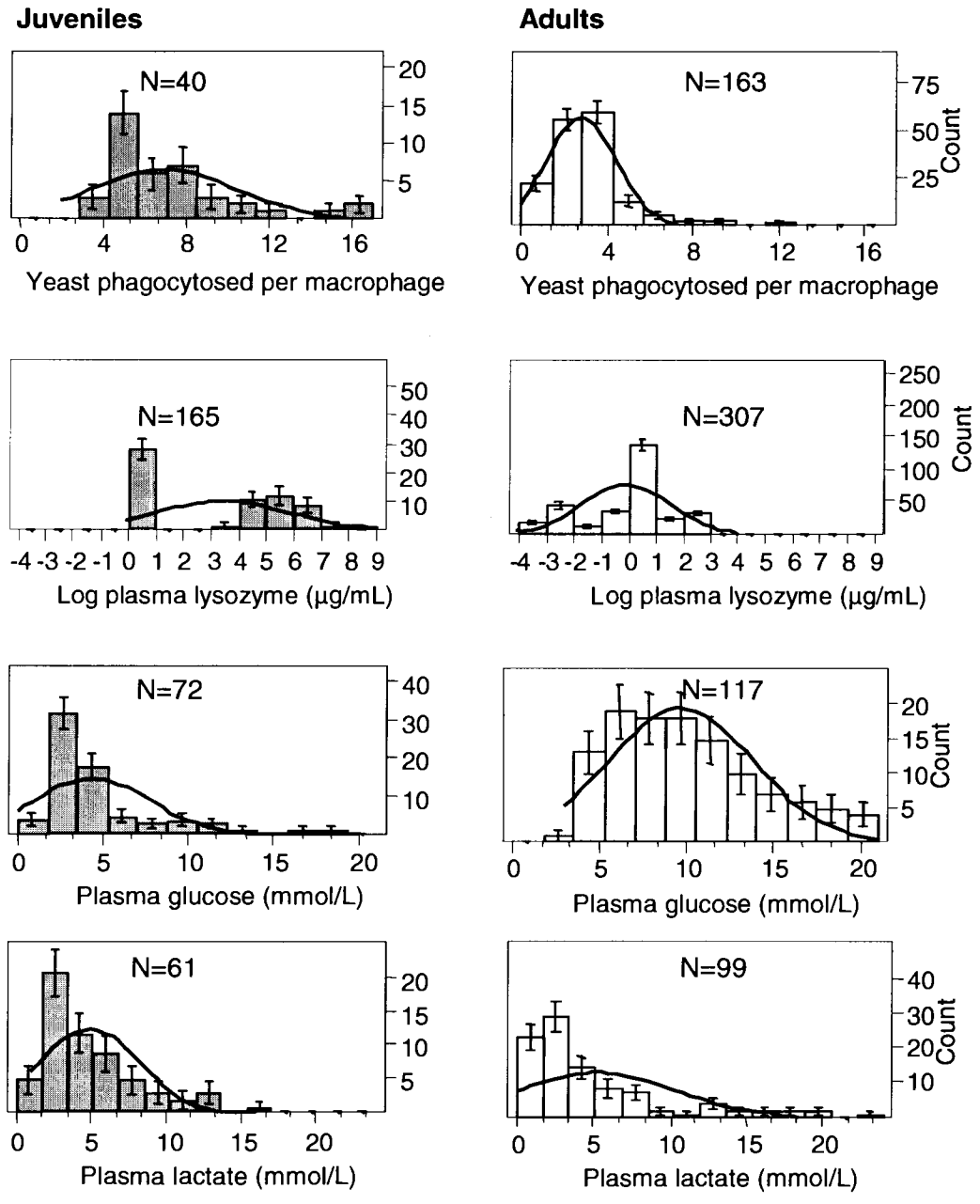


Figure 2.4. The distribution of yeast phagocytosed per macrophage, plasma lysozyme, glucose, and lactate concentration among juvenile and adult Pacific herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

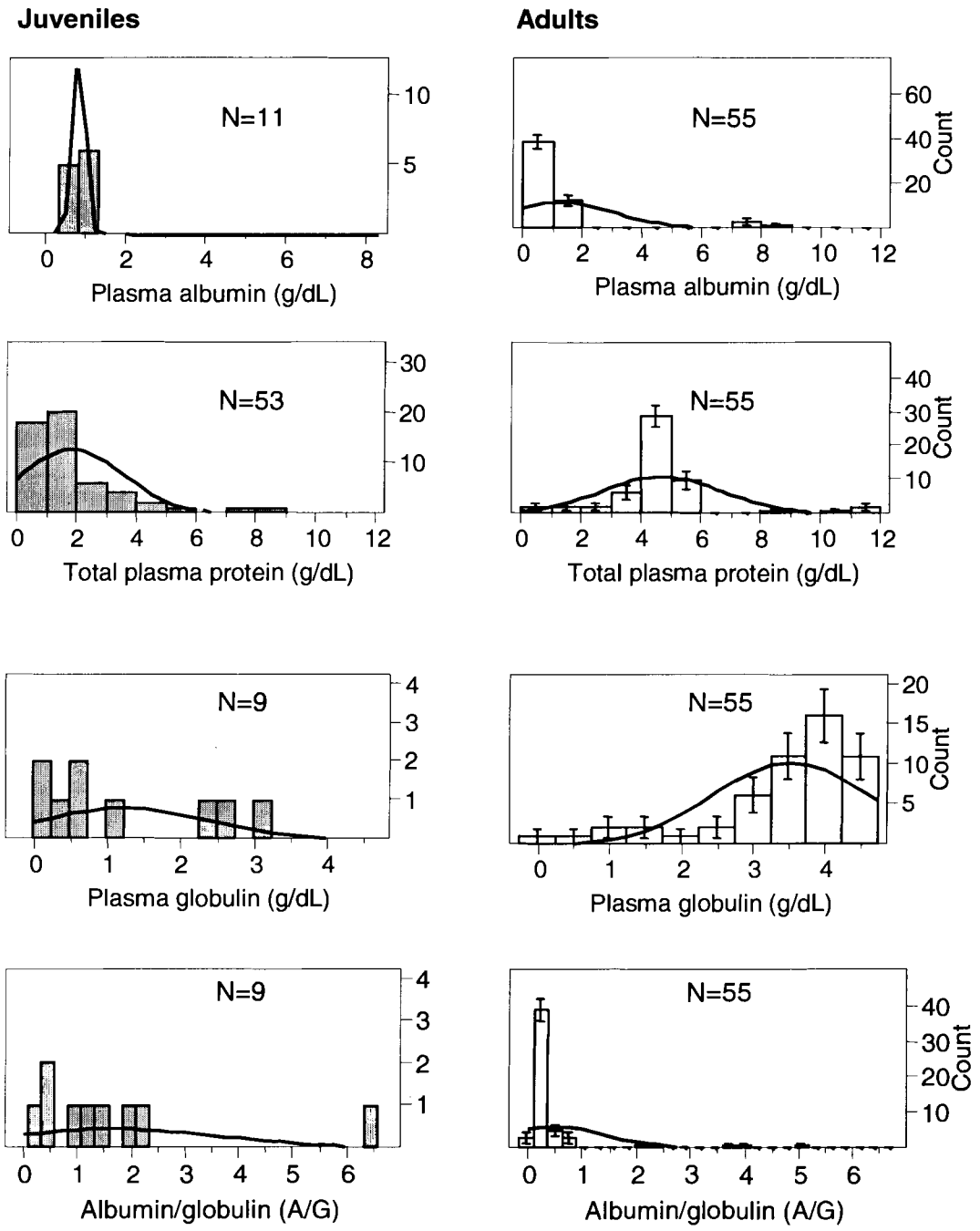


Figure 2.5. The distribution of plasma albumin, total protein, and globulin concentration and the albumin/globulin ratio (A/G) for juvenile and adult herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars arthe corresponding SE for each level.

It was hypothesized that the differences in variables between the two age classes may be a function of size and that this may be acting as a factor within each age class. Indeed, CF, Hct, and lysozyme were all positively correlated with both body mass and length in 0+year herring (Table 2.9). Conversely, the proportion of lymphocytes was negatively correlated with length. In adult herring, CF, LSI, GSI, Hct, and lysozyme were all positively correlated with both body mass and length while the percentage of lymphocytes, neutrophils, and thrombocytes were positively correlated with only length. Also in adult herring, index SSI was negatively correlated with body mass, which is interesting since Hct was positively correlated with body mass. Plasma glucose and lactate were negatively correlated with body mass and length in adult herring (Table 2.10).

Table 2.9. Variables that were correlated with either body mass or fork length in juvenile herring using Spearman's non-parametric correlation at the 95% confidence level. The Spearman Rho value (S-R) is the correlation coefficient.

		Body Mass		Fork length	
Variable	N	p-value	S-R value	p-value	S-R value
CF	195	<0.0001	0.5028	0.0068	0.1933
Hct (%)	192	<0.0001	0.3158	<0.0001	0.3139
Lymphocytes (%)	60	0.4386	-0.1019	0.0334	-0.2751
Lysozyme (µg/mL)	65	<0.0001	06111	<0.0001	0.6541

Table 2.10. Variables that were correlated with either body mass or fork length in adult herring using Spearman's non-parametric correlation at the 95% confidence level. The Spearman Rho value (S-R) is the correlation coefficient.

		Body Mass		Fork length	
Variable	N	p-value	S-R value	p-value	S-R value
CF	373	0.0011	0.1687	<0.0001	-0.3056
LSI	179	0.0005	0.2576	0.0008	0.2495
SSI	152	0.0223	-0.1853	0.0730	-0.1459
GSI	140	<0.0001	0.4918	<0.0001	0.4172
Hct (%)	366	<0.0001	0.2116	0.0003	0.1903
Lymphocytes (%)	87	0.4097	0.0895	0.0038	0.3074
Neutrophils (%)	87	0.1528	0.1546	0.0204	0.2482
Thrombocytes (%)	87	0.1260	-0.1653	<0.0001	-0.4192
Lysozyme (µg/mL)	307	<0.0001	0.5895	<0.0001	0.6145
Glucose (mmol/L)	117	<0.0001	-0.4425	<0.0001	-0.4343
Lactate (mmol/L)	98	<0.0352	-0.2130	0.0228	-0.2298

There was further support for the division of herring based on age/size within the 0+year age class. The bimodal distribution of Hct in 0+year herring appeared to be partially explained by size/age differences within the 0+year age class. A comparison between

<11 and >11 month-old juvenile herring showed that Hct became unimodal by 11 months of age with a right shift in the skew of the distribution curve (Figure 2.6). Hct was significantly greater in the older 0+year herring group (median value 42.0%) compared with the younger fish (median value 24.5%). The bimodality of Hct remained within the <11 month subset and did not disappear with further subdivision by month, lesion status or any variable. Comparison of other variables showed that LSI, Lct, and plasma glucose were all significantly lower in the older juveniles while lactate and protein were higher (Table 2.11). In addition, the prevalence of lesions and of low Hct (<13%) was higher in the <11 month old juveniles. The endpoints shown to vary within the juvenile age class are subdivided within the juvenile reference range profile.

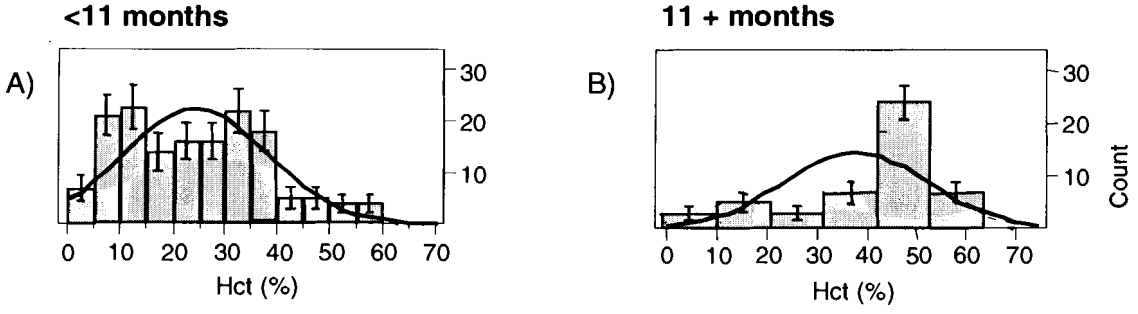


Figure 2.6. The distribution of Hct for A) <11 and B) 11 + month subsets of 0+year herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

Table 2.11. Measures that varied significantly among juvenile herring of different age and size using the Wilcoxon-Kruskal/Wallis test or Likelihood Ratio test (where indicated*) at the 95% confidence level. Values are medians with sample size in parentheses. Hct=hematocrit, Lct=leucocrit.

Variable	<11 months	11 + months	p-value	Relative change with age
Body mass (g)	4.1 (158)	5.7 (37)	<0.0001	+ 39.0%
Fork length (cm)	8.1 (158)	9.0 (37)	<0.0001	+ 11.1%
Liver-somatic index	1.21 (72)	0.79 (11)	<0.0001	- 34.7%
Hct (%) (modes)	24.5 (155) (12.8 & 32.5)	42.0 (50)	<0.0001	+ 71.4%
Lct (%)	0.93 (155)	0.54 (50)	0.0479	- 41.9%
Glucose (mmol/L)	3.59 (60)	2.32 (12)	0.0011	- 35.4%
Lactate (mmol/L)	3.31 (51)	10.88 (10)	<0.0001	+ 228.7%
Protein (g/dL)	1.41 (44)	2.00 (9)	0.0492	+ 41.8%
Lesion prevalence	30% (29/96)	0% (0/12)	<0.0001*	- 30 fold
Hct <13%	25.8% (401155)	10%(5/50)	0.0124 *	- 61.2%

2.4.3. Gender effect

There were few differences in measured variables between males and females. Female herring had lower median Hct and Lct values compared to their male counterparts, and higher median cortisol, SSI, and prevalence of *I. hoferi* infection (Table 2.12, Figure 2.7). These differences were not large relative to the ranges observed (< 30% difference), with the exception of *I. hoferi* infection and lesion prevalence. The difference in SSI, Hct, Lct, and cortisol suggests separation for males *vs.* females in the adult reference ranges for these variables if gender ratio is not equal.

Table 2.12. The median value of variables in male and female adult herring. Gender was compared using the Wilcoxon-Kruskal/Wallis test, unless indicated otherwise, at the 95% confidence level. Values are medians with sample size in parentheses. LR=Likelihood Ratio test, mø=macrophage, an asterisk (*) means values are from BS fish only.

Variable	Female (N)	Male (N)	p-value	Statistical outcome & percent difference
Body mass (g)	53.9 (119)	57.9 (112)	0.92	No difference
Fork length (cm)	19.4 (119)	19.5 (112)	0.50	No difference
CF	0.78 (119)	0.79 (112)	0.41	No difference
LSI	0.91 (95)	0.85 (80)	0.09	No difference
SSI	0.14 (77)	0.10 (71)	0.02	Female 28.6% ↓
GSI	0.61 (72)	0.64 (64)	0.67	No difference
Lymphocytes (%)	42.5 (26)	38.7 (17)	0.88	No difference
Monocytes (%)	0.35 (8)	0.00 (8)	0.37	No difference
Neutrophils (%)	29.5 (26)	28.6 (17)	0.36	No difference
Thrombocytes (%)	16.9 (26)	24.5 (17)	0.08	No difference
Hct (%)	37.4 (118)	41.8 (109)	0.01	Female 11.8% ↓
Lct (%)	1.00 (110)	1.26 (100)	0.01	Female 26.0% ↓
Cell count	1.61×10^7 (17)	1.31×10^7 (16)	0.77	No difference
Yeast per mø	2.54 (83)	2.82 (71)	0.30	No difference
Respiratory burst	0.19 (14)	0.25 (13)	0.33	No difference
Cell viability (%)	77.3 (14)	33.3 (9)	0.45	No difference
Glucose (mmol/L)	8.83 (60)	8.98 (53)	0.87	No difference
Lactate (mmol/L)	3.28 (47)	2.50 (48)	0.36	No difference
Albumin (g/dL) *	0.67 (31)	0.77 (32)	0.96	No difference
Protein (g/dL) *	4.62 (32)	4.69 (34)	0.99	No difference
Globulin (g/dL) *	3.56 (31)	3.87 (31)	0.33	No difference
A/G*	0.20 (31)	0.19 (31)	0.86	No difference
Cortisol (ng/ml) *	14.8 (28)	11.2 (31)	0.03	Female 32.1% ↑
Chloride (mEq/L)*	119 (24)	119 (24)	0.73	No difference
Phosphorus (mg/dL)*	2.79 (23)	2.33 (27)	0.17	No difference
Calcium (mmol/L)*	18.3 (23)	18.8 (27)	0.90	No difference
Lesion prevalence	37% (44/119)	23% (26/112)	0.02 LR	Females 37.8% ↑
I. hoferi positive*	60% (6/10)	20% (4/20)	0.03 LR	Females 66.7% ↑

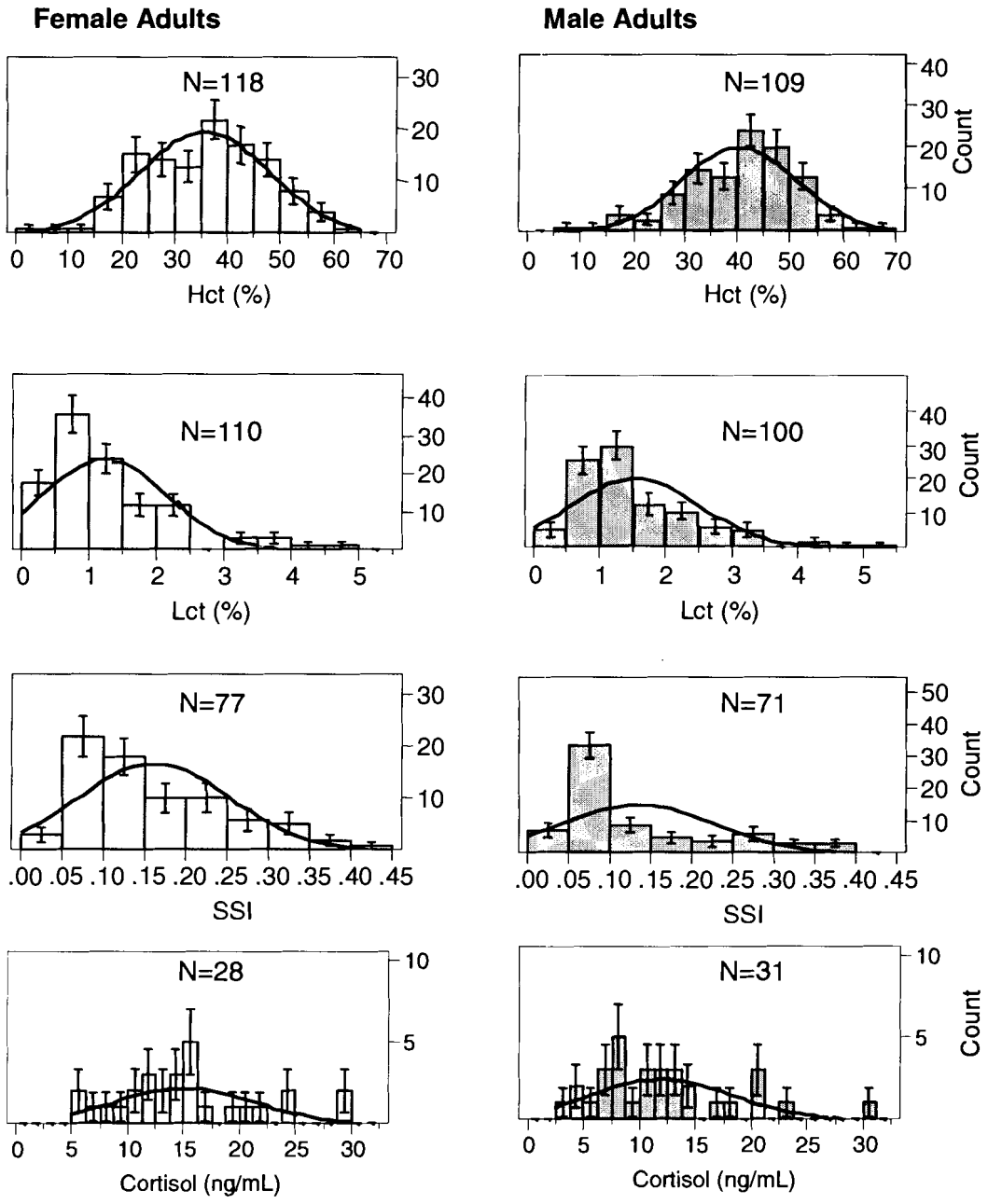


Figure 2.7. The distribution of variables that differed significantly among gender in adult herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

2.4.4. Population and regional effects

The effect of population source on mortality was only examined for the BS and QI adult populations, as mortality records were not available for the PS populations. Standardized daily holding mortality rates ranged from 0.1 to 1.1%/day for BS herring and from 1.4 to 4.9%/day for QI herring. Acclimation period mortality rates ranged from 0.4 to 2.2 and from 0.9 to 1.0%/day for BS and QI herring, respectively. The combined (holding plus acclimation) standardized preexperimental daily mortality ranged from 0.3 to 1.1 and from 1.1 to 1.8%/day for BS and QI herring, respectively. There was no significant difference in the daily mortality rate during the holding, acclimation, or total pre-experimental periods between BS and QI adults, ($p>0.05$ ANOVA).

Several variables differed significantly between the BS and PS populations of 0+year, juvenile herring (Table 2.13). These were Hct, presence of detectable Lct, percentage of lymphocytes and thrombocytes, and plasma lysozyme activity. Age differences, as shown above, accounted for some of the differences seen in Hct, lymphocytes and plasma lysozyme, since PS herring were significantly larger. However, the proportion of thrombocytes was also different between these two populations, which was not correlated with size. Lesion prevalence was significantly greater in the PS population. Figures 2.8 and 2.9 illustrate the distribution of variables in each population of juveniles.

The differences previously identified between juvenile herring that were <11 months old and those 11+ months of age (Table 2.9) supported a comparison between populations within each of these subcategories. BS juveniles were significantly smaller than those from PS in both age subcategories (Table 2.14). Significant differences between the populations were evident in Hct, Lct, Lct detection, and lesion prevalence. Hct was lower in the BS population in both age categories compared with PS fish, with the greatest difference occurring in the younger fish. The previously noted bimodal distribution of Hct was evident in both populations of <11 month old herring and was also evident in the 11+ month old BS herring. Lct was significantly higher in BS 11+ month old herring, but there was no difference in Lct between populations in herring <11 months old. The prevalence of undetectable Lct was significantly higher in BS <11 month old herring. This illustrates that although there was no difference in the median value among populations, the distribution pattern differed (Figure 2.10). The prevalence of undetectable leucocrit did not vary between populations in the 11+ month old fish.

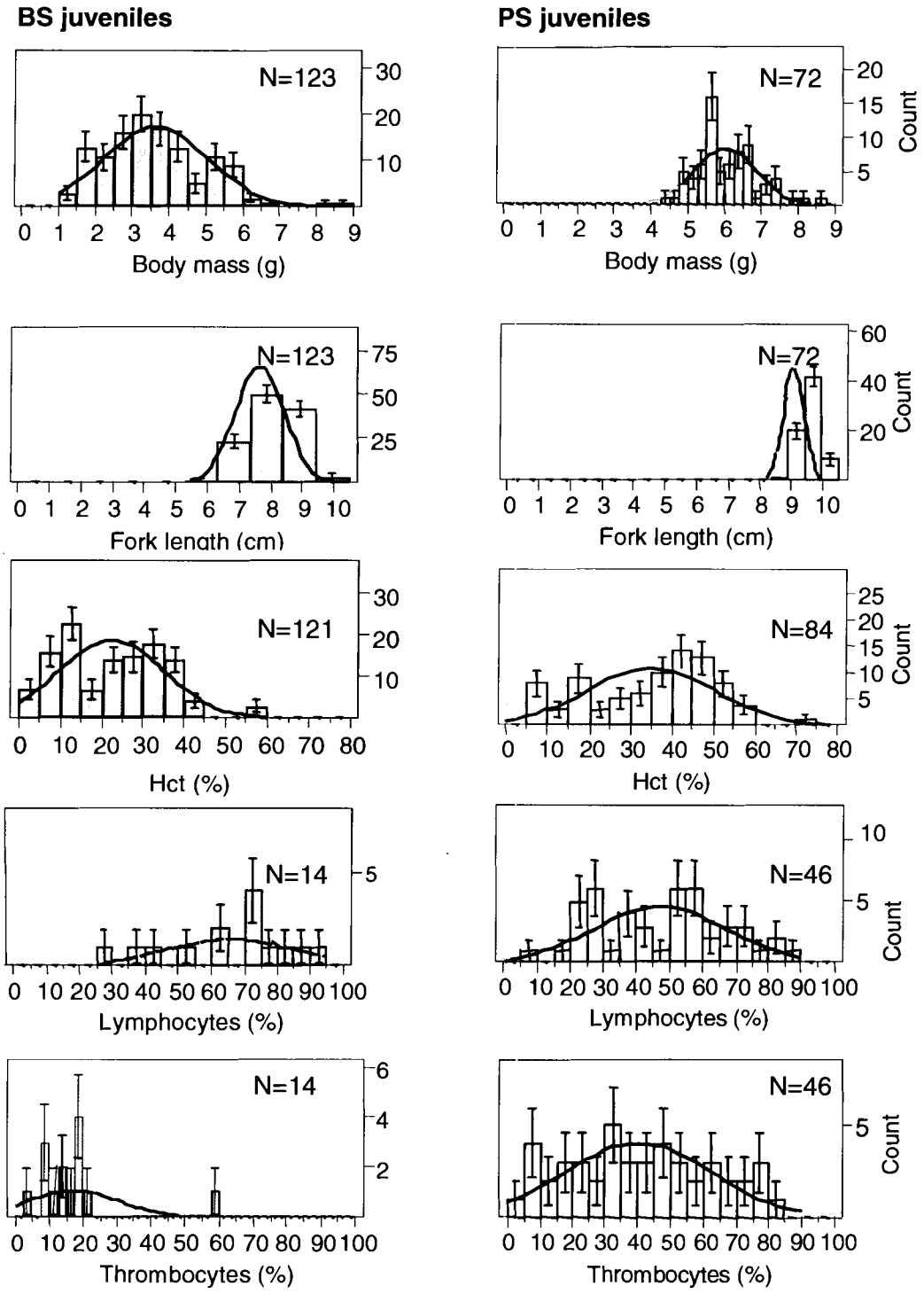


Figure 2.8. The distributions for morphometric and hematological variables that differed significantly among source populations of juvenile herring. The normal curve is superimposed on the distributions. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

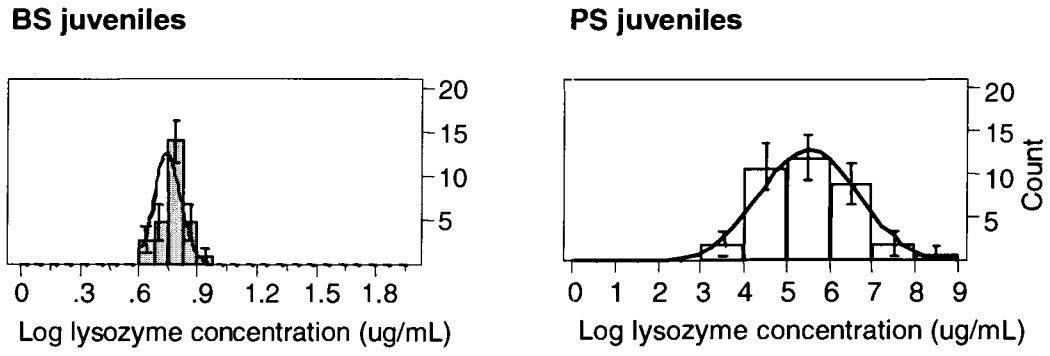


Figure 2.9. The distributions for plasma lysozyme, which varied significantly among source populations in juvenile herring. The normal curve is superimposed on the distributions. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

Table 2.13. Median values for variables measured in juvenile herring from BS and PS populations. The Wilcoxon-Kruskal/Wallis test was used at the 95% confidence level to compare populations, unless otherwise indicated. LR=Likelihood Ratio test. Sample size for each variable is in parentheses. *Previously bimodal when all 0+year combined. **Still bimodal when separated by population. Hct=hematocrit, Lct =leucocrit.

Variable	BS	PS	p-value	% Change: PS *vs.* BS
Body mass (g)	3.48 (123)	5.9 (72)	0.0007	69.5% ↑
Fork length (cm)	7.50 (123)	9.0 (72)	<0.0001	20.0% ↑
CF	0.77 (123)	0.79 (72)	0.1282	2.6% ↑
Hct (%)*	23.3**(121)	393** (84	<0.0001	68.7% ↑
Lct (%)	0.84 (121)	0.89 (84)	0.6462	6.0% ↑
Lct undetectable	30% (36/121)	14% (12/84)	0.0085 (LR)	53.3%↓
Lymphocytes (%)	72.2 (14	50.5** (46)	0.0028	30.1%↓
Monocytes (%)	0 (14)	0 (46)	0.4715	0
Neutrophils (%)	12.2 (14	5.0 (46	0.0686	59.0%↓
Thrombocytes (%)	15.7 (14)	38.5 (46)	0.0006	145.2% ↑
Lysozyme (ug/mL)	2.12 (28	265.28 (37)	<0.OuOl	124.1 fold ↑
Lesion prevalence	11%(10/89)	100% (19/19)	<0.0001 (LR)	8.1 fold ↑
Skin lesions	0% (0/123)	22% (19/85)	<0.0001 (LR)	22.0 fold ↑

Lesions were only seen in herring < 11 months old. Hemorrhagic skin lesions were significantly more prevalent in the PS population. Most of the statistical differences among populations were attributable to size. Thus, except for thrombocytes, the juvenile reference range profiles were not separated by population.

Table 2.14. The median value for Tier 1 variables among BS and PS populations in < 11 month and 11+ month old juvenile herring for variables shown to vary within these two subcategories. The Wilcoxon-Kruskal/Wallis test and the Likelihood Ratio test (where indicated) were used at the 95% confidence level. Sample size is in parentheses, including the denominator sample size when proportional values are given. The p-value and the relative magnitude of change in the median (%) is shown below. Hct=hematocrit, Lct=leucocrit.

Variable	**<11 month juveniles**		**11 + month juveniles**	
	BS	PS	BS	PS
Body mass (g)	3.4 (111)	5.8 (47)	4.7 (12)	6.2 (25)
	p-value <0.0001, 70.6% ↑		p-value = 0.0020, 31.9% ↑	
Fork length (cm)	7.5 (111)	9.0 (47)	7.9 (12)	9.1 (25)
	p-value <0.0001, 20.0% ↑		p-value = 0.0003, 15.2% ↑	
Hct (%)	22.4 (109)	31.6 (46)	37.4 (12)	43.6 (38)
	p-value = 0.0001, 41.1% ↑		p-value =0.0481, 16.6% ↑	
Lct (%)	0.84 (109)	0.89 (46)	1.52 (12)	0.47 (38)
	p-value = 0.1148, 6.0% ↑		p-value = 0.0317, 69.1% ↓	
Lct undetectable	30% (33/109)	2% (1/46)	25% (3/12)	30% (11/38)
	p-value <0.0001, 93.3% ↓, 15-fold difference		p-value =0.7890, 20.0% ↑	
Lesion prevalence	13% (10/77)	100% (19/19)	No lesions	No lesions
	p-value <0.0001, 669.2% ↑; 6.7 fold difference			
Skin lesion prevalence	0% (0/111)	41% (19/46)	No lesions	No lesions
	p-value <0.0001, 41-fold difference			

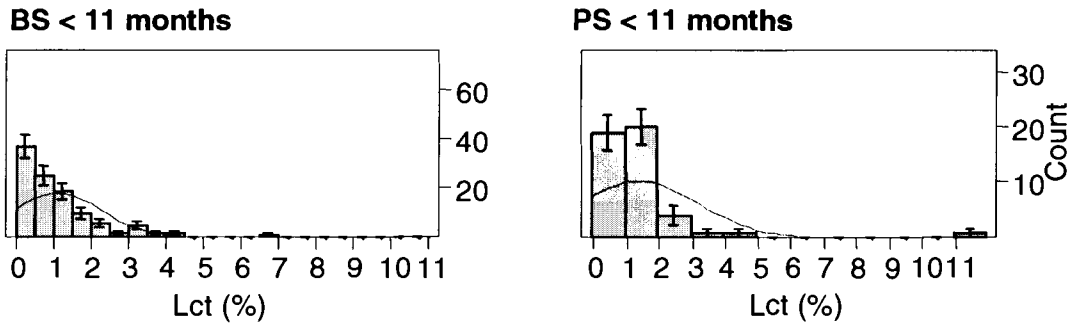


Figure 2.10. The distributions for Lct that varied significantly between source populations of < 11 month old juvenile herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and error bars are the corresponding SE for each level.

The three populations (BS, QI and PS) of adult herring were compared to determine if population source significantly affected the measured variables. QI adults were significantly smaller than both the BS and PS adults (Table 2.15). All variables differed significantly among the populations with the exception of macrophage cell count and macrophage phagocytosis. Several of these differences (CF, SSI, GSI, Hct, lysozyme, glucose, and lactate) may be attributed to the significant size difference in fish between

populations, and therefore could not be considered definitive of a population difference. However, the significantly lower Lct in the PS adults was not consistent with a size-related effect. In addition, the WBC differential count varied significantly between the BS and PS populations, which were of similar size. In BS adults, lymphocytes were the most frequent cell type compared with thrombocytes in herring from PS. These differences in WBC differential count likely account for the previously noted bimodal distribution of thrombocytes when all adult control fish were considered as one pool and compared with juvenile fish.

Table 2.15. The median value of Tier 1 and 2 variables among adult herring from BS, PS, and QI. The Wilcoxon-Kruskal/Wallis test and the Likelihood Ratio test (*) were used at the 95% confidence level to compare populations. The sample size is in parentheses, including the denominator sample size when proportional values are given. Letter pairs indicate populations that are significantly different from each other. LSI=liver-somatic index; SSI=spleen-somatic index; GSI=gonad-somatic index, mø=macrophage; Hct=hematocrit; Lct=leucocrit.

	BS	PS	QI	p-value
Body mass (g)	64.2[a] (139)	61.6[b] (61)	27.5[ab] (160)	<0.0001
Fork length (cm)	20.8[a,b] (138)	20.3[a,c] (75)	15.0[c,b] (160)	<0.0001
CF	0.72[a,b] (138)	0.91[a, c] (75)	086[b, c] (160)	<0.0001
LSI	1.00 (99)	-	0.82 (80)	<0.0001
SSI	0.10 (99)	-	0.13 (80)	0.0144
GSI	0.79 (60)	-	0.43 (80)	<0.0001
Hct (%)	39.1 (136)	40.5[a] (75)	37.5[a] (156)	0.0104
Lct (%)	1.23[a] (136)	0.72[a,b] (56)	1.05[b] (156)	<0.0001
Undetectable Lct	41% (16/139)	8% (6/75)	18% (28/160)	0.0931•
Lymphocytes (%)	55 (27)	31 (60)	-	<0.0001
Neutrophils (%)	29 (27)	23 (60)	-	0.0412
Thrombocytes (%)	16 (27)	45 (60)	-	<0.0001
Glucose (mmol/L)	7.82 (86)	-	11.90 (31)	<0.0001
Lactate (mmol/L)	2.14 (67)	-	4.64 (31)	0.0010
Lysozyme (µg/mL)	2.59[a] (86)	1.26[b] (68	074[a, b] (153)	<0.0001
Macrophage cell count	-	1.54 x 10^7 (30)	1.33 x 10^7 (7)	0.6982
Yeast per mø	2.78 (60)	2.91 (23)	2.62 (80)	0.7702
Lesion prevalence	38% (49/129)	19% (14/75)	24% (20/84)	0.0294*

The effect of population on Tier 1 variables was also evaluated for each gender. Size did not differ among populations in the gender-known subset, thus eliminating any potential confounding effect. Significant differences among populations only occurred in males and only for Hct and Lct. The median Hct was lower in QI males (38.0%) than in BS (43.5%) and PS (43.3%) males. In addition, the median Lct was higher for BS males (1.52%) compared with PS (0.92%) and QI (1.09%).

The plasma biochemistry of adult herring held at BMSC was compared with biochemistry data from PWS herring. All variables differed significantly ($p<0.05$) between Pacific Northwest (PNW) herring (QI and BS combined), n=117, and those from PWS, (n= 125) (Table 2.16). Plasma glucose, total protein, calcium, and globulin concentrations were higher in herring from the PNW. Plasma lactate, albumin, chloride, phosphorus, alkaline phosphatase (ALP) concentrations, and the A/G ratio were higher in PWS herring. Although all variables were statistically different between the two regions, only plasma lactate and calcium had 95th PE ranges that did not overlap and plasma phosphorus barely overlapped. PWS herring exhibited significantly higher plasma chloride and lactate while PNW herring had notably higher plasma glucose. The differences in these biochemical variables are suggestive of a difference in physiological stress among the two groups of herring at the time of sampling. Size and gender were not known for the PWS data, limiting the comparisons.

Table 2.16. The median values for plasma biochemistry variables among PNW herring and PWS herring. The Wilcoxon/Kruskal-Wallis test was used at the 95% confidence level to compare PNW herring to PWS herring. PWS data from C.J. Kennedy, SFU. Laboratory tests performed at SFU under similar conditions with same reagents as at BMSC. * Plasma chloride values >200 mEq/L were excluded from the analysis as they exceeded physiologically feasible levels. A/G=albumin/globulin ratio, ALP=alkaline phosphatase.

Variable	PNW		PWS		p-value
	Median (N)	95th PE	Median (N)	95th PE	
Glucose (mmol/L)	9.2 (117)	6.5 – 12.5	6.6 (299)	4.6 – 8.4	<0.0001
Lactate (mmol/L)	3.2 (99)	1.7 – 6.6	9.4 (299)	7.2 – 10.8	<0.0001
Albumin (g/dL)	0.77 (62)	0.21 – 12.2	2.2(299)	1.5 – 3.0	<0.0001
Total protein (g/dL)	4.6 (66)	0.4 – 11.5	3.7 (299)	2.8 – 4.9	0.0003
Chloride (mEq/L)	119 (48)	75 – 171	149 (123)*	124 – 171	<0.0001
Phosphorus (mg/dL)	2.7 (50)	0.9 – 4.6	5.6 (299)	4.3 – 7.9	<0.0001
Calcium (mg/dL)	18.4 (50)	15.8 – 22.0	11.8 (299)	10.9 – 12.8	<0.0001
Globulin (g/dL)	3.72 (61)	0.0 – 4.79	1.39 (299)	0.96 – 1.92	<0.0001
ALP (U/L)	20.7 (9)	3.8 – 27.6	35.0 (299)	22.3 – 48.6	<0.0014
A/G ratio	0.20 (61)	0 – 99.9	1.62(299)	0.99 – 2.63	<0.0001

Condition indices and hematological values of adult herring held at BMSC (n=374) were compared with adult herring (n=32) held at the Vancouver Aquarium (VA), which were originally from QI. These comparisons enabled comparison of the BMSC herring with

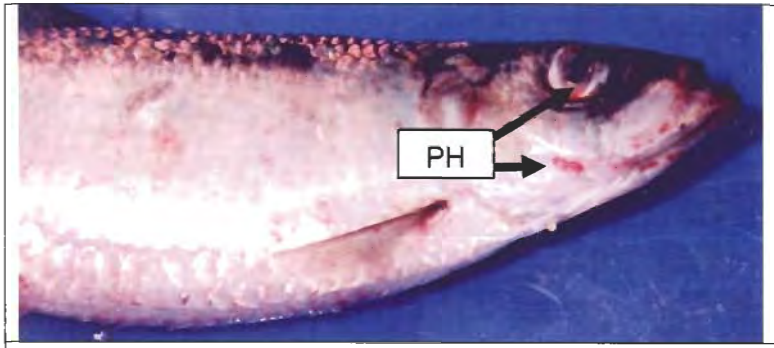
another captive reference population. VA adult herring were considerably larger and had a statistically higher median CF compared with either QI or BMSC herring (Table 2.17). SSI was almost three times greater in all BMSC herring, including the QI subset. The VA herring had a significantly lower Hct compared with the total pool of BMSC herring but there was no difference when compared with the QI subset of the BMSC herring.

Table 2.17. The median hematological and condition indices of VA adult herring, adult herring held at BMSC, and the BMSC QI subset. The Wilcoxon/Kruskal-Wallis test was used at the 95% confidence level to compare the VA herring to the Quadra Island subset and then all BMSC herring in two separate analyses. The sample size is in parentheses. LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, Hct=hematocrit, Lct=leucocrit.

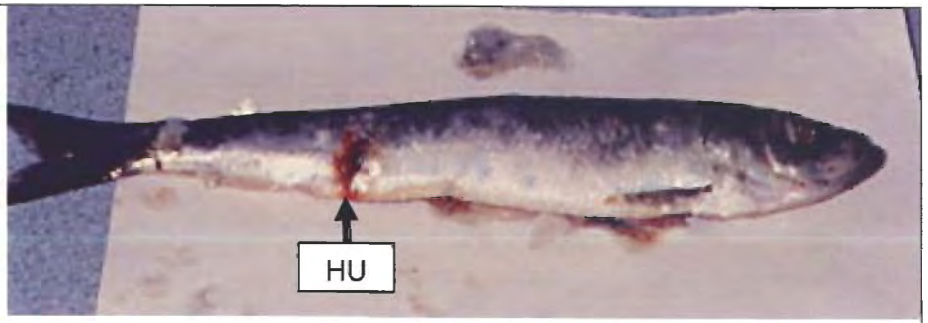
Measure	VA (N)	QI subset at BMSC (N)	BMSC (N)	p-values VA-QI / VA-BMSC
Mass (g)	110.2 (32)	27.5 (160)	49.5 (374)	<0.0001 / <0.0001
Fork length (cm)	22.8 (32)	15.0 (160)	18.0 (373)	<0.0001 / <0.0001
Condition factor	1.00 (32)	0.86 (160)	0.84 (373)	<0.0005 / <0.0001
LSI	0.89 (32)	0.82 (80)	0.89 (179)	0.1088 / 0.7532
SSI	0.04 (32)	0.13 (80)	0.11 (152)	<0.0001 / <0.0001
GSI	0.51 (32)	0.43 (80)	0.60 (140)	0.0683 / 0.5098
Hct (%)	32.3 (32)	37.49 (156)	39.11 (367)	0.2081 / 0.0190
Lct (%)	1.37 (25)	1.14 (132)	1.09 (324)	0.1320 / 0.0191

2.4.5. Prevalence and effect of lesions

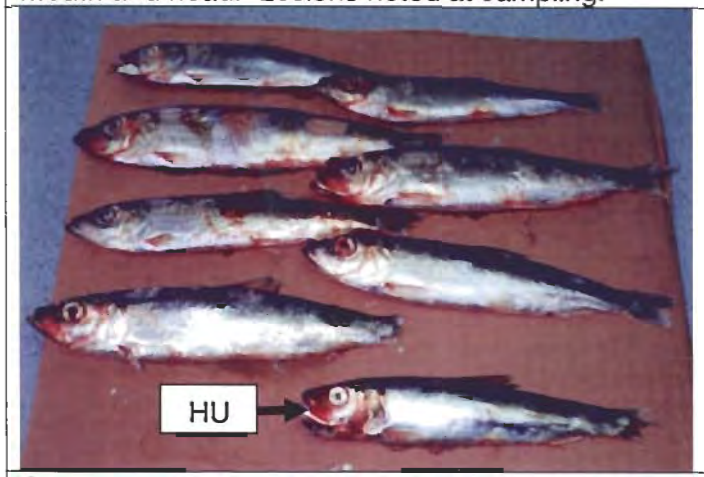
In these studies, there was the potential for scale damage during capture, transportation, and transfer to tanks, which could develop into pathological lesions. Skin abrasions and scale loss were unavoidable given the fragile nature of herring skin and scales. Adult herring from QI had less skin and scale damage than those obtained directly from seine boats in BS, but there was no appreciable difference either among the QI trips or between the different BS collections. Beach-seined juvenile herring had relatively little scale loss or skin damage. Commonly seen lesions included hemorrhagic skin erosions, discoloured livers, focal white nodules, and ascites. The prevalence of internal and external lesions varied significantly among populations of juvenile and adult herring. Lesion prevalence was highest in the PS juveniles (100%) and in the BS adults (38%) (Table 2.18). Lesions were significantly more prevalent in females from BS than other sources but there was no difference for males. Lesions were seen in live and dead herring (Figures 2.11 A-D).



A) Adult BS Pacific herring with petechial hemorrhage (PH) of the eye, and multifocal areas of petechiation around the mouth and head. Lesions noted at sampling.



B) Adult QI Pacific herring with a severe hemorrhagic ulcer (HU) of the caudal body wall with evidence of some generalized scale loss. This lesion was noted at the time of sampling.



C) Adult BS Pacific herring with multifocal hemorrhagic lesions involving the mouth, head, fin bases and body wall.

D) Adult QI Pacific herring with severe generalized erosion of skin and tail rot (TR). These lesions were seen in a tank experiencing a large die-off at 96-hours post-capture.

Figure 2.11 A-D. Examples of the lesions typically seen in adult Pacific herring during sampling (A,B) and when tank die-offs occurred (C,D).

Table 2.18. The prevalence of gross lesions in herring in the various populations. The Likelihood Ratio was used at the 95% confidence level to compare prevalence levels among populations within each age class and gender.

		BS		QI		PS		p-value
Total number		262 (45.0%)		160 (27.5%)		160 (27.5%)		
		Lesion -free	Lesions	Lesion -free	Lesions	Lesion -free	Lesions	
Adults (374/582)		80/129 (62%)	49/129 (38%)	64/84 (76%)	20/84 (24%)	61/75 (81%)	14/75 (19%)	0.0062
Gender known	Males (112)	49/68 ♂ (72%)	19/68 ♂ (28%)	26/32 ♂ (81%)	6/32 ♂ (19%)	11/12 ♂ (92%)	1/12 ♂ (8%)	0.2187
	Females (119)	31/61♀ (51%)	30/61 ♀ (49%)	34/44♀ (77%)	10/44♀ (23%)	10/14♀ (71%)	4/14♀ (29%)	0.0155
0+year (208/582)		79/89 (89%)	10/89 (11%)	-	-	0	19/19 (100%)	<0.0001
<11 months		67/77 (87%)	10/77 (13%)	-	-	0/19 (0%)	19/19 (100%)	<0.0001
11+ months		12/12 (100%)	0/0 (0%)	-	-	Internal status unknown but no skin lesions		-
1+year (50/582)		-	-	6/50 (12%)	44/50 (88%)	-	-	-

The presence of lesions had a significant effect on the median value of some Tier 1 and 2 variables in both juvenile and adult herring. Juveniles with lesions had significantly higher Hct and plasma lysozyme. In adults, LSI, plasma lactate and lysozyme were significantly higher in those with lesions, whereas thrombocytes, albumin and globulin were lower (Table 2.19). None of the lesion-free herring tested positive for *I. hoferi* while 55.6% of the herring with lesions tested positive. Across all populations, female herring had more lesions (33.9%) than males (25.7%).

2.4.5.1. The effect of specific lesions on variables - juveniles

Juvenile herring had lesions involving the skin, liver and eye. Only skin lesions were significantly associated with changes in Tier 1 and 2 variables. External lesions were associated with significantly higher Hct (38.6%), compared with the lesion-free fish (22.2%), which could signify fish with lesions were dehydrated. Plasma lysozyme was also extremely higher in herring with external lesions (335.2 µg/mL) compared with lesion-free herring (2.24 µg/mL) (Table 2.20). Hemorrhagic, full thickness skin lesions were associated with both elevated Hct and plasma lysozyme when compared with all juvenile herring with no skin lesions (Table 2.21).

Table 2.19. The median value for variables for juvenile and adult herring with and without lesions. Comparisons were made separately within age class, using the Wilcoxon/Kruskal-Wallis test at the 95% confidence level, unless otherwise indicated. Sample size is in parentheses. The first p-value is for the juvenile comparison, the second for the adults. No lesions existed in the 11 + month old juveniles. Therefore, this age category was excluded from the analysis. c/a= could not assess, CF=condition factor, LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, Hct=hematocrit, Lct=leucocrit, A/G=albumin-globulin ratio, n/e=not evaluated, *=adults only, LR=Likelihood Ratio test, Abs.=absorbance.

Variable	Lesion-free Juveniles (N)	Lesions Juveniles (N)	Lesion-free Adult (N)	Lesions Adult (N)	p-values
Body mass (g)	3.5 (67)	5.3 (29)	55.1 (205)	63.0 (83)	<0.0001/0.01
Fork length (cm)	7.5 (67)	8.8 (29)	19.0 (205)	19.5 (83)	<0.0001/<0.0001
CF	0.81(67)	0.81 (29)	0.82 (205)	0.83 (83)	0.70 / 0.65
LSI	1.21 (67)	1.54 (5)	0.86 (131)	1.03 (81)	0.15/0.0005
SSI	-	-	0.12 (117)	0.10 (35)	- / 0.32
GSI	-	-	0.61 (107)	0.59 (33)	- / 0.98
Lymphocytes (%)	n/e	52 (19)	32.6 (62)	38.9 (25)	- / 0.07
Neutrophils (%)	n/e	7.0 (19)	24.6 (62)	24.2 (25)	- / 0.98
Monocytes (%)	n/e	0 (19)	0 (48)	0 (12)	- / 0.90
Thrombocytes (%)	n/e	37.0 (19)	42.4 (62)	24.5 (25)	- / 0.03
Macrophage cell number	-	-	$1.46x10^7$ (30)	1.66×10^7 (7)	- / 0.51
Lysozyme (µg/ml)	2.10 (21)	306.9 (19)	1.06 (175)	2.48 (58)	<0.0001/ 0.01
Glucose (mmol/L)	3.50 (56)	3.95 (4)	9.02 (76)	9.59 (41)	0.77/ 0.89
Lactate (mmol/L)	3.31 (47)	3.99 (4)	2.48 (64)	5.71 (34)	0.70/ 0.0006
Albumin (g/dL)	0.85 (11)	N=0	0.63 (41)	0.21 (21)	- / 0.0047
Protein (g/dL)	1.38 (41)	1.56 (3)	4.75 (44)	4.45 (22)	0.44 / 0.63
Globulin (g/dL)	0.72 (9)	N=0	4.0 (40)	3.07 (21)	- / 0.0003
Hct (%)	19.5 (65)	26.7 (29)	39.8 (203)	38.6 (81)	0.01 / 0.31
Lct (%)	0.99 (65)	1.31 (29)	1.04 (188)	1.02 (77)	0.29 / 0.97
Yeast engulfed per macrophage	6.99 (30)	4.30 (4)	2.65 (126)	2.90 (37)	0.20 / 0.12
Respiratory burst (Abs./10^5cells)			0.20 (19)	0.20 (12)	/ 0.60
A/G	1.13 (9)	N=0	0.19 (49)	0.30 (21)	- / 0.08
Cortisol (ng/ml)	-	-	12.2 (43)	13.1 (16)	- / 0.38
Chloride (mEq/L)	-	-	119 (36)	122 (12)	- / 0.95
Phosphorus (mg/dL)	-	-	2.37 (41)	2.77 (9)	- / 0.25
Calcium (mg/dL)	-	-	17.88 (41)	19.93 (9)	- / 0.49
ALP (U/L)	-	-	23.4 (3)	19.9 (6)	- / 0.61
Gender ratio *	-	-	75 ▯ 86 ▯	26 ▯ 44▯	0.02 (LR)
I. hoferi positive *	-	-	0% (0/12)	55.6% (10/18)	0.0002 (LR)

The sub-group of juvenile herring with internal lesions had a significantly lower Hct (12.4%) compared with those with external lesions, but not with the lesion-free class (Table 2.20). Separation of lesions into the separate sub-groups showed that the effects

seen in the previous section can be attributed solely to external (skin) lesions, but it did not identify additional differences.

Table 2.20. The effect of lesion sub-groups on Tier 1 and 2 variables in juvenile herring. Sub-groups were compared using the Wilcoxon/Kruskal-Wallis test followed by Dunnett's test to determine which pairs of sub-groups differed significantly, at the 95% confidence level. Similar letters indicate sub-groups that are significantly different from each other. Values are medians with sample size in parentheses. Hct=hematocrit, LSI=liver-somatic index.

Variable	No lesions (N)	External lesions (N)	Internal lesions (N)	p-value
Body mass (g)	3.5 [a,] (67)	5.6[a, b] (21)	4.3 [b] (8)	<0.0001
Fork length (cm)	7.5 [a,] (67)	9.0 [a, b] (21)	8.0 [b] (8)	<0.0001
CF	0.81 (67)	0.81 (21)	0.83 (8)	0.6978
LSI	1.20 (67)	1.44 (2)	1.54 (3)	0.2481
Hct (%)	19.5 [a] (65)	38.6 [a, b] (21)	12.4 [b] (8)	<0.0001
Lymphocytes (%)	None	52.0 (19)	None	-
Thrombocytes (%)	None	37.0 (19)	None	-
Lysozyme (µg/ml)	2.10[a] (21)	335.2[a] (18)	2.56 (1)	<0.0001
Glucose (mmol/L)	3.5 (68)	10.2 (1)	3.8 (3)	0.3322
Lactate (mmol/L)	3.31 (57)	7.19 (1)	3.24 (3)	0.3399
Albumin (g/dL)	0.85 (11)	None	None	-
Globulin (g/dL)	0.72 (9)	None	None	-
A/G ratio	1.13 (9)	None	None	-

Table 2.21. The median Hct and lysozyme values in juvenile herring with respect to skin lesion status. Comparison between the sub-groups was made using the Wilcoxon/Kruskal-Wallis test at the 95% confidence level. Sample size is in parentheses. Hct=hematocrit.

	Skin Lesion Status		p-value
	Skin lesions (N)	No skin lesions (N)	
Hct	38.6 (19)	26.6 (186)	0.02
Lysozyme (µg/ml)	335.2 (18)	2.24 (47)	<0.0001

2.4.5.2. The effect of specific lesions on variables – adults

In adult herring, most lesions involved the skin, or liver or combinations of both these systems. Lesions involving the eye, gallbladder, gills, kidney, spine, and peritoneal cavity were seen infrequently. Separation of adult herring with lesions into the sub-groups identified skin and/or liver lesions as being significantly associated with alterations in Tier 1 and 2 variables. In addition to the measures that varied when all lesions were pooled, CF, thrombocytes, glucose and A/G were shown to vary between specific lesion categories. External lesions alone were associated with significantly higher plasma lysozyme concentration (2.5 µg/mL) compared with the lesion-free group (1.06 µg/mL).

Adult fish with only internal lesions had higher LSI, lymphocytes, plasma lactate and albumin, but less thrombocytes and globulin compared to the lesion-free sub-group. Plasma glucose was significantly higher (10.9 mmol/L) than the other two lesion sub-groups but did not differ from the lesion-free group (9.02 mmol/L). Adult fish with external lesions plus internal lesions were significantly associated with an increase in CF, LSI, plasma lactate, albumin and lysozyme concentration, and a decrease in thrombocytes compared to the lesion-free fish (Table 2.22).

Table 2.22. The effect of lesion sub-groups on Tier 1 and 2 variables in adult herring. Sub-groups were compared using the Wilcoxon/Kruskal-Wallis test primarily, and the Likelihood Ratio test where indicated (*), at the 95% confidence level. Similar letters indicate sub-groups that are significantly different from each other. Values are medians with sample size in parentheses, including denominator size for proportional values.

Variable	No lesions (N)	External lesions (N)	Internal lesions (N)	External and internal lesions (N)	p-value
Body mass (g)	55.1[a,b] (205)	72.8[a,c,] (29)	52.4[c,e] (38)	80.3 [b, d, e] (16)	<0.0001
Fork length (cm)	20.7 [a, b] (205)	20.4 [a] (29)	19.0 [c] (38)	20.2 [b] (16)	0.0085
CF	0.82 [a] (205)	0.86 [b] (29)	0.76 [b, c] (38)	1.00 [a, c] (16)	0.0009
LSI	0.86[a,b] (131)	1.03 (9)	1.00[a] (6)	1.17[b] (6)	0.0037
Hct (%)	39.8 (203)	39.0 (28)	40.5 (37)	34.7 (16)	0.3033
Lymphocytes (%)	32.6 [a] 62)	31.9 [b] (13)	54.8 [a, b] (7)	44.4 (5)	0.0414
Thrombocytes (%)	42.4[a,b] (62)	40.3 (13)	19.2[a] (7)	17.8[b] (5)	0.0382
Lysozyme (µg/ml)	1.06 [a, b] (175)	2.56 [a, c] (21)	2.20 [c] (31)	4.4 [b] (6)	0.0002
Glucose (mmol/L)	9.02 (76)	6.47 [a] (8)	10.9[a,b] (28)	5.41[b] (5)	0.0230
Lactate (mmol/L)	2.48 [a, b] (64)	1.48 [c, d] (5)	5.89 [a, c] (27)	12.28[b,d] (2)	<0.0001
Albumin (g/dL)	0.63 [a, b] (41)	0.84 [c] (5)	0.81 [a] (15)	8.4 [b, c] (1)	0.0122
Globulin (g/dL)	4.0[a] (40)	3.22 (5)	3.07[a] (15)	2.5 (1)	0.0039
A/G ratio	0.17 (40)	0.27 (5)	0.36 (15)	0.17 (1)	0.0476
I. hoferi positive	0% (0/12)	20% (1/5)	100% (4/4)	56% (5/9)	0.0001*

2.4.6. Anemia and Hct covariance

Low Hct is generally associated with an anemic state that can be considered physiologically detrimental. Based on this premise, the presence of low Hct in herring was considered a potential indicator of sub-optimal condition and it was postulated that low Hct might be associated with alterations in other variables. In addition, an examination of the Hct distribution pattern in juvenile herring had identified a lower mode centered at 12.8% and a higher mode, similar to adults at 35.0%. Therefore, 13.0% was considered a reasonable threshold for defining herring as anemic, especially in the context of the literature values in other teleosts (Table 2.3 and 2.4, Gallaugher and Farrell 1998). Low Hct was more prevalent in juveniles and alteration of Tier 1 variables

in association with low Hct was only seen in this age class. Tier 2 variables were not associated with low Hct in either age class.

The prevalence of a Hct <13.0% was 22.0% in juvenile herring and 4.9% in adult herring. Herring with a Hct <13.0% were smaller in both age classes, with length being a significant covariate in the juveniles and both length and mass being significant covariates in adults. Anemic juveniles and adults also had significantly lower Lct and plasma lactate (Table 3.23, Figure 2.12). The proportion of herring with undetectable Lct was significantly greater in those with low Hct in both age classes. No other variables differed significantly in the adult herring (Table 2.23). Dividing juveniles into those <11 months and those 11+ months eliminated the effect of size as a potential confounder of the effects of low Hct. Lct remained significantly lower in the <11 months old group with Hct <13% (Table 2.24). A significant difference in the prevalence of undetectable Lct was seen only in the < 11-month group. In the 11+ month group, only plasma lactate varied significantly, being lower in the low Hct herring.

Table 2.23. Evaluation of the impact of anemia on immunological variables in herring using the Wilcoxon/Kruskal-Wallis test at the 95% confidence limit or the Likelihood Ratio test, where indicated (*). Values are medians with the sample size in parentheses. The first p-value in each cell represents the juvenile comparison; the second represents the adult comparison. LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, mø=macrophage, *LR=likelihood ratio test, Hct=hematocrit, Lct=leucocrit.

Variable	Hct<13.0%	Hct≥13.0%	Hct<13.0%	Hct≥13.0%	p-value
	Juvenile	Juvenile	Adult	Adult	Juvenile / Adult
Mass (g)	4.0 (45)	5.0 (147)	26.2 (18)	50.6 (349)	0.06 / 0.0009
Fork length (cm)	8.0 (45)	8.5 (147)	15.0 (18)	18.2 (348)	0.0009 / 0.0045
CF	0.80 (45)	0.78 (147)	0.84 (18)	0.84 (348)	0.43 / 0.38
LSI	1.28 (29)	1.02 (52)	1.09 (5)	0.89 (172)	0.06 / 0.42
SSI	-	-	0.11 (4)	0.11 (146)	- / 0.70
GSI	-	-	0.96 (4)	0.59 (135)	- / 0.30
Lct (%)	0.26 (45)	0.95 (160)	0.80 (18)	1.00 (349)	0.0014 / 0.24
Lct undetected (%)	40% (18/45)	19% (30/160)	39% (7/18)	10% (36/349)	0.0043/ 0.0022*
Lymphocytes (%)	50.0 (7)	54.7 (52)	57.6 (1)	33.6 (86)	0.64 / 0.21
Monocytes (%)	0 (7)	0 (52)	None	None	0.79
Neutrophils (%)	3.0 (7)	6.0 (52)	20.3 (1)	24.2 (86)	0.36 / 0.78
Thrombocytes (%)	46.0 (7)	29.5 (52)	22.0 (1)	39.0 (86)	0.31 / 0.60
Yeast /mø	5.72 (13)	6.60 (27)	1.81 (4)	2.76 (158)	0.32 / 0.08
Lysozyme (µg/ml)	2.45 (12)	85.4 (52)	1.18 (16)	1.16 (287)	0.21/ 0.85
Glucose (mmol/L)	3.29 (23)	3.34 (48)	9.05 (3)	9.26 (114)	0.81 / 0.81
Lactate (mmol/L)	3.49 (22)	4.84 (38)	1.83 (2)	3.22 (96)	0.03 / 0.27
Albumin (g/dL)	0.81 (2)	0.85 (9)	0.49 (2)	0.79 (60)	0.81 / 0.15
Protein (g/dL)	1.30 (17)	1.49 (35)	3.65 (2)	4.64 (60)	0.69 / 0.09
Vibrio titre	0 (5)	10 (4)	-	-	0.56

Table 2.24. Variables that differed significantly with low Hct within the age sub-groups of juvenile herring. Comparisons were made using the Wilcoxon/Kruskal-Wallis and Likelihood Ratio tests, where indicated (*) at the 95% confidence level. Values are the median with sample size in parentheses, including the denominator sample size when proportional values are given. The first p-value in each cell represents the <11 month comparison; the second represents the 11+ month comparison. Hct=hematocrit, Lct=leucocrit, mo.=month.

Variable	Hct < 13%	Hct ≥13%	Hct < 13%	Hct ≥13%	p-value
	< 11 mo.	< 11 mo.	11 + mo.	11 + mo.	<11 mo./11 + mo.
Lct (%)	0.25 (27)	1.16 (115)	0.26 (5)	0.60 (45)	0.0008 / 0.30
Lct undetectable	40% (16/40)	16% (18/115)	40% (2/5)	27% (12/45)	0.0021 / 0.54 *
Lactate (mmol/L)	3.49 (20)	3.90 (28)	6.64 (2)	11.63 (8)	0.16 / 0.04

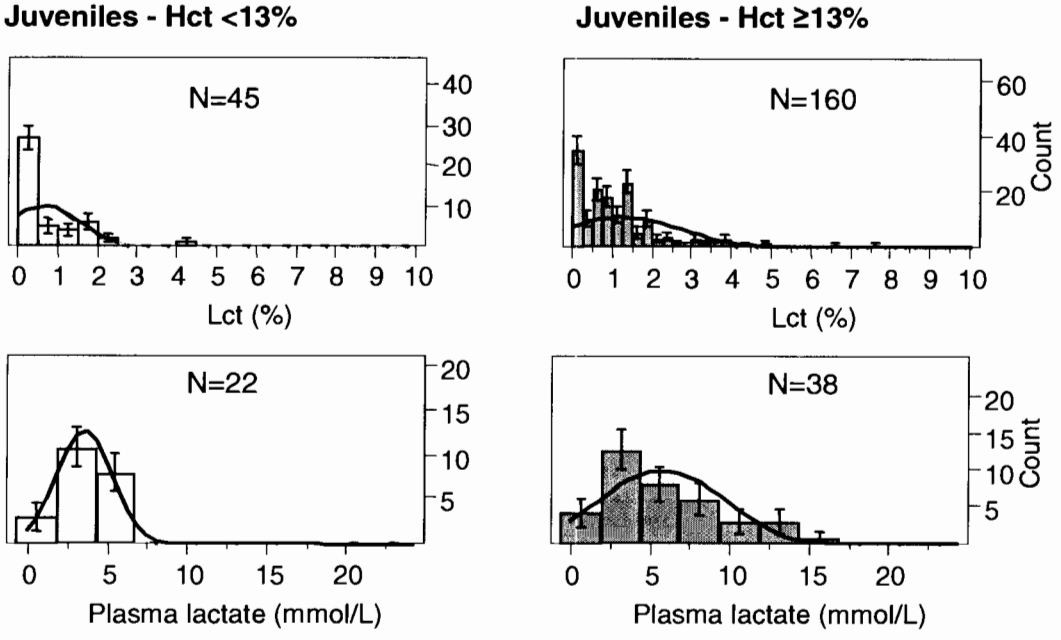


Figure 2.12. The distribution of variables that differed significantly with Hct < 13% or ≥13% in juvenile herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

2.4.6.1. Covariance of variables with Hct

In juvenile herring, Lct, plasma lactate and lysozyme concentration were positively correlated with Hct, while LSI was negatively correlated (Table 2.25). The correlation of LSI and lysozyme with Hct in the absence of a difference in the median values in Table 3.19 may reflect the smaller sample sizes used in the comparison between Hct

categories. In adults, thrombocytes, plasma glucose, globulin and phagocytosis activity were all positively correlated with Hct. Conversely, SSI, lymphocytes, plasma chloride and lactate were all negatively correlated with Hct (Table 2.26).

Table 2.25. Significant correlation of variables with Hct in juvenile Pacific herring using Spearman's non-parametric correlation at the 95% confidence level. The S-R value is the Spearman Rho correlation coefficient. Lct=leucocrit.

Variable	N	S-R value	p-value
LSI	81	-0.0349	0.0022
Lct (%)	205	0.2221	0.0014
Lactate (mmol/L)	60	0.3483	0.0064
Lysozyme (µg/mL)	64	0.3858	0.0016

Table 2.26. Significant correlation of variables with Hct in adult Pacific herring using Spearman's non-parametric correlation at the 95% confidence level. The S-R value is the Spearman Rho correlation coefficient. SSI=spleen-somatic index.

Variable	N	S-R value	p-value
SSI	150	-0.3714	<0.0001
Lymphocytes (%)	60	-0.3814	0.0003
Thrombocytes (%)	60	0.4928	<0.0001
Glucose (mmol/L)	117	0.3308	0.0003
Lactate (mmol/L)	98	-0.2235	0.0270
Chloride (mEq/L)	48	-0.3578	0.0125
Globulin (g/dL)	61	0.3933	0.0017
Yeast per macrophage	162	0.2534	0.0011

2.4.7. Captivity effects (holding time (T_H) and experimental sampling time (T_E))

2.4.7.1. Evaluation of experimental sample time differences using pooled controls vs. time zero only controls on median values.

Time zero controls (T_0) were defined as fish sampled after the acclimation period but prior to initiation of experiments. Pooled controls (T_P) are all controls used in experiments at varying sample times minus the T_0 controls. In juvenile fish, the effect of pooling all but the T_0 controls *vs.* using T_0 only controls was a significant decrease in body mass, length, Lct, Hct, thrombocytes, and plasma lysozyme, and an increase in lymphocytes and the prevalence of lesions and anemia in the pooled group compared to the T_0 only fish. CF, monocytes, neutrophils and eosinophils did not vary (Table 2.27). It is possible that the significant decrease in both Hct and Lct between T_0 and T_P juvenile herring may reflect a VHSV epizootic effect. It was not possible to evaluate the

remainder of the juvenile variables due to a lack of T_0 data for several variables. In the adults, mass, length, CF, GSI, phosphorus, plasma lysozyme and percentage active macrophages were all significantly decreased in the pooled group while LSI, plasma lactate, albumin, total protein, calcium and prevalence of lesions were increased (Table 2.28). Both age classes showed a decrease in both length and plasma lysozyme in the respective pooled group compared with the T_0 subset.

Table 2.27. Comparison of median values between T_0 controls *vs.* T_P for juvenile herring using the Wilcoxon/Kruskal-Wallis test at the 95% confidence limit or the Likelihood Ratio test, where indicated (*). Sample size is in parentheses. *LR=likelihood ratio test. T_P=time pooled controls, T_0=time zero controls, Hct=hematocrit, Lct=leucocrit.

Variable	T_P controls (N)	T_0 controls (N)	p-value
Body mass (g)	4.3 (155)	5.6 (40)	0.0180
FL (cm)	8.0 (155)	8.8 (40)	0.0037
CF	0.78 (155)	0.79 (40)	0.4349
Hct (%)	25.0 (165)	35.8 (40)	0.0004
Lct (%)	0.64 (165)	1.33 (40)	<0.0001
Lymphocytes (%)	62.1 (30)	42.5 (30)	0.0228
Monocytes (%)	0.3 (30)	0.0 (30)	0.1044
Neutrophils (%)	6.0 (30)	6.0 (30)	0.8648
Thrombocytes (%)	19.8 (30)	42.5 (30)	0.0087
Eosinophils (%)	0.0 (9)	0.0 (30)	0.3375
Lysozyme (µg/ml)	2.15 (36)	306.85 (29)	<0.0001
Lesions present	11.2 (10/89)	100 % (19/19)	<0.0001
Lct undetectable	29.1 % (48/165)	0.0 % (0/40)	<0.0001
Anemia present	26.7 % (44/165)	2.5 % (1/40)	0.0001*

Table 2.28. Comparison of median values between time zero controls *vs.* entire pool of controls for adult herring using the Wilcoxon/Kruskal-Wallis test at the 95% confidence limit or the Likelihood Ratio test, where indicated (*). Sample size is in parentheses. *LR=Likelihood Ratio test. Mø=macrophage. T_P= time pooled controls, T_0= time zero controls. Hct=hematocrit, Lct=leucocrit.

Variable	T_P controls (N)	T_0 controls (N)	p-value
Body mass (g)	37.9 (282)	72.4 (92)	<0.0001
Length (cm)	16.8 (282)	19.8 (91)	<0.0001
CF	0.79 (282)	0.94 (91)	<0.0001
LSI	0.92 (157)	0.77 (22)	0.0080
SSI	0.11 (130)	0.12 (22)	0.9541
GSI	0.57 (130)	0.88 (10)	0.0086
Hct (%)	39.1 (278)	39.0 (89)	0.6543
Lct (%)	1.00 (278)	0.97 (89)	0.9391
Lymphocytes (%)	38.0 (57)	31.9 (30)	0.0770
Monocytes (%)	0.0 (30)	0.0 (30)	0.1735
Neutrophils (%)	26.6 (57)	24.00 (30)	0.7922
Thrombocytes (%)	25.9 (57)	24.00 (30)	0.1140
Glucose (mmol/L)	8.92 (107)	10.48 (10)	0.2643
Lactate (mmol/L)	3.78 (88)	1.78 (10)	0.0043
Albumin (g/dL)	0.83 (54)	0.41 (8)	0.0025
Total protein (g/dL)	4.78 (58)	3.64 (8)	0.0048
Globulin (g/dL)	3.83 (53)	2.90 (8)	0.1463
Albumin/globulin	0.20 (53)	0.16 (8)	0.4292
Cortisol (ng/ml)	13.0 (49)	11.3 (10)	0.1297
Phosphorus (mg/dL)	2.05 (40)	3.16 (10)	0.0018
Chloride (mEq/L)	119 (40)	125 (8)	0.3540
Calcium (mg/dL)	19.5 (40)	15.9 (10)	0.0045
Lysozyme (µg/ml)	1.09 (273)	9.34 (34)	<0.0001
Macrophage cell count	1.53×10^7 (27)	1.48×10^7 (10)	0.6080
% Active mø (NBT)	33.3 (17)	96.0 (10)	0.0002
Yeast/macrophage	2.75 (146)	1.84 (17)	0.5164
Lesions present	23.8 % (49/206)	41.5 %(34/82)	0.0034
Lct undetectable	110.8% (30/278)	14.6 % (13/89)	0.3405
Anemia present	5.0 % (14/278)	4.5 % (4/89)	0.8354

2.4.7.2. Degree of variation attributable to the variation in T_H using only T_0 controls

Differences in the length of T_H (from capture to initiation of an experiment) were shown to account for significant proportions of the variation of some Tier 1 and 2 variables. These were mass, length, CF, Hct and Lct in juveniles and Lct, yeast phagocytosed per

macrophage and plasma lysozyme in adult herring when only T_0 fish were considered (Tables 2.29 and 2.30). T_H was not significantly associated with the prevalence of lesions (p=0.0830), anemia (p=0.8512) or undetectable Lct (p=0.7206) in adult fish. Only the prevalence of anemia could be evaluated for the effects of T_H in juvenile fish and there was no significant association (p=0.7663) either.

Table 2.29. The proportion of variation attributable to a difference in T_H for variables measured in T_0 juvenile herring using multivariate correlation and Spearman's non-parametric association test at the 95% confidence limit. T_H=holding time, T_0=time zero, CF=condition factor, Hct=hematocrit, Lct=leucocrit, r^2 = correlation coefficient, S-R value designates the Spearman Rho correlation coefficient.

Proportion of variation attributable to T_H				
Variable	N	r^2	S-R value	p-value
Body mass (g)	40	0.89	0.7513	<0.0001
Length (cm)	40	0.89	0.7495	<0.0001
CF	40	0.66	0.6102	<0.0001
Hct (%)	40	0.29	0.3251	0.0407
Lct (%)	40	0.23	0.4528	0.0033

Table 2.30. The proportion of variation attributable to a difference in T_H for variables measured in T_0 adult herring using multivariate correlation and Spearman's non-parametric association test at the 95% confidence limit. T_H=holding time, T_0=time zero, CF=condition factor, Hct=hematocrit, Lct=leucocrit, r^2 = correlation coefficient, S-R value designates the Spearman Rho correlation coefficient.

	Proportion of variation attributable to T_H			
Variable	N	r^2	S-R value	p-value
Body mass (g)	92	0.15	0.1546	0.1412
Length (cm)	91	0.12	0.1435	0.1747
CF	91	0.03	0.0933	0.3793
Hct (%)	89	-0.08	-0.0867	0.4191
Lct (%)	89	0.50	0.5158	<0.0001
Yeast /mø	17	-0.94	-0.8539	<0.0001
Lysozyme (µg/ml)	34	-0.89	-0.7897	<0.0001

2.4.7.3. Degree of variation attributable to the variation in T_H for T_P controls

Evaluation of the effect of T_H on Tier 1 and 2 variables common to at least two different holding periods using the T_P, showed an increase in the number of variables with a significant proportion of variation attributable to T_H. In the juvenile fish, variation in body

mass, length, CF, Hct, Lct, the percentage of lymphocytes, neutrophils and thrombocytes, and lysozyme was significantly attributable to T_H differences (Table 2.31).

Table 2.31. The proportion of variation attributable to a difference in T_H for variables measured in T_P, control juvenile herring using multivariate correlation and Spearman's non-parametric association test at the 95% confidence limit. T_H=holding time, CF= condition factor, Hct=hematocrit, Lct=leucocrit, r^2 =correlation coefficient. S-R value designates the Spearman Rho correlation coefficient.

	Proportion of variation attributable to T_H			
Variable	N	r^2	S-R value	p-value
Body mass (g)	155	0.59	0.6046	<0.0001
Fork length (cm)	155	0.52	0.5930	<0.0001
CF	155	0.34	0.2602	0.0011
Hct (%)	165	0.20	0.2757	0.0003
Lct (%)	165	-0.08	-0.1584	0.0421
Lymphocytes (%)	30	-0.50	-0.5198	0.0032
Monocytes (%)	30	0.29	0.2373	0.2067
Neutrophils (%)	30	-0.46	-0.4636	0.0099
Thrombocytes (%)	30	0.53	0.5190	0.0033
Lysozyme (µg/ml)	36	0.19	0.5821	0.0002

In the adult fish variation in body mass, length, CF, percentage lymphocytes, neutrophils and thrombocytes, LSI, SSI, GSI, Hct, plasma glucose, lactate, lysozyme and yeast phagocytosed per macrophage was significantly attributable to differences in T_H to varying degrees. Contrary to the findings for T_0 adults, the variation in Lct in the T_P control adults was not attributable to T_H (Table 2.32).

Table 2.32. The proportion of variation attributable to a difference in T_H for variables measured in T_P, control adult herring using multivariate correlation and Spearman's non-parametric association test at the 95% confidence limit. T_H=holding time, CF=condition factor, Hct=hematocrit, Lct=leucocrit, Mø=Macrophage, r^2 =correlation coefficient. S-R value designates the Spearman Rho correlation coefficient.

	Proportion of variation attributable to T_H			
Variable	N	r^2	S-R value	p-value
Body mass (g)	282	0.72	0.7545	<0.0001
Length (cm)	282	0.82	0.8273	<0.0001
CF	282	-0.49	-0.5351	<0.0001
Hct (%)	278	0.25	0.2082	0.0005
Lct (%)	278	-0.06	0.0273	0.6504
Lymphocytes (%)	57	0.61	0.6087	<0.0001
Neutrophils (%)	57	0.33	0.2905	0.0284

	Proportion of variation attributable to T_H			
Variable	N	r^2	S-R value	p-value
Thrombocytes (%)	57	-0.83	-0.2764	0.0374
LSI	157	0.32	0.3615	<0.0001
SSI	130	-0.39	-0.3691	<0.0001
GSI	130	0.09	0.2568	0.0032
Glucose (mmol/L)	107	-0.45	-0.4886	<0.0001
Lactate	88	0.07	-0.2618	0.0138
Lysozyme (µg/ml)	273	0.48	0.6073	<0.0001
Yeast /mø	146	0.14	0.1628	0.0496
Macrophage cell count	27	0.19	0.1194	0.5532

2.4.7.4. Interaction between holding time (T_H) and experimental time (T_E)

A significant interaction effect ($p<0.05$) between T_H and T_E was identified using the GLM for Hct, Lct, plasma glucose and lactate, and yeast phagocytosed per macrophage for both the juvenile and adult herring. Additionally, percentage lymphocytes showed a significant interaction in juvenile fish only while CF, LSI and plasma lysozyme showed a significant interaction between T_H and T_E in the adult fish. The suite of variables affected by the combination of differences in T_H and T_E showed considerable similarity between age classes. This suggests that time in captivity is a significant influencing factor upon establishing reference ranges from control fish.

2.4.8. Reference range profiles

Several demographic factors (age, size, gender, population) and disease indicators (lesions, anemia) were examined to determine their influence on hematological, biochemical, and immunological parameters in Pacific herring. The presence or absence of statistically significant differences in the measured variables used in this study between the levels of each factor is summarized in Table 2.33. These findings were used to aid in developing the reference range profiles (Tables 2.34 and 2.36). The established differences in measured variables between the age classes supported separate profiles for juveniles and adults. Based on earlier results, some measures were also further subdivided within the juvenile class.

The variables changing with gender were not separated as male *vs.* female in the adult reference range profile table used in my experiments (Table 2.36), as there were no gender ratio differences among treatment groups within experiments and gender was

not always known. However, if gender ratio is significantly different among treatment groups in other situations, then the appended reference ranges for SSI, Hct, Lct, and cortisol should be used (Table A2.8).

Most of the statistical differences seen between populations may have been purely attributable to the size differences within each age class. Without a larger sampling of size-matched herring from each population, it was not possible to support separate profiles for each population. Therefore, the profiles were pooled across populations within each age class, with the exception of variables differing with T_H. Two profiles were produced for each age class, one included all control fish except the T_0 fish, and the other used only the T_0 controls. This was done because there were significant differences in the median value of the variables measured between the T_0 subset of controls and the T_P controls.

Variables in which differences in T_H accounted for a significant proportion of the spread of a variable were subdivided into reference ranges for each specific T_H for both the T_0 and the T_P reference range profiles. These two sets of reference range profiles are used in Chapter 3 to assess the response of control and exposed fish over time (captivity effects) and to assess the impact of statistically significant treatment effects in exposed fish, but only for variables that did not demonstrate a change over experimental time alone. Variables that did change with experimental time were instead assessed against their time-matched controls for the specific experiment.

The observation of lesions and anemia in both age classes raised the question of whether or not to exclude these individuals from the reference range database. None of the anemic fish were subsequently excluded from the reference range database due to the lack of association of anemia with any other measurement variables. It was considered unlikely that their exclusion would provide additional resolution. Excluding fish with lesions from the reference range profiles only significantly affected the variability (median, 95th PE, and the coefficient of variance, CV) of Hct and plasma lysozyme and then only among juvenile fish ($p<0.0001$, ANOVA) in the <11 month old sub-group. The erosive skin lesions in the < 11 month old juveniles were most likely driving these differences as the median plasma lysozyme and Hct both decreased once these individuals were excluded. It may be that this type of skin lesion led to

dehydration and subsequent hemoconcentration. Therefore, overall, the presence of gross lesions did not impact the 95th PE ranges. Consequently, a separate reference range for fish with no lesions was considered valuable only for the < 11 month old juvenile fish when Hct and plasma lysozyme were considered (Table 2.35).

Two profiles were established for each age class: one uses only the T_0 fish and the other uses the T_P controls, with differences due to T_H and gender where appropriate. Determining which profile to use to assess whether or not a statistical change due to WSFO exposure and pathogen challenge falls within the reference range for captive herring depends upon whether or not a given variable changes with time in the controls of the specific experiment or with T_H. Variables that are resistant to the effects of T_H and T_E can be scored using the pooled profile, while those that are sensitive to time effects require use of the specific time-matched controls only. The discussion section addresses the comparability of the reference ranges established in this study with the previously published values for herring, and for other fish species, where known.

Table 2.33. Summary of the impact of demographic factors and disease status indicators that were considered in the establishment of reference range profiles in Pacific herring using $p<0.05$ to designate a "yes" classification. Shaded cells under the source column indicate variables that also covaried significantly with size such that differences could not be deemed population source specific. CF=condition factor, LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, TP=total protein, A/G=albumin/globulin, ALP=alkaline phosphatase, Hct=hematocrit, Lct=leucocrit, Mø=macrophage, ITP=*Ichthyophonus hoferi*; na=not assessed, n/a=not applicable, J=juvenile, A=adult, T_0= time zero, T_P= time pooled.

	AGE	SIZE		M/F	SOURCE		LESIONS		LOW HCT (<13%)		HOLD TIME				EXPT. TIME	
Variable		J	A	A	J	A	J	A	J	A	J		A		J	A
											T_0	T_p	T_0	T_p		
Body mass	Y	n/a	n/a	N	Y	Y	Y	Y	N	Y	Y	Y	N	Y	Y	Y
Length	Y	n/a	n/a	N	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y
CF	Y	Y	Y	N	N	Y	N	N	N	N	Y	Y	Y	Y	N	Y
LSI	Y	N	Y	N	na	Y	N	Y	N	N	na		-	Y	Y	Y
SSI	na	na	Y	Y	na	Y	na	N	na	N	na		-	Y	na	N
GSI	na	na	Y	N	na	Y	na	N	na	N	na		-	Y	na	Y
Hct	Y	Y	Y	Y	Y	Y	Y	N	na	na	Y	Y	N	Y	Y	N
Lct	Y	N	N	Y	N	Y	N	N	Y	N	Y	N	Y	N	Y	N
Lymphs	Y	Y	Y	N	Y	Y	na	N	N	N	-	Y	-	Y	Y	N
Monocytes	Y	N	N	N	N	N	na	N	N	na	-	N	-	-	N	N
Neutrophils	Y	N	Y	N	N	Y	na	N	N	N	-	N	-	Y	N	Y
Thrombos	N	N	Y	N	Y	Y	na	Y	N	N	-	Y	-	Y	Y	Y
Glucose	Y	N	Y	N	na	Y	N	N	N	N	na		-	Y	Y	Y
Lactate	N	N	Y	N	na	Y	N	Y	Y	N	na		-	Y	Y	Y
Albumin	N	N	N	N	na	na	ca	Y	N	N	na		na		N	Y
TP	Y	N	N	N	na	na	N	N	N	N	na		na		N	Y
Globulin	Y	N	N	N	na	na	na	Y	na	N	na		na		N	Y
A/G ratio	Y	N	N	N	na	na	na	N	na	N	na		na		N	Y
Cortisol	na	na	N	Y	na	na	na	N	na	N	na		na		na	Y
Chloride	na	na	N	N	na	na	na	N	na	N	na		na		na	Y
Calcium	na	na	N	N	na	na	na	N	na	N	na		na		na	Y
Phosphorus	na	na	N	N	na	na	na	N	na	N	na		na		na	Y
ALP	na	na	N	N	na	na	na	N	na	N	na		na		na	na
Lct status	Y	N	N	N	Y	N	N	N	Y	Y	Y	Y	N	Y	Y	Y
Lysozyme	Y	Y	Y	N	Y	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y
Hct < 13 %	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y	N	N
Mø count	Y	na	N	N	na	N	na	N	na	na	na			N	na	N
Respiratory burst	na	na	N	N	na	na	na	N	na	na	na		na		na	N
Yeast/mo	Y	N	N	N	na	N	N	N	N	N	-	-	Y	N	Y	Y
Lesions	N	Y	Y	Y	Y	Y	na	na	N	N	Y	N	Y	Y	N	N
ITP +ve	na	na	Y	Y	na	na	na	Y	na	N	na		na		na	na

Table 2.34. The median, 95th PE, and CV for a profile using all control juvenile herring *vs.* one using only the time zero controls. PE=percentile estimation, CV=coefficient of variation, CF=condition factor, LSI=liver-somatic index, Hct=hematocrit, Lct=leucocrit, mo.=month, TP= total protein, mø=macrophage, BS=Barkley Sound, PS=Puget Sound. For use with juvenile herring ranging from 1.6 – 7.8 g and 6.4-9.8 cm. Variables are subdivided for differences in holding time (T_H) in days (d) and age (mo.).

Variable	T_H (d)	All controls				Time zero controls			
		Median	N	95th PE	CV	Median	N	95th PE	CV
CF	BS, 6 d	0.63	39	0.53 – 0.90	15.5	0.63	10	0.58 – 0.84	12.7
	BS, 61 d	0.82	84	0.59 –1.04	14.8	---	---	---	---
	PS, 77 d	0.79	72	0.65 – 0.98	9.8	0.81	30	0.60 – 0.94	9.1
LSI	<11 mo.	1.21	72	0.56 – 2.33	34.2	---	---	---	---
	11+ mo.	0.79	11	0.28 – 0.97	29.9	---	---	---	---
Hct (%)	<11 mo. 6 d	25.4	39	2.9 – 55.4	44.5	30.9	10	13.6 –37.3	27.2
	<11 mo. 61d	18.0	70	0.82 –46.2	62.7	---	---	---	---
	<11 mo. 77d	18.7	16	5.6 – 56.0	57.2	38.7	30	7.7 – 56.1	36.9
	11+ mo.	42.0	50	7.0 – 68.2	38.2	---	---	---	---
Lct (%)	<11 mo. 6 d	0.71	39	0 – 3.76	112.6	0.82	10	0.65 – 2.48	54.9
	<11 mo. 61 d	0.95	70	0 – 4.89	105.6	---	---	---	---
	<11 mo. 77 d	1.17	46	0.01 – 10.4	126.7	1.41	30	0.68 – 11.6	101.8
	11+ mo 61d	1.52	12	0 – 7.66	113.1	---	---	---	---
	11+ mo 77d	0.47	38	0 – 1.89	91.2	---	---	---	---
Lympho-cytes (%)	BS, 6 d	72.2	14	29.5 – 94.7	28.9	---	---	---	---
	PS, 77d	50.5	46	10.2 – 87.0	42.2	42.5	30	16.0 – 88.0	40.3
Monocytes (%)		0	60	0 – 9.3	210.0	0.0	30	0.0 – 1.0	209.7
Neutro-phils (%)	BS, 6 d	12.2	14	0 – 48.1	94.2	---	---	---	---
	PS, 77d	5.0	46	0 – 53.9	128.9	6.0	30	1.0 – 57.0	124.0
Thrombo-cytes (%)	BS, 6 d	15.7	14	2.7 – 59.5	76.8	---	---	---	---
	PS, 77d	38.5	46	2.5 – 81.3	55.8	42.5	30	28.3 – 58.5	50.2
Eosinophils (%)		0	39	0 – 4.0	511.8	0.0	30	0.0 – 1.0	547.7
Yeast/mø		6.00	40	2.70 –16.9	50.4	---	---	---	---
Vibrio titre		0	9	0 – 1600	267.2	---	---	---	---
Lyso-zyme (µg/mL)	BS, 61 d	2.12	28	1.88 – 2.56	---	---	---	---	----
	PS, 77 d	265.3	37	40.0 – 6.85 x 10^3	---	---	---	---	197.9
Glucose (mmol/L)	<11 mo.	3.59	60	1.64 – 17.4	---	---	---	---	----
	11+ mo.	2.32	12	1.20 – 3.41	---	---	---	---	----
Lactate (mmol/L)	<11 mo.	3.31	51	1.34 – 8.84	---	---	---	---	----
	11+ mo.	10.88	10	6.57 – 15.3	---	---	---	---	----
Albumin (g/dL)		0.85	11	0.50 –1.00	18.4	---	---	---	---
TP (g/dL)	<11 mo.	1.41	44	0.14 – 7.34	---	---	---	---	----
	11+ mo.	2.00	9	0.96 – 8.29	---	---	---	---	----
Globulin (g/dL)		0.72	9	0.11 –3.09	89.7	---	---	---	---
Albumin/globulin		1.13	9	0.28 –6.50	114.7	---	---	---	---

Table 2.35. Supplemental to Table 2.34 for juvenile herring, using only control fish with no lesions for variables significantly impacted by the presence of lesions. No time zero juvenile fish were examined by post-mortem so their gross lesion status was unknown.

All controls - no gross lesions				
	Median	N	95th PE	CV
Hct (%) < 11 mo.	19.5	65	0.81 – 48.2	62.2
Lysozyme (µg/mL)	2.12	27	1.88 –2.34	5.9

Table 2.36. The median, 95th PE, and CV for a profile using all control adult herring *vs.* one using only the time zero controls. PE=percentile estimation, CV=coefficient of variation, LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, Hct = hematocrit, Lct= leucocrit, ALP=alkaline phosphatase. Applicable for adult herring ranging from 16.5 – 117.4 g and from 12.9 –24.0 cm. Variables are subdivided for holding time (T_H) differences in days (d) where appropriate

Variable	T_H (days)	All controls pooled				Time zero controls			
		Median	N	95th PE	CV	Median	N	95th PE	CV
CF	19 d	0.89	129	0.67 – 1.04	10.8	---	---	---	---
	78 d	0.79	31	0.60 – 1.02	10.5	---	---	---	---
	90 d	0.91	75	0.69 – 1.25	14.3	0.95	30	0.90 – 1.02	12.1
	124 d	0.69	108	0.51 – 1.14	20.2	0.78	31	0.47 – 1.39	5.1
	153 d	0.99	30	0.88 – 1.43	11.5	0.99	30	0.88 – 1.43	1.5
LSI	19 d	0.87	49	0.42 – 1.66	28.6	---	---	---	---
	78 d	0.78	31	0.19 –1.14	20.1	---	---	---	---
	124 d	1.00	99	0.51 – 2.10	33.7	0.77	22	0.46-1.46	31.2
SSI	All -124 d	---	---	---	---	0.12	22	0.02 – 0.30	58.9
	19 d	0.20	49	0.04 – 0.41	50.7	---	---	---	---
	78 d	0.09	31	0.04 – 0.26	45.0	---	---	---	---
	124 d	0.10	72	0.02 – 0.34	64.1	---	---	---	---
GSI	19 d	0.52	49	0.05 – 6.23	131.4	---	---	---	---
	78 d	0.32	31	0.00 – 24.9	258.3	---	---	---	---
	124 d	0.79	60	0.26 – 24.9	258.3	0.88	10	0.71– 27.21	238.7
Hct (%)	All	---	---	---	---	39.0	89	6.6 – 57.7	31.1
	19 d	35.3	125	5.3 – 59.1	42.1	---	---	---	---
	78 d	41.1	31	27.8 – 55.0	16.7	---	---	---	---
	90 d	40.5	75	23.2 – 52.7	18.9	---	---	---	---
	124 d	39.2	108	8.5 – 62.3	34.3	---	---	---	---
	153 d	39.0	28	19.3 – 57.8	27.2	---	---	---	---
Lct (%)	All	1.00	367	0.0 – 3.97	82.3	---	---	---	---
	PS (90 d)	---	---	---	---	0.68	30	0.03 – 1.39	60.7
	124 d	---	---	---	---	0.56	31	0.0 – 4.63	120.0
	153 d	---	---	---	---	2.03	28	1.35 – 2.93	49.1

Variable	T_H (days)	All controls pooled				Time zero controls			
		Median	N	95th PE	CV	Median	N	95th PE	CV
Lymphocytes (%)	90 d	31.1	60	6.8 – 57.3	39.4	31.9	30	6.92–58.62	4.2
	124 d	54.6	27	24.1 – 80.0	33.4	---	---	---	---
Neutrophils (%)	90 d	22.6	60	1.8 – 73.4	61.7	24.0	30	1.8 – 77.2	64.1
	124 d	29.0	27	0.0 – 61.5	61.4	---	---	---	---
Thrombo-cytes (%)	90 d	45.1	60	3.2 – 73.9	37.8	42.5	30	1.8 – 74.0	43.9
	124 d	16.3	27	5.0 – 25.9	41.6	---	---	---	---
Glucose (mmol/L)	78 d	11.9	31	6.5 – 20.4	25.6	---	---	---	---
	124 d	7.8	86	3.5 – 18.5	46.0	10.5	10	5.8 – 14.4	24.9
Lactate (mmol/L)	78 d	4.6	31	2.3 – 8.9	35.5	---	---	---	---
	124 d	2.1	67	0.7 – 23.4	116.7	1.78	10	1.08 – 3.15	31.0
Albumin (g/dL)		0.77	62	0.21 – 12.2	157.5	0.41	8	0.15 – 1.10	59.3
Total protein (g/dL)		4.64	66	0.36-11.46	47.0	3.64	8	0.83 – 5.30	6.8
Globulin (g/dL)		3.72	61	0 – 4.79	47.7	2.90	8	0.48 – 4.82	3.8
Albumin/globulin		0.20	61	0 – 99.9	379.2	0.16	8	0.04 – 0.73	6.7
Cortisol (ng/mL)		12.6	59	4.0 – 30.2	47.6	11.3	10	4.9 – 18.9	47.7
Chloride (mEq/L)		119	48	75 –171	18.8	125	10	98 – 150	14.5
Phosphorus (mg/dL)		2.72	50	0.94 – 4.55	38.1	3.16	8	2.66 – 4.62	18.0
Calcium (mg/dL)		18.4	50	12.9 –33.1	24.1	15.9	10	13.9 - 18.6	8.9
ALP (U/L)		20.7	9	3.8 – 27.6	38.9	---	---	---	---
Lysozyme (µg/mL)	19 d	1.01	127	0.07 – 1.44	---	---	---	---	----
	78d	0.04	26	0.02 – 0.08	---	---	---	---	----
	90d	1.26	68	0.67 – 14.01	68	10.07	24	7.96– 20.03	22.2
	124 d	2.59	86	1.93 – 17.65	92.3	2.21	10	2.12 – 2.71	9.8
Macrophage (mø) count		1.33 x 10^7	7	1.18 x 10^7 – 2.59 x 10^7	33.2	1.48 x 10^7	10	3.13 x 10^6 – 3.46 x 10^7	58.0
Yeast/ mø	19 d	2.32	49	1.13 – 4.07	34.8	---	---	---	---
	78 d	3.46	31	1.63 – 11.0	54.4	---	---	---	---
	90 d	2.91	23	0.11 – 6.65	77.4	5.03	7	4.00 – 6.65	19.1
	124 d	2.78	60	0.75 – 3.95	31.8	---	---	---	---
	153 d	---	---	---	---	1.27	10	0.68 – 1.98	32.2
% Phagocytosis		81.2	9	76.47 –87.3	3.96	---	---	---	---
Respiratory burst		0.20	31	0.03 –1.36	102.7	---	---	---	---

2.5. DISCUSSION

Reference ranges are available for hematological, blood biochemistry, and immunological parameters for many species. This facilitates evaluating responses to environmental stressors, including toxicant exposure. Establishing reference ranges for captive Pacific herring proved to be challenging. Ideally, normal ranges are based on a sample of healthy individuals that are not compromised in terms of their potential to provide baseline information (Brunden *et al.,* 1970; Sandnes *et al.,* 1988). However, defining 'normal" for wild fish populations, such as Pacific herring, is difficult due to inherent capture bias towards less fit individuals, stress (changes in plasma biochemistry and immunosuppression) and injury associated with capture, transport and holding, and a lack of knowledge of their pathogen and toxicant exposure history. These aspects of the transfer of wild herring into captivity had the potential to influence the reference ranges of the measurement variables in this study.

At the BMSC, fish did not thrive over periods of several months or longer, as revealed by the high, pre-experimental mortality rates and by the presence of skin lesions. Scale loss and abrasions were commonly seen. Measures were taken where possible to decrease mortality by limiting the density of fish during transport, supplying supplemental oxygen, using saltwater ice to maintain transport water at a temperature and salinity comparable to that of the sea storage pens, reducing vibration during transport and minimizing transport time. Nevertheless, it is possible that survivors used to determine reference ranges (control fish) were not a cross-section of the wild population. Pre-experimental mortality may have selected for more fit survivors, or more likely, may have resulted in mostly fitter survivors remaining plus a small percentage of unwell fish. Regardless, pre-experimental mortality during the overall holding period (including acclimation) had the potential to bias the reference ranges determined from using the surviving fish. The approach taken to determine reference ranges was conservative and inclusive rather than exclusive to restrict ranges and thus contributed to broad ranges for the majority of variables.

The mortality rate within a group was used as an indicator of the overall health of those individuals. There was the potential for mortality during collection, transport, transfers to tanks, holding and acclimation periods and during the experiments, and so differences could have existed between populations and age classes. However, crude mortality

rates prior to starting experiments were not statistically different between populations or age classes and were, on average, 1.68 to 1.97%/day; with up to 10-fold variation within each population/age group. This implies that background pre-experimental mortality was comparable and therefore not a confounding factor on variables. The ongoing mortality in this study suggested that capture and holding herring in captivity could be a sufficient stressor in itself and may be detrimental to herring health (supported by the loss of body mass over time) and the cause of mortality was not known. Therefore, differences in the cause of death among populations and age classes may have had a differential effect on the immune status of the surviving fish and on reference ranges.

Captivity has been reported to reduce feeding and to alter condition indices, and hematological and biochemical variables in other fish species. Cunners (*Tautogolaburs adspersus*) maintained in captivity showed decreased feeding and CF (Walton *et al.,* 1983), while plaice (*Pleuronectes platessa*) showed hyperglycemia and elevations in Na^+, K^+ and Cl^-, Ca^{2+} and Mg^{2+} of varying duration (Bourne, 1986). Confinement stress in dogfish *(Scyliorhinus canicula*) held for 4 d in 100 L closed circulation tanks resulted in decreased Hct and Lct (Torres *et al.,* 1986). In captive herring, parasite loads, trauma from bumping into tank walls, pre-existing infection with pathogens, confinement-induced VHSV, and inadequate nutrition may have contributed to morbidity and mortality. Sea lice (*Caligus sp.*) were observed on the skin and gills, and peritoneal cavity worms (*Anisakis sp.*) were found within the peritoneal cavity, on the surface of the liver and adjacent to intestines in some fish. Some lesions were consistent with trauma and/or pathogen damage, and some fish did not feed as readily as others. It is possible that full acclimation was never reached prior to an individual fish entering into an experiment and that the fish experienced conditions prior to the experiment that reduced their overall health status and affected reference ranges. However, it was beyond the scope of this work to determine definitive etiologies for mortality.

Several studies (Munkittrick and Dixon, 1989; Shuter, 1990; Sibly, 1996; Power, 1997; Stehr *et al.,* 2004) have emphasized the importance of examining fish of different ages with criteria specific to that age group. In addition, age affects the normal ranges of hematological, biochemical and immune function measures in other fish species (Dethloff *et al.,* 2001; Svetina *et al.,* 2002; Duffy *et al.,* 2003; Khallaf *et al.,* 2003). This study validated this age effect for Pacific herring by showing that hematological, biochemical and immunological measures varied significantly with age. The fact that the

median values of most variables differed significantly between age classes supported separate analyses within an age class.

Age-dependent changes suggested that the adults were probably in better overall health, having a higher CF, Hct, Lct, total plasma protein, globulin and macrophage cell count (all variables in which changes can have a functional consequence). In juvenile fish, the lower total protein, globulin and Lct compared with adult fish had the potential to increase their susceptibility to disease. However, phagocytic activity of juvenile macrophages and plasma lysozyme activity was higher than in adults. It was possible that the macrophages of juvenile herring were more efficient, perhaps because they had recently experienced more primary immune responses compared with adults that had immunological memory. Marty *et al.* (2003) found age-dependent differences in macrophage aggregates in several rockfish species in PWS. The higher plasma lysozyme concentration in juvenile herring is suggestive of an up-regulation of this component of the non-specific immune system (Mock and Peters, 1990). Again, this may have been a response to antigenic stimulation. In contrast, juvenile Japanese medaka (*Oryzias latipes*) injected with the polychlorinated biphenyl congener 126 had less antibody forming cells than similarly treated aged fish thereby showing a reduction in specific immunity (Duffy *et al.,* 2002). While it appears that juvenile herring have a greater non-specific immune response than adult fish, their specific immunity may not be as effective as adults if their antibody forming cells respond similarly to those of juvenile medaka.

Gender has been associated with significant differences in condition indices, biochemical and hematological parameters, and disease prevalence in other fish species. CF was lower in female lake whitefish (*Coregorius clupeaformis*) exposed to heavy metals (Cooley *et al.,* 2002) and GSI was lower in female rainbow trout exposed to pulp and paper effluent compared with males (van den Heuvel and Ellis, 2002). Blood chemistry profiles varied in feral lake trout (*Salvelinus namaycush),* with calcium lower in males and liver enzymes lower in females (Edsall, 1999). Hct and leucocyte counts were lower in female rainbow trout (Miguel *et al.,* 1988), similar to the findings for the herring in this study. Hct and Lct were lower and SSI and cortisol were higher in female herring compared with male herring. These gender differences in herring suggested that gender might need to be considered when these variables are examined in addition to

examining parameters separately for age. However, in the present study, gender bias was minimized, as gender ratios were similar among treatment groups.

In other fish, population origin has been reported as a source of variation (Niimi, 1990; Kortet *et al.,* 2002; Stehr *et al.,* 2004). In Nile tilapia (*Oreochromis niloticus*), significant differences in CF were found in fish from distinct geographic locations, but there was concurrent variation in size, which determines CF (Khallaf *et al.,* 2003). This was similar to the findings in herring. Differences in several variables occurred among herring from different source populations in both juveniles and adults. However, there were also significant differences in size among populations, which confounded the assessment of the variables shown to differ with size. Sample sizes were not large enough to adequately control for size and consequently differences in variables due to population alone could not be established definitively. There were no population differences in the variables studied in juveniles that could not be attributed to size alone, except the percentage of thrombocytes. In adults, all population differences, with the exception of Lct, thrombocytes and lymphocytes, may also have been purely attributable to size alone because the variables had been shown to vary with size. Lct was significantly lower in PS adults compared to the other two populations. Further examination of the gender-identified adults showed that this difference in Lct was significant in males, but not in females. The prevalence of lesions did vary significantly among populations for each age class (Table 2.18). This may also be attributable to a variation in size as both body mass and length were associated with lesion prevalence. However, because the prevalence of skin lesions specifically varied between populations for both age classes ($p<0.0001$) it may be that differences due to capture and handling efficiency also played a role.

In this study, Hct was also significantly different between sources of male adult herring that were of comparable size, and was considered a true population difference. However, this difference in Hct was considered insufficient evidence to conclude that the BS, QI and PS populations required separate reference ranges for all variables because none of the other variables differed significantly. Therefore, Lct and Hct (both in adult males) and thrombocytes (only in juveniles) were the only variables that can be recognized as being population-specific based on the results of this study with a fairly narrow spread between populations for Hct and Lct but a > 2-fold variation in

thrombocytes. Thus, reference ranges were not specifically divided for population source for the majority of the variables. However, differences in holding time, which varied among experiments and thus populations, did necessitate subdivision of the reference range profiles for several variables. This occurred in both the pooled profile and the time zero profile. Therefore, data from the source populations were pooled for some variables and separated for others for both juvenile and adult herring. Experimental exposures did not mix population sources and hence, for the most part, size and source did not vary among treatment groups. It is possible that more variables are truly population-specific but larger samples of size-matched herring in both age classes for each source population with identical holding times would be needed to determine this.

While regional differences in all plasma biochemistry variables were clearly demonstrated, differences in handling or capture methods may also have contributed to differences. The data suggests that these PWS herring were more stressed than those from the PNW at the time of sampling, based on the direction and magnitude of the difference in some values (higher plasma lactate and chloride levels). In addition, cortisol levels for PNW herring were low (Table 3.26). PWS fish were sampled within 4 hours of purse or beach seine or gill net capture (Marty *et al.*, 1998), unlike PNW herring, which were transported back to holding tanks, and sampled after a holding and acclimation period. Therefore, if anything the PNW reference range may be more representative of unstressed fish than their PWS counterparts. The contribution of fish size to these differences in biochemistry could not be evaluated because morphometric data were not available for the PWS group for comparison. Therefore, although these median plasma biochemistry values suggest that PWS and PNW herring should be considered separately, uncertainties exist which warrant further study. Comparison of the plasma biochemical reference ranges for the PNW herring in this study with those that are published for herring showed overlap between the ranges although the ranges are generally wider in the PNW herring (Table 2.3, Marty *et al.*, 1998).

The prevalence of lesions and low Hct are considered potential indicators of disease. Pathological gross lesions can indicate trauma, inflammation, and/or infection and may imply that an individual is compromised physiologically (Kent and Fournie, 1993; Vethaak, 1996; Khan, 2003). Therefore, it was hypothesized that herring with either

lesions or low Hct had the potential to significantly affect the variability in the measurement variables that were used in the present study. Furthermore, it was hypothesized that the presence of skin lesions, had the potential to result in either an elevation or reduction of Hct due to either dehydration or blood loss. In the former, there is an increase in the concentration of blood components due to an influx of ions and loss of water, while in the latter a decrease in Hct is expected (Khan *et al.,* 1980; Rodger and Richards, 1998; Law, 2001; Nylund *et al.,* 2003).

I found that the presence of erosive, hemorrhagic skin lesions was associated with significant increases in Hct in younger juvenile herring, thus supporting the dehydration hypothesis, not blood loss, in these moribund fish. Damage to skin can lead to dehydration if critical cell membrane damage occurs disturbing the homeostasis of fluid and ionic balance (Law, 2001). Dehydration has also been observed in starry flounder *(Platichthys stellatus)* due to suppression of drinking following exposure to ionic silver (Hogstrand *et al.,* 1999).

Erosive, hemorrhagic skin lesions were also associated with greatly elevated lysozyme in juvenile herring. Lysozyme is found in the mucus layer that overlies the skin and is considered a first line of defense (Ellis, 2001). Therefore, the elevation in plasma lysozyme in juvenile herring with skin lesions may be a protective response against pathogens. The presence of skin lesions resulted in different responses among juveniles compared with adults. Hemorrhagic, erosive skin lesions elevated Hct in juvenile but not adult herring. This suggests that adult herring are probably more resistant to dehydration when skin integrity is broken down. This may simply reflect a more favourable surface area:volume ratio for adults *vs.* juveniles as the size of lesions relative to the total surface area was greater for juveniles. Alternately, adult herring may have the capacity to up-regulate thrombocyte production in response to skin lesions and withstand the deleterious effects of skin damage and scale loss because thrombocytes are important in the clotting and healing processes in fish (Murad and Houston, 1988; Fänge, 1992).

The bimodal distribution of lymphocytes in the <11 month old PS fish occurred concurrently with a high prevalence of skin lesions and was accompanied by a greater percentage of thrombocytes compared with the < 11 month old BS fish that had no skin

lesions. As elevation in lymphocytes can indicate inflammation (Slauson and Cooper, 1990), it is possible that differences in the severity of lesions, and hence the inflammatory response, could account for the bimodal lymphocyte distribution. However, there was no concurrent histopathology to confirm this possibility. Assessment of gross lesion status was only conducted at the time of sampling. Therefore, some individuals that were classified as lesion-free may have had gross external or internal lesions during the captive period that went undetected and were grossly resolved by the time of sampling. This could underestimate the prevalence of lesions and their effect on measurement variables.

The evidence linking skin lesions with elevated Hct and plasma lysozyme in juvenile fish could be a useful diagnostic tool in the future to evaluate if skin lesions are impacting fish. Intuitively, it would seem that lesions causing holes and erosions of the skin would result in the loss of RBCs and decreased Hct. Yet, this was definitely not the case with my juvenile herring. The juvenile fish in my experiments responded to the skin damage on some level (possibly by splenic release or increased hematopoiesis overcompensation or water redistribution). Therefore, at some threshold, skin lesions are non-lethal but are associated with a response at the physiological level. The increase in lysozyme concentration signifies activation of a first-line immune response to ward off infection.

All internal lesions seen in juvenile herring involved an abnormal colour of the liver, which may have been due to hemosiderin within melano-macrophage aggregates (Hinton and Lauren, 1990). Hemosiderin is a byproduct of degraded hemoglobin and its occurrence can be consistent with blood loss and damaged red blood cells (Khan, 1995, Agius and Roberts, 2003). In adult fish, internal lesions included abnormal liver colouration, white focal nodules in the liver, kidney abnormalities, and ascites. LSI was significantly elevated in adult herring with liver lesions, which is indicative of liver hypertrophy that could be associated with infiltration of the liver with inflammatory or other cells (Hinton and Lauren, 1990; Wester and Canton, 1992; Boorman *et al.*, 1997; Mikaelian *et al.*, 1998).

The increase in lactate to between 6.5 to 23.6 mmol/L seen in the upper 25th percentile of adult herring may reflect either muscle exertion that occurred as a handling response or be indicative of hypoxia (McDonald and Milligan, 1992). Since fish were sampled

quickly and increases in lactate due to handling reach a peak over 1 h, hypoxia was more likely responsible for the increases in plasma lactate concentration. The statistically significant elevation in albumin in adults with internal lesions could reflect dehydration as this plasma protein aids in determining oncotic pressure and influences hydration status in mammals (Duncan and Prasse, 1986), yet a concurrent significant increase in Hct was not apparent. Moreover, in rainbow trout, it has been shown that plasma oncotic pressure is not a factor in the transcapillary, fluid balance such that a change in albumin would not result in dehydration (Olson *et al.,* 2003). It is unknown if the same is true in herring, but if so then elevated albumin could be due to its increased synthesis in the liver (Sandnes *et al.,* 1988).

Despite the statistically significant impact of lesions on the median value of Tier 1 and 2 variables, the presence or absence of gross pathology (lesions) had no impact on the 95th PE reference ranges for most variables. Only the reference ranges of Hct and lysozyme in the <11 months old juveniles were affected by the presence of lesions. Thus, the reference range profiles included fish with lesions although a supplemental range is given for <11 month old juveniles for Hct and lysozyme using only lesion-free fish.

Baseline ranges for Hct does vary considerably between species. They can range from 1% in Antarctic icefish species *(Chionodraco kathleenee and Gyodraco antarcticus)* (Wells *et al.*, 1990) to >50% in actively swimming species such as the mackerel (*Scomber scombrus*; Fänge, 1994). In a study of 52 teleost species, values of 41.0%± 14.3 were reported (Wilhelm-Filho *et al.*, 1992) and 23 to 33% is considered the normal range for Hct in rainbow trout (Gallaugher *et al.*, 1995). Baseline Hct ranges have not previously been established for Pacific herring but teleosts usually have Hct values >20% (Gallaugher and Farrell, 1998; Table 2.3). Hct of 20% allows sufficient O_2 carrying capacity to maintain cardiovascular homeostasis in teleosts. Therefore, it suggested that the lower Hct mode (12.8%) in juvenile herring might signify an anemic subset of fish as a low Hct generally means an underlying anemic condition (Duncan and Prasse, 1986; Lim and Klesius, 2003).

Anemia has been extensively studied in the starry flounder (Wood *et al.*, 1979), in which Hct of 6% was considered moderately anemic and was observed frequently, with many

fish <20%, but only 1/97 below 6%. In induced-anemia experiments, flounder with a Hct of 5% had to make extreme compensations to maintain resting O_2 consumption by increasing arterial oxygen tension to maintain O_2 transport. It was suggested that this mechanism could only be expected in benthic fish as they can saturate their hemoglobin at low arterial oxygen tension when at rest (Wood *et al.*, 1979). Based on this information, it seemed reasonable to hypothesize that herring, a mobile, pelagic, species cannot compensate to the same degree as flounder and that low Hct values (<13%) did signify anemia.

The key physiological consequence of anemia is reduced O_2 carrying capacity, and thus reduced oxygenation of all tissues occurs if cardiac output is not increased to compensate (Gallaugher *et al.*, 1995). In rainbow trout, anemia increased resting heart rate and cardiac output while decreasing exercise performance and O_2 transport (Gallaugher *et al.*, 1995; Gallaugher and Farrell, 1998). Similar findings were reported in brown bullhead (*Ameiurus nebulosus*) following experimentally induced anemia (Gilmour *et al.*, 2003). In a schooling fish such as herring, these cardiovascular responses secondary to anemia could mean a decreased capacity to maintain swimming speed of the school, which would increase predation risk (Billerbeck *et al.*, 2001). Anemia also has the potential to be associated with changes in other variables because this indicator of abnormal red blood cell production or blood loss implies a lower overall condition, physiological compromise, and increased disease susceptibility (Lim and Klesius, 2003). For example, anemia occurred in conjunction with altered plasma biochemistry and WBC differential counts in roach exposed to bleached kraft pulp and paper mill effluent (Jeney *et al.*, 1996) and in conjunction with reduced *in vitro* macrophage chemotaxis in channel catfish (*Ictalurus punctatus*) when macrophages were exposed to the exoantigen of *Edwardsiella ictaluri* (Lim and Klesius, 2003).

If the lower Hct mode represented anemia in juvenile herring, then it seems reasonable to hypothesize that this may be associated with alterations of other variables in herring. However, few variables were significantly associated with low Hct. In fact, other than significantly lower Lct and a greater prevalence of undetectable Lct in the <11 months old juveniles with low Hct, and lower plasma lactate in the 11 + months juveniles, there were no significant differences in the other variables. Therefore, although lower Lct in the younger juveniles had the potential to increase disease susceptibility, there were no

other adverse consequences associated with low Hct. It is possible that the low Hct and Lct indicated severe hemodilution or poor haemopoeisis.

Age-dependent hematopoietic development could be an alternate explanation for the low Hct and Lct seen in many juvenile herring. The absence of a lower Hct mode in the older juveniles (11 + months) may indicate that hemopoeisis was fully developed in this sub-group, but not in the younger sub-group. The younger sub-group (< 11 months) had two distinct modes for Hct suggesting the capacity to produce red blood cells may have been lower in some fish. The 11 + month sub-group had no skin lesions to explain the higher Hct. In carp, Hct and hemoglobin values were shown to be age-dependent with a lower Hct and hemoglobin observed in the younger fish (Svetina *et al.*, 2002). It appears that a similar age dependency for Hct may also exist in juvenile herring. The overall lower Hct in juveniles relative to adult herring also supports reduced hematopoiesis as a partial explanation for the observed differences with age and size. Lct would also be expected to be lower in juveniles relative to adults if hematopoiesis were the sole explanation for the Hct differences, but this was not the case. Therefore, it can be concluded that low Hct in juvenile herring is likely a function of both age/size plus anemia of unknown causes.

Hct was significantly correlated with several variables in both age classes of herring. Lactate was the only variable that was correlated with Hct in both age classes, but positively for juveniles and negatively for adults. Covariance of other variables (LSI, Lct, lactate, and lysozyme in juveniles; SSI, lymphocytes, thrombocytes, glucose, lactate, chloride, globulin and phagocytosis in adults) with Hct could influence detection of a treatment effect in these variables if significant differences in Hct exist between treatment groups regardless of the cause. The changes seen in the aforementioned variables in conjunction with increasing Hct are all indicators of ill health supporting an association between increased Hct and the presence of skin lesions as previously discussed.

Differences in T_H and T_E were correlated to the variability in some Tier 1 and 2 variables in both juvenile and adult herring. In addition, there was significant interaction between these two time factors for both age classes. The variables (Hct, Lct, plasma glucose and lactate, and yeast phagocytosed per macrophage) common to both ages of herring that

were significantly affected are some of the standard measures of secondary stress. It may be that the instability in these variables over time reflected an intermittent secondary stress response. As a social, schooling fish it is feasible that holding herring in tanks in smaller numbers than would occur under natural conditions places an ongoing strain on the fish as reflected in the changes seen in controls with time.

The reference range profiles that have been established for juvenile and adult herring were compared with the published values for other fish species, and adult herring where known. Juvenile herring showed wider ranges of Hct and Lct with a lower minimum Hct and a higher maximum Lct (Table 2.4). The ranges for plasma glucose and lactate overlap with those of rainbow trout (Nielsen *et al.*, 1994), but only in the < 11 month old sub-group. The older juvenile herring have lower glucose and higher lactate ranges compared with juvenile rainbow trout. The range for total protein overlaps with that of juvenile snapper for both age sub-groups of herring, although the ranges in herring are wider (Table 2.4). It appears that in general the reference ranges for the above variables are wider in herring than in the other fish species.

The condition indices ranges in adult herring in this study are generally comparable with those seen in other herring studies (Elston *et al.*, 1997), with dab (*Limanda limanda*, Tahir *et al.*, 1993; Hutchinson and Manning, 1996) and with catfish (Martin and Black, 1998) and overlap with rainbow trout (Barnhart, 1969). The Hct ranges for herring held for 19 d or 124 d were considerably wider, with a lower minimum value than for all other species except dab (Table 2.36, Table 2.3), while the Hct range for the other groups of herring were comparable to those reported in other fish species. Literature Lct ranges are somewhat sparse but the median values in herring are comparable to the means reported for Atlantic salmon (*Salmo salar*, Sandnes *et al.*, 1988) and roach (*Rutilus rutilus*, Jeney *et al.*, 1996). The range in lymphocytes in herring overlaps with that of several fish species (Table 2.3), but a lower minimum value is evident in herring. Neutrophils show a considerably wider range compared with Atlantic salmon (Blaxhall and Daisley, 1973) but similarity with dab (Secombes *et al.*, 1991) and bass (*Dicentrarchus labrax*, Alvarez-Pellitero and Pintó, 1987), while the range in percentage of thrombocytes in herring is greater than in dab (Secombes *et al.*, 1991) and rainbow trout (Lamas *et al.*, 1994).

Reference ranges for adult PWS herring have been previously published for plasma glucose, total protein, albumin, chloride, ALP, calcium and phosphorus for fish sampled immediately upon capture *via* gill net, beach seine or purse seine and MS-222 anesthesia (Marty *et al.*, 1998, Table 2.3). The T_0 reference ranges for the current study show similar ranges in plasma glucose, albumin and calcium compared with the PWS herring, overlap in total protein but with a wider range, overlap in plasma chloride and phosphorus but with lower values, and show a considerable reduction in ALP compared with the PWS fish. Examination of the T_P reference range shows an increase in the range for total protein, albumin, and calcium compared with PWS herring, emphasizing that additional time in captivity increases the variability of these variables. Relative to other fish species, the ranges in plasma biochemistry variables are comparable with, or overlap, those of rainbow trout, Atlantic salmon, dab, and pike but are generally of greater width (Table 2.3). Plasma lysozyme in adult herring was lower in this study than the values previously reported for herring (Fänge *et al.*, 1976). In summary, while the median values for herring in this study were similar to other fish species, and to other herring studies, for the most part they demonstrated wider ranges.

It can be concluded that age, size, gender to some extent, skin lesions (<11 month juveniles only), holding time, and experimental time contribute to population variability. The following recommendations are intended to control for these factors during future experiments with herring:

1) Comparison between groups of herring during experimental exposures requires that the age/size of the fish be homogeneous among treatment groups. Ideally, controlling for size (especially juveniles < 1 year of age) should be done at the time of experimental set up by grading based on length/mass. However, due to the adverse impact that additional handling has on herring this is not always practical. Therefore, alternately an assessment of homogeneity of size among treatments groups should be performed for each exposure experiment to determine if size differences exist among groups and should be controlled for during analysis.

2) Gender ratio differences among adult groups can confound Hct, Lct, SSI and cortisol, especially if sample sizes are small. Therefore, gender should be identified by post-mortem, if possible, when these variables are measured to allow gender comparison.

Alternately an equal number of male and female fish can be used for pooled analyses or gender-controlled for by stratified analysis.

3) Ideally, juvenile herring with visible lesions should not be used in experiments. However, lesions may develop after an experiment has started. Therefore, to minimize any confounding effects, the prevalence of gross skin lesions in experimental exposures should not differ significantly between treatment and control groups when evaluating Hct and lysozyme, as these variables can change significantly in association with skin lesions. If the prevalence of lesions varies among treatment groups then either herring with these lesions could be excluded to prevent potential confounding of treatment effects or the results need to acknowledge the difference in lesion prevalence. In this study, significant differences in lesion prevalence among treatment groups of juvenile experimental fish were recognized when significant differences in Hct or lysozyme were observed.

4) The distribution pattern of Hct in juvenile herring should be compared among treatment groups as changes in modality may be more informative than changes in measures of central tendency (median and mean). Future studies with juvenile herring should continue to delineate the age/size-dependent variation in Hct and Lct observed in the current study.

5) Differences in the length of T_H (from capture to initiation of experiments) accounted for some of the variability in Tier 1 and 2 variables. Therefore, when conducting several experiments, T_H should either be identical to eliminate effects due to differences in captivity time prior to starting each experiment or be included as an analytical variable. Several variables in both age classes (Hct, Lct, plasma glucose and lactate, and yeast phagocytosed per macrophage) showed a significant interaction effect between T_H and T_E.

6) If T_H accounts for a significant proportion of the spread of a variable then it should be subdivided into reference ranges for each specific T_H for both T_0 and T_P reference range profiles. The T_0 reference profile (divided by T_H when appropriate) should be used to evaluate for captivity effects in control and exposed/challenged fish in

experiments over time and for differences in the extent of captivity effect between control fish and treated fish.

7) Variables that do not change with T_E, can be evaluated using the T_0 or T_P reference ranges (T_P has larger sample size and generally decreased variance) to assess response of exposed/challenged fish in experiments to a treatment. However, variables that do differ with T_E must be assessed only against the time-matched ranges of control fish within the specific experiment.

8) The pooling of T_0 controls between experiments can only occur if the T_H is the same. Future studies to identify the length of T_H at which variability stabilizes may result in establishing narrower reference ranges for the variables measured in the current study.

Variability was high in most of the hematological, biochemical and immunological variables studied in both age classes. This suggested that small changes in these variables during experimental exposures might not be detected statistically. Examination of factors affecting the median and 95th PE reference range for the variables measured identified those that are more and less stable in captive herring. The resulting reference range profiles provide data for investigators using PNW herring of similar age/size to determine if a specific fish falls within the 95th PE reference range. Despite the noted differences seen with age, size, gender, the presence of skin lesions, holding time and experimental time, it was possible that this study did not identify other important determining factors of normality in Pacific herring. Therefore, these profiles should only be used as general guidelines for comparing to other herring.

Chapter 3

Changes in immunotoxicological states in Pacific herring (*Clupea pallasi*) over time and in response to sub-lethal exposure to crude oil.

"What is it that is not poison? All things are poison and nothing is without poison.
It is the dose only that makes a thing not poison."
(Paracelsus, 1538)

3.1. INTRODUCTION

3.1.1. Pollution due to Petroleum Hydrocarbons

Five million tonnes of crude oil per year enters the marine environment globally (Neff, 1990). Sources of petroleum hydrocarbon pollution include marine transportation, offshore oil production, coastal oil refineries, sewage outfalls, natural seeps, and atmospheric fallout (Lee, 1977; Perry, 1980; Cerniglia and Heitkamp, 1989; Varanasi *et al.*, 1989; McElroy *et al.*, 1989; Albers, 1994; White *et al.*, 1994; Simpson *et al.*, 1996; Holladay *et al.*, 1998). While marine crude oil transportation, including losses during regular ship operations; oil spills due to accidents at sea and spills during operations at oil terminals are all key sources of petroleum hydrocarbon pollution, chronic leakage and pollution from natural seeps are also significant sources (Dey *et al.*, 1983; Betton, 1994; Spies *et al.*, 1996; Rudolph *et al.*, 2002).

3.1.2. *Exxon Valdez* Oil Spill (*EVOS*)

One of the worst spills of crude oil into a pristine marine environment occurred on March 24th, 1989 when the oil tanker, *Exxon Valdez*, ran aground on Bligh Reef in Prince William Sound (PWS), Alaska. The *Exxon Valdez* spilled between 42,000,000 and 46,000,000 L of Alaskan North Slope crude oil into surrounding waters (Norcross *et al.*, 2001; Brown *et al.*, 1996a; Barber *et al.*, 1995). It was estimated that 35% of the oil evaporated during the first two weeks after the spill, 40% oiled shorelines within PWS and 25% spread from PWS to the Gulf of Alaska (Galt *et al.*, 1991). By August 1989, 450 km of shoreline throughout western PWS was contaminated with 17,600,000 L of oil (Brown *et al.*, 1996b).

The initial spill led to the closure of the 1989 herring fishery and commercial fishing closures for all areas with visible evidence of oil contamination (Saxton *et al.*, 1993). The closures reflected a concern for food safety, as well as concerns for the potential adverse impact of fishing upon an already depleted herring population (Pearson *et al.*, 1999). The herring fishery was re-opened in the fall of 1989 and continued until the spring of 1993.

3.1.3. Exposure of PWS Pacific herring to crude oil from the EVOS

During the *EVOS*, all life stages of Pacific herring in PWS were potentially exposed to oil because adult fish were in the region at the time of the spill and they spawned only a few weeks after the spill (Carls *et al.*, 1997). The *EVOS* occurred approximately three weeks

prior to the peak in herring spawning in 1989 on April 14th. Eggs would have hatched after 10 to 21 days and larvae would have remained in the region for at least 70 days (see section 2.1.1)

The National Oceanic and Atmospheric Administration (NOAA) monitored hydrocarbon concentrations in PWS during 1989 and 1990 (Short and Rounds, 1993; Neff and Stubblefield, 1995; Short and Harris, 1996). Measurements made during the five weeks post-spill reported water concentrations of TPAH of 0.83 to 6.24 ppb near oiled beaches (Short and Harris, 1996) and 0.4 to 1.5 ppb at the margins of the spill trajectory (Pearson *et al.*, 1999). More specifically, the TPAH concentration of water samples was measured at 11 stations in PWS in 3 periods from 31st March to 4th April, 12th to 15th April and 2nd to 8th May. These water samples were taken at 1, 3, and 5 weeks after the spill, from near-shore sites (i.e. larval rearing areas), at 1 and 5 m depths and ranged from 0.83 to 6.24 ppb (Short and Harris, 1996). Concentrations in the second sampling period ranged from 0.83 to 4.00 ppb and by the third sampling period were only strongly attributable to *Exxon Valdez* oil (*EVO*) at 2/11 stations and only at 1 m depth in the range of 0.92 to 1.59 ppb (Short and Harris, 1996). The concentration of TPAH reported in open water areas in Montague Strait, PWS ranged from 0.91 to 1.11 ppb and was only detected during the first sample period (Short and Harris, 1996). Therefore, chronic exposure concentrations ranged from 0.83 to 6.24 ppb.

These post-*EVOS* TPAH exposure concentration ranges were much lower than reported acutely toxic concentrations of 200 ppb for eggs (Pearson *et al.*, 1985), larvae (2300 ppb), and adults (2200 ppb) (Rice *et al.*, 1987). Concentrations of TPAH peaked during April 1989 and were at background (pre-spill) levels by August 1989 (Pearson *et al.*, 1995). Short and Babcock (1996) reviewed data from water sampled at 8 sites in PWS from 1977 to 1980 and in 1989 before the *EVOS*. They concluded that petroleum hydrocarbons were typically not present in PWS water prior to the *EVOS* except for elevations in naphthalene and methylnaphthalene in 1989. The concentration of TPAH at background sites in PWS increased to 0.1 ppb during the month after the *EVOS* (Maki, 1991). As such, the *EVOS* resulted in a pulse oil exposure with levels of hydrocarbons that were greatest during the initial exposure (Pearson *et al.*, 1999).

Estimates of the distance covered by herring spawn (spawn length) affected by the *EVOS* vary between industry and Natural Resource Damage Assessment (NRDA) reports. Initial industry reports stated that 96% of the spawn length in PWS (158 km) was not oiled, and that <1% of its length (0.9 km) experienced moderate to heavy (visibly oiled) oiling (Pearson *et al.*, 1995). In contrast, NRDA estimates of non-oiled spawn length were notably lower at 56.4% (89.8 km), with 34.6% of the length oiled and 9% of the length being inadequately assessed to be categorized as oiled or not (Brown *et al.*, 1996b). A recent reassessment of the spawn length, spawn exposure, water TPAH concentration data from samples collected from late March to August 1989, and aqueous TPAH toxicity has been conducted by Carls *et al.* (2002). They concluded that 25 to 32% of the spawn biomass was exposed to TPAH concentrations that exceeded the lowest observed adverse effect concentrations (0.4 ppb) in laboratory experiments that exposed eggs for 16 days and that the *EVO* was biologically available in areas with no visible shoreline oiling. In addition, abnormalities observed in larvae after the *EVOS* (Marty *et al.*, 1997a) have been attributed to aqueous polyaromatic hydrocarbon (PAH) exposure and not direct oiling effects as originally hypothesized (Carls *et al.*, 2002).

While the concentrations of aqueous TPAH (0.83 to 6.24 ppb) following the *EVOS* were well below previously published toxicity thresholds (200 ppb for eggs) (Pearson *et al.*, 1999), evidence now exists to show acute toxic effects (malformation, mortality, ascites) occurring in larvae exposed as eggs to only 0.4 to 1 ppb (Carls *et al.*, 1999). Thus, there was potential for herring larvae to be exposed in large numbers (up to 32% of the spawn mass) to toxic concentrations (Carls *et al.*, 2002). The longevity of spilled petroleum hydrocarbons in coastal environments, plus the restricted inshore nature of the spawning grounds may have had the potential for significant effects on herring in this area.

In the first few years following the *EVOS*, there was no recorded decline in returning numbers of spawning herring in PWS. In fact, for the 3 years immediately following the spill (1990 to 1992) there were record high numbers of returning herring (Pearson *et al.*, 1995). By 1991, there was evidence of a herring population recovery in PWS, with the observed biomass (1.17×10^8 kg) being 20% greater than predicted (9.67×10^7 kg) (Funk, 1991). Historically, a strong year class occurs every four or five years in PWS (Funk and Sandone, 1990). The biomass returning in 1992 was 9.8×10^7 kg (Marty *et al.*, 1998). However, the adults returning in 1992 (1988 year class) were likely never

exposed to *EVO* given their behaviour and geographic location as 1+ year old fish (see Section 2.1.1.) at the time of the spill.

In contrast in 1993, (four years post-spill) drastic reductions in the number of returning herring were seen, with only one-third (4.5×10^7 kg) of the anticipated numbers returning to PWS (Meyers *et al,* 1994). By 1994, the biomass returning had dropped to 1.5×10^7 kg (Marty *et al.*, 1998). Those that returned were primarily 5-year olds (1988 year class). The population declines in 1993 resulted in a closure of the commercial seine fishery in PWS until 1997 (Quinn *et al.*, 2001). Since larvae were exposed following the *EVOS* in 1989 and were due to return to spawn at 4-years of age, the lack of a return of this 1989-year class in 1993 generated a hypothesized link to prior sub-lethal oil exposure.

Even so, several alternate hypotheses were put forward to account for the decline in the number of returning Pacific herring in PWS in 1993. These included 1) the direct effects of the oil spill in 1989, 2) declines in food biomass available to herring, 3) changes in water temperature, 4) increases in disease, and 5) increases in predation and harvesting effects (Pearson *et al.*, 1999). With the exception of the first hypothesis, all of these hypotheses could be a result of changes in natural cycles. Pearson *et al.* (1999) concluded that the 1989 oil spill did not contribute to the 1993 decline because of the record high population levels and harvests in PWS in the years following the spill, the lack of change from expected age class distribution and the low level of oil exposure documented for herring in 1989 and the following years. They concluded that natural factors, such as poor nutritional status, alone or in combination with disease, or other factors, were the most likely causes. However, eggs and larvae were exposed during the *EVOS*, and are now known to be sensitive to TPAH in the 0.4 to 9.1 ppb range (Carls *et al.*, 1997; Carls *et al.*, 1999), and the *EVOS* concentrations were in the 0.83 to 6.24 ppb range. Therefore, there was a possibility that toxic effects on herring eggs in 1989 were underestimated.

Mortality in herring populations is highest during their first winter as young-of-the-year due to starvation and predation (Stokesbury *et al.*, 2002). Therefore, while the *EVOS* may not directly be responsible for the declines in Pacific herring observed since 1993, its impact on the entire food chain within PWS may indirectly have resulted in either increased predation upon herring and/or a decrease in the food sources available to

them. Lowering of their overall nutritional status may have contributed to a reduction in the tolerance threshold for disease and their ability to avoid predators.

3.1.4. Crude Oil Toxicity

Crude oil is a complex liquid mixture of thousands of different compounds (Moles *et al.*, 1979), which can be classified into aliphatic, alicyclic, and aromatic hydrocarbons (Clark and Brown, 1977; Albers, 1994). Aliphatic hydrocarbons are open-chain compounds that can be either unsaturated or saturated. Alicyclic hydrocarbons contain some carbon atoms arranged in either an unsaturated or a saturated ring. Aromatic petroleum hydrocarbons contain at least one six-carbon benzene ring and include the polyaromatic hydrocarbons (PAHs) and comprise 0.2 to 7% of crude oils (Albers, 1994, McElroy *et al.*, 1989). The nonhydrocarbon constituents of crude oil include: oxygen (<3%), nitrogen (<1%), sulfur (0.1 to 10%), and varying amounts of heavy metals such as vanadium, nickel, iron and copper (Perry, 1980). For example, Prudhoe Bay Crude Oil (PBCO), produced on Alaska's North Slope has high-sulfur, high-nitrogen, and a high percentage of aromatics (Clark and Brown, 1977).

These various fractions of crude oil have different properties and toxicities. For example, relatively non-toxic non-hydrocarbons typically found in petroleum include cresols, xylenols, naphthols, carboxylic acids, quinoline and substituted quinolines, hydroxybenzoquinolins and substituted pyridines (Clark and Brown, 1977). Low molecular weight aromatic hydrocarbons such as benzene, toluene, and xylene are the most water-soluble, and they can readily dissolve and/or suspend in the water column (Khan *et al.*, 1995). The water-solubility of aromatic hydrocarbons can make these compounds lethal by direct contact or in dilute solutions, however due to a high volatility, they may evaporate rapidly (Neff, 1990).

In general, PAHs are hydrophobic and tend to bind to particulates and sediments, forming a PAH reservoir (McElroy *et al.*, 1989). It appears that much of the acute toxicity of crude oil is a function of its di-aromatic and tri-aromatic hydrocarbon content. It is generally agreed that the soluble aromatics of oil contribute the most to its acute aquatic toxicity (Anderson *et al.*, 1974). The potential for chronic toxicity is from non-volatile, persistent components of the crude oil such as PAHs (Perry, 1980; Varanasi, 1989; Livingstone *et al.*, 1992).

Many environmental factors determine the ultimate fate of PAHs in a crude oil spill and they include wave action, water currents, temperature, and winds at the time of the spill (Wasik and Brown, 1973; Clark and Macleod, 1977). Lighter, more volatile components tend to evaporate quickly, while the fate of the less volatile components is governed by emulsification, dissolution, photo-oxidation, biodegradation and uptake by marine organisms, and adsorption to suspended particles (Lee, 1977). In general, the concentration of toxicants decreases with time due to dilution, evaporation, and biodegradation (Moles *et al.*, 1979).

Residence time for some components of crude oil in the water column is up to 6 months. However, retention in coastal environments can vary from a few days to more than 10 years, depending on sediment and substrate characteristics (Albers, 1994). For example, one month after a spill of 2000 tons of Iranian crude oil, hydrocarbon concentrations of 0.2 to 0.3 ppm were detectable in seawater 30-km from the site of the spill. Following the *EVOS*, water column hydrocarbon concentrations peaked at < 10 ppb for one month after the spill (April) and were at background levels by five months post-spill (August) (Pearson *et al.*, 1999).

3.1.5. Crude Oil Toxicity to Fish

Crude oil can have a wide array of physiological, histopathological and behavioural effects on fish. Components of hydrocarbons can enter the circulatory system and lead to physiological changes such as osmoregulatory dysfunction, hyperglycemia (McKeown and March, 1978) and changes in blood chemistry (Payne *et al.*, 1978). Crude oil toxicity also includes decreased growth and reproduction (Kiceniuk and Khan, 1987), decreased "off-bottom" swimming activity (Berge *et al.*, 1983), histopathological changes (Khan and Kiceniuk, 1984), and structural changes to the liver (Mikaelian *et al.*, 2003). The immunosuppressive toxicity of the PAH fraction of crude oil is considered in detail in a subsequent section.

In the event of an oil spill, the immediate effect of oiling can lead to fish mortality due to anoxia from gill damage. The more gradual effects from oil exposure depends on the degree of uptake and excretion of oil components by an aquatic organism, which in turn varies with such factors as age, species and gender (Varanasi *et al.*, 1989). Larval and juvenile fish are generally more sensitive to xenobiotic exposure than their adult counterparts (Moore and Dwyer, 1974; McKim, 1985; Malins and Hodgins, 1981).

Petroleum hydrocarbons from an oil spill may persist long enough to cause significant larval mortality and reduced growth depending on the timing of the spill relative to spawning. For example, Marty *et al.* (1997b) observed ascites and premature emergence in pink salmon *(Oncorhynchus gorbuscha)* larvae exposed to oil-contaminated gravel. Species differences in toxicity are likely due to variation in the capacity to metabolize PAHs and in the tissue lipid percentage. In particular, the liver lipid content plays a role in toxicity as PAHs are primarily metabolized in the liver and this organ is a primary lipid storage site (Varanasi *et al.*, 1989). Similarly, differences in toxicity between male and female fish appear to stem from differences in PAH metabolism and differences in the accumulation of PAHs in the liver *vs.* the gonads during gonadal development (Varanasi *et al.*, 1989).

The route of chemical uptake by fish can be *via* either one or all of the following: gill, oral, and dermal absorption (Kennedy, 1995). Uptake of crude oil hydrocarbons *via* the gills has been observed and damage to fish gills is thought to be a good index of the impact of toxins and irritants on fish populations (Blanton and Robinson, 1973; Lee *et al.*, 1972, Brand *et al.*, 2001). Crude oil can specifically enter fish *via* the mucus cells of the gill epithelia (Prasad, 1988) and disrupt gill function. Haensley *et al.* (1982) have shown that mucus cell hyperplasia is a common response to crude oil exposure. An impairment of gill ion regulation leads to abnormal plasma electrolyte concentrations (McDonald *et al.*, 1989). Rainbow surfperch (*Hypsurus canji*) and rubberlip surfperch (*Rachochilus toxodes*) showed severe gill lesions when living near a natural petroleum seep (Spies *et al.*, 1996). In addition, gill citrate synthase and cytochrome C oxidase activities were affected by exposure to the water accommodated fraction (WAF) of oil in Atlantic salmon (Gagnon and Holdway, 1999). An increase in oxygen consumption was observed in sheepshead minnow (*Cyprinodon variegates*) exposed to South Louisiana crude and Kuwait crude at 19.8 ppm and 10.4 ppm, respectively (Anderson *et al.*, 1974). Exposure of pink salmon fry to the water soluble fraction (WSF) of PBCO increased ventilatory effort (Thomas & Rice, 1975). While the majority of studies have examined the uptake of oil *via* the gills, oral and dermal absorption may also occur. Accumulation of naphthalene in the skin of rainbow trout (*Oncorhynchus mykiss*) exposed to PAHs has been reported (Varanasi *et al.*, 1978) and Clay hoplo (*Hoplosternum littorale*) exposed orally to crude oil showed toxicity with impairment of ion regulation following exposure (Brauner *et al.*, 1999).

Histological changes following oil and PAH exposures have been observed in several fish species and in various organs including, the liver, kidney, gills, and spleen. Rockfish (*Sebastes spp.*) from previously oil-exposed sites in PWS had more hepatic fibrosis, megalocytosis, and macrophage aggregates than fish from reference sites in 1991 (Marty *et al.*, 2003). Flatfish species from PAH-contaminated areas had histological changes in the liver and spleen (Khan, 2003). English sole (*Pleuronectes vetulus*) developed toxicopathic liver lesions in association with sediment aromatic hydrocarbons. (Stehr *et al.*, 2004). Holladay *et al.* (1998) found histological changes in the head kidney of tilapia following exposure to benzo(a)pyrene (B(a)P), an oil component. Chronic exposure of winter flounder (*Pleuronectes americanus*) to crude oil resulted in gill hyperplasia, and hemosiderosis in the spleen, liver, and kidney (Khan, 1995). Liver neoplasia has been reported in English sole exposed to high concentrations of PAHs (Myers *et al.*, 1991) and in flounder *(Platichthys flesus)* from PAH-contaminated sites (Vethaak and Jol, 1996). In addition to histopathological changes, exposure to crude oil can also alter behaviour. For example, decreased schooling has been observed with experimental exposure of bream (*Abramis brama*) to 2600 ppb hydrocarbons (Patten, 1977) and an avoidance response was elicited at 700 ppb in crescent perch (*Therapon jarbua*), and striped mullet (*Mugil cephalus*; Patten, 1977) and at 3200 ppb in mature Pacific salmon *(Oncorhynchus sp.*; Weber *et al.*, 1981).

3.1.6. Crude Oil Toxicity in Pacific herring

The effects of oil exposure have been examined in Pacific herring eggs, embryos, larvae, and adults (Rosenthal and Alderdice, 1976; Smith and Cameron, 1979; Carls, 1987; Rice *et al.*, 1987; Marty *et al.*, 1997a). During the *EVOS,* eggs, larvae, and adults were exposed to oil (Carls *et al.*, 2002). The adverse effects of petroleum hydrocarbon exposure on Pacific herring eggs and larvae have been clearly demonstrated (Brown *et al.*, 1996b; Kocan *et al.*, 1996a; Kocan *et al.*, 1996b; Hose *et al.*, 1996; Norcross *et al.*, 1996; Marty *et al.*, 1997a; Middaugh *et al.*, 1998). Herring eggs and larval stages may be most at risk for direct and indirect exposure following an oil spill due to the tendency for oil to stay in shallow inshore waters and to penetrate below the surface. Eggs that hatch following exposure to crude or fuel oil produce deformed larvae (Perry,1980; Kocan *et al.*, 1996a). Eggs collected from PWS in 1992 from a previously *EVOS* oiled site had a lower percentage hatch and a greater incidence of morphological abnormalities than those from reference sites (Kocan *et al.*, 1996a). A comparison of herring studies by Pearson *et al.* (1999) suggested 200 ppb as the threshold for toxic

effects in herring eggs although the duration of exposures is not stated. However, Carls *et al.* (1997) found morphological defects in herring eggs at 9 ppb after a 21 to 28 days exposure to weathered oil.

Pacific herring embryos exposed to an oil-water dispersion (OWD) of PBCO containing 9.2 mg/L high molecular weight and 64 mg/L low molecular weight hydrocarbons resulted in significant physical deformities, genetic damage, reduced mitotic activity, lower hatch rate and weight, and premature hatching. This OWD closely resembles the mixture of oil and water that occurs after an oil spill (Kocan *et al.*, 1996a), although the actual concentration of TPAH was higher than that reported following the *EVOS*. Embryos placed in PWS 3 years after the *EVOS* produced 12% more abnormal larvae and larvae of lower weight at sites that were oiled in 1989 compared to un-oiled sites (Kocan *et al.*, 1996a). Both genetic and physical deformities were seen, as well as a 37.5% lower hatch weight, premature hatching and decreased mitotic activity.

Exposure of herring larvae to the WSF of crude oil at 1300 ppb resulted in high acute mortality at both 4 and 48 hours post-fertilization (Middaugh *et al.*, 1998). Exposure to 300 to 900 ppb WSF of PBCO resulted in 100% herring larval mortality within 21 days, decreased swimming ability (50% by day 4) and rapid reductions in feeding rate (50% by day 1), with TPAH concentration an order of magnitude greater than the reported concentrations following the *EVOS,* (Carls 1987). Abnormal larvae and premature hatch have also been reported following a 15 to 20 d continuous exposure to OWD of PBCO above 240 ppb (Kocan *et al.*, 1996a).

Herring fry exposed to 5000 ppb dissolved hydrocarbons have 70 to 100% mortality within 4 days. Rice *et al.* (1987) found that the LC_{50} for the WSF of Cook Inlet crude oil for exposures varying from 1.5 to 12 days was 2200 ppb for adult Pacific herring. Collectively these toxicity data suggest that eggs are the most sensitive life-stage for exposure to the WSF of crude oil as they are affected at concentrations at least an order of magnitude lower than larvae and adults. There is a general lack of information, however, regarding the effects of exposure on juvenile Pacific herring.

3.1.7. Immunosuppression due to PAHs

PAHs, as a constituent of crude oil, can be immunosuppressive in Pacific herring given the known effects in other species, but this has not been studied extensively. PAHs

have been indicated as mammalian immunotoxicants (Hodgins *et al.*, 1977; Holladay *et al.*, 1998). B(a)P and 7,12-dimethyl-benz(a)anthracene (DMBA) are two of the most commonly studied single PAHs. These PAHs are immunotoxic to both the humoral and cell-mediated immune systems and are potent carcinogens (Anderson *et al.*, 1995). Malmgren *et al.* (1952) first documented immunosuppression due to PAH exposure in mice. Mice exposed to B(a)P at 40 mg/kg for 7 days *via* subcutaneous injection exhibited a 50 to 66% reduction in polyclonal antibody response (Blanton *et al.*, 1986).

It has been demonstrated that constituents of crude oil are also immunosuppressive in a variety of fish species including flounder (Alkindi *et al.*, 1996), striped mullet (Thomas *et al.*, 1980), coho salmon (*Oncorhynchus kisutch;* McKeown, 1981), cutthroat trout (*Salmo clarki* ; Woodward *et al.*, 1983), Atlantic cod (*Gadus morhua*) and winter flounder (Dey *et al.*, 1983). Dunier and Siwicki (1993) suggested that chronic sublethal PAH challenge predisposes fish to disease. Both DMBA and B(a)P can decrease resistance to pathogens and neoplasia (White *et al.*, 1994). Exposure to DMBA by intraperitoneal (i.p.) injection at 1 to 100 mg/kg has been shown to be immunosuppressive in the oyster toadfish (*Opsanus tau;* Seeley and Weeks-Perkins, 1997). Macrophage phagocytosis activity was suppressed and nonspecific cytotoxic cell activity was almost completely inhibited in the toadfish. Similar to the toadfish, juvenile European seabass *(Dicentrarchus labrax)* showed decreased phagocytosis and inhibited respiratory burst activity following injection with B(a)P at 20 mg/kg (Lemaire-Gony *et al.*, 1995). In contrast, tilapia exposed to DMBA by i.p. injection exhibited no alteration in phagocytosis unless concentrations causing mortality and decreased swimming were used (Hart *et al.*, 1998). These examples illustrate that the non-specific immune response to PAHs can vary with fish species.

Field studies have correlated immunosuppression with tissue PAH concentration, although the exposure history of the fish is often unknown (Arkoosh *et al.*, 1991; 1994). Suppression of phagocytic activity has been shown in fish from polluted rivers containing PAHs (Weeks *et al.*, 1986). There are several possible means by which PAHs may suppress the immune system, including 1) interaction with the aryl hydrocarbon (*Ah*) receptor by binding to this receptor and activating the *Ah* gene complex, 2) membrane perturbation effects, 3) altered interleukin (IL) production, 4) disruption of intracellular calcium mobilization, and 5) metabolic activation to reactive metabolites (White *et al.*,

1994). If PAHs are immunosuppressive then the potential for increased susceptibility to disease from opportunistic pathogens exists.

3.1.8. Observations of disease in PWS Pacific herring

During the spring of 1993, 15 to 43% of the returning herring in PWS had external ulcers or sub-dermal fin and skin hemorrhages (Meyers *et al.*, 1994; Marty *et al.*, 1998). It is not clear if this prevalence range was unusually high, as lesion prevalence in prior years had not been reported. The rhabdovirus, viral hemorrhagic septicemia virus (VHSV) (North American strain) was isolated from some affected fish in 1993 (Meyers *et al.*, 1994). This was the first identification of VHSV in Alaskan herring. VHSV was also found in adult Pacific herring stocks sampled in other Alaskan locations and in BC (Meyers *et al.*, 1994; Meyers and Winton, 1995). VHSV was first isolated in Alaska during the summer of 1990 and 1991 from skin lesion material taken from two Pacific cod (*Gadus macrocephalus*; Meyers *et al.*, 1992) and in North America from spawning chinook salmon (*Oncorhynchus tshawytscha*) at Orcas Island, Washington (WA) and from adult coho salmon, in 1988 (Amos *et al.*, 1998). VHSV is now considered endemic in Pacific herring in North America but it was not reported in herring prior to 1993 (Meyers and Winton, 1995). Since 1993, the North American strain of VHSV has also been isolated from captive, "healthy" juvenile herring from Auke Bay, Alaska (Meyers *et al.*, 1994) from Campbell River and Sechelt, BC (Traxler and Kieser, 1994) and from PS, WA (Kocan *et al.*, 1997).

In 1994, Marty *et al.* (1998) sampled returning PWS herring for various pathogens. This work showed that 4.7% of fish tested were positive for VHSV (virus isolation from kidney/spleen tissues) and 29.2% were positive (isolation of spores from multiple tissues) for *Ichthyophonus hoferi* (*I. hoferi*). The *I. hoferi* prevalence was at least twice that of fish sampled from 1989 to 1993 (2.1 to 15.0%). Consequently, both of these pathogens, *I. hoferi* (Rahimian and Thulin, 1996) and VHSV (Meyers and Winton, 1995), were suggested as potential contributors to the PWS population declines.

The identification of VHSV in wild adult herring returning to PWS in the years following the *EVOS* led to the hypothesis that VHSV may be significantly pathogenic to Pacific Northwest fishes (Meyers *et al.*, 1994). Kocan *et al.* (1997) demonstrated that specific pathogen free (SPF) laboratory-reared Pacific herring were susceptible to VHSV *via* direct exposure to naturally infected herring fry. The SPF herring had similar clinical

signs to those associated with naturally infected wild fish. It has been hypothesized that prior exposure of the 1989 year-class to petroleum hydrocarbons during the March 1989, *EVOS* had resulted in long-term immunosuppression and that this was manifested as an increased susceptibility to these specific pathogens. Although there was no confirmed mass mortality in PWS during 1994, the actual cause of the population decline remained unknown, but VHSV was implicated as a contributing factor (Meyers *et al.*, 1994; Meyers and Winton, 1995), along with several ecological variables (predation, food availability, and water temperature). Similarly, Mellergaard and Spanggaard (1997) suggested that the 50% decrease in spawning biomass of North Sea herring during 1990 to 1995 might have been due to increasing fishing pressures in combination with concurrent *I. hoferi* epizootics (0.4 to 10.6%). During 1997 to 1998, there was another increase in the prevalence of VHSV (14 to 15%) in PWS herring. Research into the association between pathogens and population cycles in PWS is on-going (Quinn *et al.*, 2001).

3.1.9. VHSV overview

VHSV is an opportunistic pathogen that is endemic to the PNW herring populations, which are the primary natural host species for the North American strain of the virus (Meyers *et al.*, 1994; Meyers *et al.*, 1992; Meyers and Winton, 1995). The North American strain of VHSV is considered moderately pathogenic to herring compared with the more virulent European strain (Meyers and Winton, 1995). Kocan *et al.* (1997) confirmed the pathogenicity of VHSV in PNW herring. VHSV is presently considered endemic in the Pacific sandlance (*Ammodytes hexapterus*), English sole, and shiner perch (*Cymatogaster aggregata*) in the PNW (Hershberger *et al.*, 1999). This strain of VHSV was recently isolated from sardines (*Sardinops sagax*), mackerel, eulachon *(Thaleichthys pacificus*) and surf smelt (*Hypomesus pretiosus pretiosus*) from southern California and the PNW (Hedrick *et al.*, 2003). VHSV has also been isolated from pike (*Esox lucius*), whitefish, and grayling (*Thymallus thymallus*) (Meier *et al.*, 1994).

The name VHS was accepted by international agreement in 1963 (Altara 1963). Previously, VHS has been referred to with various synonyms: Egtved disease; entero-hepatic-renal syndrome; epizootic exophthalmia; infectious anemia; trout plague; new disease of trout; abdominal dropsy of trout; infectious kidney swelling and liver degenerative disease, (Post, 1987; Wolf, 1988). The causative agent, VHSV, belongs within the family Rhabdoviridae and has recently been assigned to the newly named genus Novirhabdovirus (Lorenzen *et al.*, 1999).

VHS was first seen in rainbow trout in 1938 (Schaperclaus 1938) and the virus was first isolated using monolayers of fish cell cultures (Jensen, 1963). The clinical signs may include ascites, hemorrhagic lesions on the body wall and at the base of pectoral and tail fins, and slight bilateral exophthalmus (Meier *et al.*, 1994). Other signs include ulceration of the skin leading to mortality from osmoregulatory shock, secondary microbial infections and predation due to behavioural changes (Meyers and Winton, 1995). Infected fish do not feed and behaviour can range from lethargy to hyperactivity. Their skin is usually darker and may have pinpoint hemorrhaging, while pale gills reflect anemia (Plumb, 1993).

Transmission of VHSV can be from water, infected carriers, predator birds, infected fomites and blood-sucking parasites (Roberts, 1989). Survivors of VHS go on to become carriers (Plumb, 1993). Others have also seen a low level of virus in adult carriers (Sanz and Coll, 1992). For example, brown trout can serve as carriers of VHSV for extended periods of several years (Enzmann *et al.,* 1992). Horizontal, waterborne transmission of VHSV *via* cells of the skin and fins as portals of entry has also been reported (Wolf, 1988, Yamamoto *et al.*, 1992). Therefore, the potential exists for other species in the immediate vicinity of large schools of infected herring to be at risk for infection (Meyers *et al.*, 1994). In addition, because herring are a major forage food for predator fish species they provide the potential for VHSV transmission to adult salmonids (Meyers and Winton, 1995). VHSV has been shown to survive two conventional freeze-thaw cycles (Meyers *et al.,* 1994) and remains stable at -20°C for several years (Meyers and Winton, 1995). Therefore, there is also potential for VHSV to be spread to salmonids when herring are frozen at -20°C or less and then used as bait.

Water temperature also governs transmission rate, although reports of the optimal water temperature that supports a VHSV outbreak vary. Water temperatures of between 7 to 11°C are capable of supporting the virus (deKinkelin and Dorson, 1973), and VHSV is rarely seen in rainbow trout when water temperature is between 14 to 16°C (deKinkelin and Dorson, 1973). In rainbow trout, transmission occurs easily at temperatures of 1 to 12°C. In fact, outbreaks occur mostly at water temperatures of 10°C or less (Wolf, 1988; Meier *et al.*, 1994). Seabass and turbot (*Scophthalmus maximus*) were shown to be

susceptible to experimental infection with VHSV in water <15°C (Castric and de Kinkelin, 1984). The optimal water temperature range for transmission of VHSV in herring has not been defined.

VHS can be acute or chronic in duration (Post, 1987), but the exact pathogenesis is not fully known. The route of entry and identity of the primary target cell are controversial. The gills are thought by some to be the primary site of entry (Chilmonczyk, 1980; Wolf, 1988). Replication is observed in the gills 48 h after infection and afterwards in the kidney (Neukirch, 1984). The epidermal tissues may be another entry and early replication site (Yamamoto *et al.*, 1992). This would be more likely if skin lesions already exist. The kidney is a primary target organ with extensive damage to hematopoietic tissue occurring often resulting in profound anemia (Wolf, 1988).

Bath immersion exposure to VHSV is thought to best replicate a natural exposure (Kocan *et al.*, 1997). This exposure route best mimics the mode of disease transmission of the selected pathogen, the pathogenesis, and the activation of the immune response under natural conditions (Jansson and Ljungberg, 1998). Inoculation with VHSV by bath immersion of sea bass (2 to 5 x 10^5 plaque forming units (pfu)/ml) and turbot (4 x 10^5 pfu/ml) led to 50% mortality, while injection (4 x 10^4 to 2 x 10^7 pfu; 1 x 10^6 to 6 x 10^7pfu) caused > 90% mortality following classical clinical signs (Castric and de Kinkelin, 1984). Sea bass that survived had detectable neutralizing serum antibodies.

3.1.10. VHSV infection in herring

Traxler and Kieser (1994) reported a VHSV prevalence of 22% in adult Pacific herring caught near marine net pen salmon farms on the central coast of BC in 1993 that had no external lesions. Additional sampling near net pens confirmed this prevalence level compared with 14% in open BC coastal waters (Kent *et al.*, 1998). During 1994, VHSV was isolated from spleen-kidney tissue pools and the skin of 4.7% of herring sampled in PWS (Marty *et al.*, 1998). Kocan *et al.* (1997) concluded that 0+-year PS herring are naturally infected with VHSV before they reach 6 months of age. The natural prevalence has been estimated as low as 1% in wild juvenile herring populations but, even so, significant outbreaks can occur. For example, from November 1998 to February 1999 thousands of metric tons of adult herring infected with VHSV died off the northeastern coast of Vancouver Island, Canada (Kocan *et al.*, 2001a). The 10-fold difference in

prevalence estimates between the BC adult herring and the PS juvenile herring could represent the start of an epizootic period within the BC adult herring population during the sampling period, as an epizootic, with a prevalence of 80% prevalence was also recorded one month earlier on the north coast of BC (Meyers and Winton, 1995).

VHSV is considered a significant pathogen in terms of herring mortality during times of population stress due to environmental factors (Pearson *et al.*, 1999). This implies that low endemic prevalence levels could precipitate epizootics if other stressors such as crowding, predation, low salinity, poor nutrition, pollutants such as crude oil, or other pathogens were acting on a population. An age-dependent decrease in mortality upon exposure has been reported in herring (Meier *et al.*, 1994; Meyers and Winton, 1995; Kocan *et al.*, 2001a). Kocan *et al.* (1997) saw mortality in specific pathogen free (SPF), 0+year herring challenged with VHSV within 4 to 6 days post-exposure, with the percentage of mortalities reaching 70 to 100% after 7 to 10 days and the virus titers reaching $10^{6.0}$ to $^{6.7}$ pfu/g. Virus titers in tissues peaked at 6 to 8 days post-exposure ($10^{7.7}$ pfu/g), with dispersement of the virus into the water commencing 48 h after exposure (Kocan *et al.*, 1997). The transmission rate of VHSV during natural transmission studies was related to the number of infected fish to which the SPF fish were exposed (Kocan *et al.*, 1997).

It has been postulated that 10 to 15% of wild herring are sub-clinically infected with VHSV, meaning that they show no clinical signs and express signs of disease when they are stressed (Marty *et al*, 1998). Therefore, it is possible that a sub-clinical VHSV infection could account for the anemia reported in some reference range herring (Chapter 2). It can be anticipated that sub-clinically infected individuals will either recover and be immune to re-infection, or die and effectively remove anemic individuals from the population.

In PWS herring, VHSV infection was significantly associated with liver necrosis, gastritis, mineralization of myocardium and meninogoencephalitis (Marty *et al.*, 1998). External lesions that were considered consistent with VHSV included fin base and focal skin reddening. In addition, decreased plasma albumin, ALP and cholesterol were also significantly associated with VHSV infection and the loss of albumin was attributed to leakage from the external lesions (Marty *et al.*, 1998).

3.1.11. *hoferi* overview

Hofer first reported *Ichthyophonus sp.* disease in 1893 in cultured brown trout (*Salmo trutta*) and in brook trout (*Salvelinus fontinalis*) in Germany (Post, 1987; McVicar, 1982; Athanassopoulou, 1992). This 'fungal-like' pathogen is endemic within wild populations of Pacific herring (Hershberger *et al.*, 2002). It has a very broad host range and varying virulence (Noga, 1993; Spanggaard *et al.*, 1994). Its fungal-like nature and name were first described by Plehn and Mulsow, 1911 (Rahimian, 1998) and *Ichthyophonus* disease was officially recognized as the name in 1974.

There has been much controversy regarding its classification as either a true fungus or as a protozoan. Until recently *I. hoferi* was viewed to be a fungus by most parasitologists, according to Rahimian (1998). However, cluster analysis led Spanggaard *et al.* (1996) to state that *I. hoferi* cannot be considered a fungus. Molecular phylogeny has now placed it in the Mesomycetozoa, which are a new clade of protistans that includes *Psorospermium and Rhinosporidium* (Herr *et al.*, 1999). Some of the confusion in the classification of this organism stems from the misidentification of pathologically similar disease conditions (McVicar, 1982). *I. hoferi* is an obligate parasite that grows optimally at 10°C (Post, 1987). The resting stage cyst is the stage most often seen in fish tissue. The presence of these cysts results in a systemic granulomatous infection (McVicar & McLay, 1985; Sitja-Bobadilla and Alvarez-Pellitero, 1990) characterized by white fusiform nodules in the body musculature and on the surface of the heart and liver (Mellergaard and Spanggaard, 1997; Spanggaard *et al.*, 1994).

Post (1987) states that *I. hoferi* likely occurs worldwide in both salt and freshwater and that all fish species are likely susceptible. Over 80 species of fish are known to be susceptible, 21 of these are marine, as well as some amphibians and copepods, birds and reptiles (Rand, 1994; Post 1987). This pathogen has a broad host range, but differences in susceptibility and pathogenicity varies with the species of fish (McVicar, 1982). For example, rainbow trout were infected by an experimental challenge involving water-borne transmission of *I. hoferi*, whereas goldfish (*Carassius auratus*), guppy *(Lebistes reticulatus),* squawfish *(Ptychocheilus oregonensis)* and catfish *(Ameiurus nebulosus)* were not (Gustafson and Rucker, 1956). Recently, *I. hoferi* has been reported at a prevalence of 45% in Pacific salmon (*Oncorhynchus* spp.) returning to the Yukon River (Kocan *et al.*, 2004).

Host susceptibility is thought to be the primary determinant of pathogenesis. Fish that are more resistant develop the chronic, inflammatory form with granulomas and connective tissue encapsulation of spores. Fish that are more susceptible develop the acute form with massive tissue invasion, necrosis, and death within a few weeks (Noga, 1993). This acute, systemic form involves foci of infection in the heart, viscera, red musculature and roughened skin (Sindermann, 1990).

I. hoferi is primarily transmitted *via* ingestion of amoeboblasts and most commonly occurs from ingestion of spores from contamination of fresh food (McVicar, 1982; Spanggaard *et al.*, 1996). It can also be transmitted *via* contaminated water (Gustafson and Rucker, 1956). Spanggaard *et al.* (1995) proposed the hyphal state as the invasive state in the fish gastrointestinal tract (GIT), with thick-walled multinucleate spores germinating in the stomach due to the low pH, which is essential for hyphae formation. The resulting hyphae can penetrate the GIT and rupture when they encounter blood vessels. This permits release of ameboid bodies into the peripheral blood stream, which are then carried to the vascularized organs. Quiescent cysts from the GIT can be excreted in the feces (McVicar, 1982; Post, 1987; Spanggaard *et al.*, 1996). Leucocytes within vascularized organs phagocytose the ameboid bodies and a connective tissue capsule forms that invades the leucocyte cell wall. Asexual reproduction within this capsule occurs and results in quiescent cysts (Post, 1987). These cysts remain in the host and upon death of the host they germinate and become infective by producing aseptate hyphae (Dorier & Degrange, 1960; McVicar, 1982). Definitive diagnosis of *I. hoferi* requires identification of the characteristic life stages in the tissue of a host (Wolke, 1975) such as the thick-walled "resting spore" in wet mounts or by histology.

In some individual fish, only external changes are seen, such as scoliosis in herring and salmonids due to invasion of the nervous system (McVicar, 1982) and roughening of the skin in herring and rainbow trout (Sindermann and Scattergood, 1954). In herring, the pathogenicity of *I. hoferi* appears to be due to the alteration and atrophy of infected tissues as a direct result of the growth and/or reproduction of spores. As the infection progresses, the replacement of the tissue or organ with inflammatory cells and necrotic cells further interrupts the normal functioning of the tissue or organ (Rahimian, 1998).

3.1.12. *hoferi* infection in herring

A variety of prevalence rates of *I. hoferi* infection have been found in various populations of herring. *I. hoferi* was prevalent in 10 to 27% of herring in an epizootic in the Gulf of St. Lawrence from 1955 to 1957 (Sindermann, 1958), in 3.7% of commercial herring catches in the North Sea during 1991 (Mellergaard and Spanggaard, 1997) and in 10.6% of herring in research vessel catches in the North Sea during 1991 (Mellergaard and Spanggaard, 1997). From 1990 to 1995, the spawning biomass of North Sea herring was decreased by 50%. Mellergaard and Spanggaard (1997) suggest this decline may be due to increasing fishing pressures in combination with the general effect of a concurrent *I. hoferi* epizootic. More specifically, Patterson (1996) developed a disease dynamics model that accounted for the prevalence of *I. hoferi*, stock abundance, fishing mortality and catches. Sitja-Bobadilla and Alvarez-Pellitero (1990) found *I. hoferi* in 24.4% of cultured fish and in 14% of wild fish in the Spanish Mediterranean area. On the west coast of Sweden, a prevalence of 4.2% was found in Atlantic herring (Rahimian, 1998). In BC, the *I. hoferi* prevalence in herring ranged from 10.5 to 52.5% in 2000 and 2002, (Jones and Dawe, 2002). Epizootics in herring stocks have also been seen in Denmark and Norway (Sindermann and Scattergood, 1954).

In PWS in 1993, 80,000 tons (60%) of the spawning Pacific herring failed to return and an *I. hoferi* prevalence level of 27% was identified in the fish that did return (Marty *et al.*, 1998). This led to the hypothesis that *I. hoferi* may play a role in declining Alaskan herring populations. Working with SPF juvenile Pacific herring, Kocan *et al.* (1999) confirmed Koch's postulates and proved experimentally that *I. hoferi* can cause disease during experimental infection in PS herring. The prevalence of *I. hoferi* in wild PS Pacific herring was shown to increase with age (Hershberger *et al.*, 2002). However there was no association between increased prevalence and mortality in that particular population (Kocan *et al.*, 1999).

3.1.13. *Vibrio anguillarum* (*Listonella*) overview

Vibrio anguillarum is a gram-negative bacterium and an opportunistic pathogen commonly encountered in the marine environment. Phylogenetic studies recommended placing this organism in the genus *Listonella* in 1985 (Noga, 1996) and this name change has occurred but it has not been widely accepted in the literature. Therefore the author has elected to retain the use of the historical name throughout the remainder of the thesis. *V. anguillarum* is the main causative agent of Vibriosis, a syndrome

characterized by hemorrhagic septicemia (Egidius, 1987). Increases in water temperature, especially if stocking density is high and if salinity and organic loads are high, are conducive to Vibriosis (Hjeltnes and Roberts, 1993). This pathogen is very invasive and causes hemorrhagic and ulcerative skin lesions (Fryer and Rohovec, 1993). Gross external lesions include erythema (reddening) at the base of the fins and within the mouth, hemorrhaging of the vent, and petechiae of the musculature. As the disease progresses, hemorrhagic lesions develop in the body musculature, which can also lead to ulcerative skin lesions. Swollen kidneys and congestion of the liver and the spleen can be seen internally (Fryer and Rohovec, 1993).

V. anguillarum has been previously used as a bath challenge pathogen in immunotoxicological studies (Thuvander and Carlstein, 1991; Regala *et al.*, 2001). This pathogen was selected as a preliminary Tier 3 challenge agent in this study for the following reasons: 1) herring are known to be susceptible and to develop clinically apparent disease ; 2) the bath challenge route is effective at inducing infection based on its prior use as a fish challenge pathogen; and 3) proof of infection after exposure is simple *via* a combination of clinical signs and bacteriology. Also, the clinical signs include hemorrhagic septicemia with skin lesions which can also be seen with VHSV (Noga, 1996), the challenge pathogen that was used in subsequent experiments. Furthermore, *V. anguillarum* is susceptible to the antibacterial activity of lysozyme (Grinde, 1989) and antibodies have been seen to be protective against *V. anguillarum* by passive immunization (Harrell *et al.,* 1975; Viele *et al.,* 1980). Therefore, it was considered a suitable challenge pathogen for assessing the integrated immunological system following WSFO exposure.

Bath challenge tests were essential for herring to avoid the additional handling associated with an injection challenge and the likelihood of scale loss and skin damage. Based on the findings in other fish species, this challenge route was assumed to result in uptake both through the gills and by the gastro-intestinal tract. In rainbow trout, uptake in gill tissue shortly after bath exposure to *V. anguillarum* confirmed that the gills are a route of entry (Baudin-Laurencin and Germon, 1987). Olsson *et al.* (1996) found that *V. anguillarum* can penetrate the intestinal mucus of turbot and can gain access to the intestinal tract. Bøgwald *et al.* (1994) demonstrated that *V. anguillarum* is taken up in

the intestinal tract of Atlantic salmon, and endocytosis of bacterial cells in both cod and herring fry hindgut has also been reported (Olafsen and Hansen, 1992).

3.1.14. Assessing sub-lethal immunotoxicity of exposure to crude oil in herring

Carls *et al.* (1998) hypothesized that exposure of adult Pacific herring to sublethal concentrations of oil can cause immunosuppression and pathogen expression. It has been suggested that exposure of herring to PAHs in 1989 following the *EVOS* may have damaged developing cells destined to become functional immune cells and that abnormal immune function would be evident at a later stage in life if challenged with specific pathogens (Kocan *et al.*, 1997). It was also postulated that stress could reactivate the expression of VHSV in sub-clinical VHSV carriers, resulting in viral shedding and infection of non-immune individuals (Carls *et al.*, 1998).

3.1.15. Objectives

The potential for sub-lethal impact of crude oil exposure on adult immune systems has been examined. Carls *et al.* (1998) demonstrated that pre-spawning adult herring exposed to the WSF of weathered crude oil at an initial concentration of 58 ppb for 16 to 18 days had increased susceptibility to VHSV (oil source not stated but presumed to be Alaska North Slope given the location of the laboratory in Auke Bay, Alaska). However, the concentrations used by Carls *et al.* (1998) were 10 to 100 times higher than environmental levels associated with the *EVOS* and the study appeared after I began my experiments. Furthermore, little published information exists on the immunotoxicological response of juvenile herring and the effect of sublethal WSFO exposure upon developing juvenile herring has not been thoroughly investigated. The impact of exposure to sublethal concentrations of the WSFO on Pacific herring immune responses represents a novel study. Moreover, given the differences identified in Chapter 2 between juveniles and adults, the expectation was that age classes (0+ and 2+ year) would show differences in their immune response to WSFO. The rationale for the study design is explained below.

Toxicology can be defined as the study of the adverse effects of chemical agents on biological systems (Klaassen *et al.*, 1986). Exposure concentrations, durations of exposure, routes of exposure and the life stage of an organism are all paramount in governing whether or not contact with a xenobiotic such as crude oil will result in a toxic effect. Consequently, for extrapolation purposes, it was necessary that concentrations of the WSFO in laboratory exposures were representative of actual exposure

concentrations reported in PWS. In this study, herring were exposed to WSFO. The peak exposure concentrations of TPAH used in these experiments (55.9 to 321 ppb) were initially 10 to 50 fold higher than the reported range of peak TPAH concentrations in PWS seawater following the *Exxon Valdez* oil spill *(EVOS).* However, experimental TPAH concentrations declined rapidly to 1.3 to 5.0 ppb within 4 d of initiating exposures and were comparable to those found in seawater samples taken 7, 21 and 35 d post-*EVOS,* which ranged from 0.83 to 6.24 ppb (Short and Harris, 1996). Measurements of TPAH at spawning sites just prior to, and at the time of spawning were in the 1.9 to 2.6 ppb range at approximately 21 d post-spill (Brown *et al.,* 1996a; Short and Harris, 1996; Carls *et al.,* 2002).

The duration of exposures in the present study (16 to 28 days) were similar to the potential period that adult herring were exposed in PWS, since the spill occurred 21 days before spawning. However, experimental exposures for 0+year herring were shorter than the duration of exposure in PWS. While peak exposure occurred within the first couple of weeks post-spill, PAHs from the *EVOS* were still present in samples taken between May 2nd to 8th, 1989 (Short and Harris, 1996). Pearson *et al.* (1999) states that by August 1989 background (pre-spill) levels of TPAH were evident. This implies that between mid-May to August that TPAH levels were still greater than usual, i.e. for up to 5 months. Therefore, the younger age class was potentially exposed longer than the adults in PWS because they rear in the nearshore nursery bays for at least the first year of life (Norcross *et al.,* 2001).

Preliminary studies examining Tier 1 and 2 variables used three WSFO concentrations for exposures with each age class. In the subsequent experiments, fish were exposed to only the maximum achievable PAH concentration exposure for 21 to 28 days. This was done in an effort to better understand the pattern of change seen with WSFO exposure because results from the preliminary studies showed a trend towards higher mortality at the higher WSFO concentration (120 g oil) and few effects at the lower concentrations (40 g oil and 80 g oil).

The challenge pathogens used in this study to assess Tier 3 (disease susceptibility) included VHSV and *I. hoferi* as these were epizootic in herring in PWS, Alaska post-*EVOS* and have been implicated in population declines during 1993 to 1995 (Marty *et al.*, 1998). In addition, juvenile herring were challenged with the bacteria, *Vibrio*

anguillarum, in the initial juvenile experiment. Pathogen challenges are generally thought to represent the most comprehensive test of the immune system because the ability to mount an adequate defense requires both the humoral and cellular components of the immune system to be competent (Anderson, 1990).

3.2. METHODS

3.2.1. WSFO exposures

3.2.1.1. Water Soluble Fraction of Oil (WSFO) Generation

Juvenile and adult herring were exposed in a series of experiments to the WSFO of PBCO from the Alaska North Slope which was sent from Alaska. The WSFO was produced using a polyvinyl chloride (PVC) column, two feet in length, designed to permit only the WSFO to enter the tanks (Figure 3.1). This apparatus, developed at the Auke Bay Lab, NOAA, AK, was used previously to expose fish to WSFO (Thomas *et al.,* 1989, Carls *et al.,* 1998). The internal compartment of the column was filled with silica biofilter rings (Siporax™, Aquatic Ecosystems, Apopka, FL) that had been soaked in PBCO. The biofilter rings were inert and non-toxic.

Initial tests were conducted at BMSC to determine if the contact time of the biofilter rings with PBCO affected the amount of oil absorbed by a filter ring. No significant differences were found among those that were soaked 1, 2, 4 and 6 h. Therefore, in all experiments, biofilter rings were soaked for 1 h and allowed to drain overnight through nylon mesh prior to their placement within a polyvinyl chloride column. To determine the amount of oil absorbed, 10 biofilter rings from each batch of biofilter rings were selected and weighed prior to soaking and again after overnight drainage.

The PVC columns were fitted to each tank so that inflowing seawater ran first through the column before entering an experimental tank. Control tanks received water flowing through a PVC column containing biofilter rings without oil. Water percolated through the filter rings, creating the WSFO while retaining the oil slick within the column because the outflow to the tank was positioned below the top of the water column. The PVC column was vented to allow for slick overflow outside of the experimental tank if the water flow rate changed. Seawater flow rates were 5 L/min for tanks containing adult herring and 2 L/min for tanks containing juveniles.

In preliminary experiments, three WSFO concentrations were used. This was achieved by filling the WSFO generating column with different amounts of biofilter rings as follows: 100 g of biofilter rings (40 g oil); 200 g of biofilter rings (80 g oil); 300 g of biofilter rings (120 g oil), which was the maximum achievable concentration. The remaining experiments only used the maximum amount (120 g) of oil. On average there was 1.37 biofilter rings/g oil. Effluent water was passed through a chlorination/dechlorination system and discharged onto a rock/sand substrate prior to entering the ocean.

3.2.1.2. Concentration of hydrocarbons

Determination of the total polyaromatic hydrocarbon (TPAH) concentrations was conducted in one representative multiple concentration experiment, and in two representative time series experiments (Table 3.1). One L water samples were taken at 24 h in the multiple concentration experiment and 4 L water samples were taken at 1, 2, 4, and 7 days after initiation of the time series experiments. All samples were stored at 4°C. Water was extracted by hexane (2 mL) and extracts were submitted to the Auke Bay Laboratory, NOAA, Alaska for analysis of TPAH and total hydrocarbons by gas chromatography/mass spectroscopy (Appendix 1, Protocol No. 12). TPAH concentrations in water ranged from 0.09 to 0.34 ppb (mean=0.20 ± 0.03) in control tanks during experiments (PNW background). In exposure tanks the TPAH concentration ranged from 26.0 to 321.0 ppb at the peak (day 1) to 0.19 to 13.6 ppb at the end of the exposure (day 21 to 28) (Table 3.1).

The TPAH concentration at the end of the 28-day QI trial was 100 times higher at 13.64 ± 0.90 ppb (controls at 0.34±0.03) compared with the 21-day exposures. No methodological differences existed to account for this anomaly other than a notable elevation in water temperature of 1 to 2°C at the time, which could potentially affect the rate of loss of PAHs and the composition of the WSFO. There were no earlier measurements for this experiment as the concentration was being monitored with a semi-permeable membrane device (SPMD) placed within the tanks. Unfortunately, analysis of the SPMD at the end of the experiment did not occur and thus there was no time course of TPAH recorded to aid in interpreting this result. It is possible that this was an erroneous measurement due to either error in sample processing or analysis.

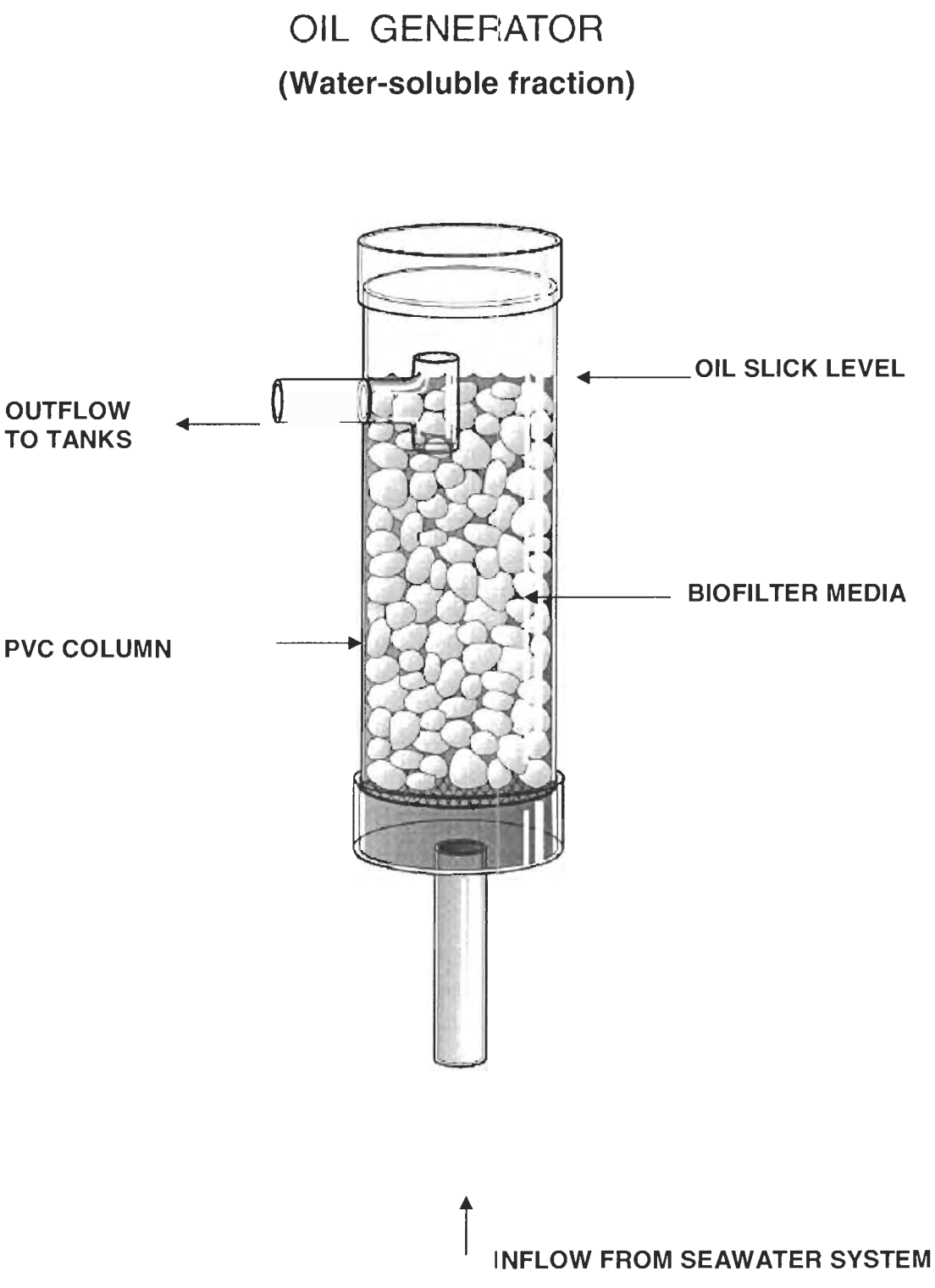


Figure 3.1. Representative PVC column used to generate WSFO.

3.2.1.3. Duration of WSFO exposure

Prior work with the oil generator had shown that hydrocarbons are discharged into the water column over a period of 21 d, with the peak discharge occurring in the first 24 to 48 h (Carls *et al.*, 1998). TPAH concentrations in water in the current study showed a similar pattern and were released as a pulse within the first 24 to 48 h. Aliphatic hydrocarbons (alkanes, isoparaffins, cycloalkanes) were not measured but were anticipated to decline similarly, as in prior studies (Carls *et al.,* 1998). After the pulse of peak exposure, hydrocarbon concentrations declined to 0.42 to 3.03 ppb over the next 7 to 14 d of the exposure period and overlapped with the range reported in PWS (0.83 to 6.24 ppb) at near-shore sites in the weeks following the *EVOS* (Short and Harris, 1996). At the end of the 21-day exposures the TPAH concentration ranged from 0.19 to 1.87 ppb and overlapped with the TPAH concentration at the *EVOS* margins of 0.4 to 1.5 ppb (Pearson *et al.*, 1999). The decay in the WSFO profile over the course of the 21-day exposures parallels the decrease in the aqueous TPAH concentrations seen in PWS following the pulse exposure from the *EVOS*. A detailed breakdown of the hydrocarbon compounds contained in each sample, as a result of the 120 g oil application, and concentrations for each time point are provided in Appendix 2, including comparative control tank data.

Table 3.1. Summary of TPAH water concentrations at specific time-points following initiation of exposure to the WSFO of Prudhoe Bay Crude Oil (reported as ppb). BS = Barkley Sound, QI=Quadra Island. Concentrations are mean parts per billion of total polyaromatic hydrocarbons ± SE. *=sampled within 10-minutes after initiation of exposure (corresponds to "immediate" in Tables A2.1 and A2.2).

Experiment	0-day*	1 -day	2-days	4-days	7-days	14-days	21-days
pControl tanks		0.21 ±0.05	0.22 ±0.03	0.30 ±0.00	0.09 ±0.02		0.12 ±0.04
Multi-concentration (BS) 40 g oil 80 g oil 120 g oil		 26.0 65.0 321.0					
Time series –BS juveniles (120 g oil)	82.82 ±1.68	9.69 ±1.02	5.00 ±0.32	6.63 ±3.60	3.03 ±2.34	0.42 ±0.11	0.19
Time series – BS adults (120 g oil)	6.00 ±0.47	55.92 ±41.71	3.40 ±0.32	1.33 ±0.19	0.99 ±0.08	n/a	0.19 ±0.02
EVOS (near-shore)	0.83 – 6.24 (Short and Harris, 1996; Carls *et al.*, 2002)						
EVOS (margins)	0.4 – 1.5 (Pearson *et al.*, 1999)						

3.2.2. Tier 3 responses – Pathogen challenges

Susceptibility to a pathogen, as assessed here by infection rate and mortality, is considered the most important and comprehensive test in immunotoxicological screening (Anderson, 1990; Wester *et al.*, 1994). The ability of fish to resist a stressor comprises Tier 3 of the classical immunotoxicological system (Wester *et al.*, 1994). Pathogen challenge is a broad test of an organism's response that can have biological significance at the individual and population level. Furthermore, by evaluating Tier 1 and 2 variables during the pathogen challenge component of specific experiments, associations were drawn between the effect of dual exposure to WSFO and VHSV on measures of health in Pacific herring. It was hypothesized that if WSFO exposure was immunosuppressive to herring, then differences in the immunotoxicological variables could be anticipated when the herring were further challenged with VHSV.

In this study, pathogen challenges, using *V. anguillarum* and VHSV, were conducted in conjunction with WSFO exposure. In the adult WSFO exposure/VHSV challenge, herring were exposed to WSFO for 21 d, followed by a VHSV-challenge. In the juvenile WSFO exposure/VHSV challenge, the VHSV challenge began 7 d into a 21-day WSFO exposure, such that exposure to the combination of WSFO and pathogen overlapped. In addition, preliminary challenges with VHSV and *I. hoferi* alone were done.

Static bath challenges involved lowering the water level in each tank and exposing herring to the specific pathogen for 1 h (Kocan *et al.*, 1997). This exposure method was used in all challenges except the oral *I. hoferi* challenge. Controls were treated similarly with respect to lowering the water level and all tanks were aerated during each bath challenge. Following challenges with *V. anguillarum*, VHSV and *I. hoferi*, herring were monitored twice daily over a six to eight week period for subsequent mortality. During the *V. anguillarum* challenge, all dead fish were cultured (see Appendix 1, Protocol No. 9 for details) for the presence of the organism in the head kidney. The response variables used to evaluate susceptibility following pathogen challenge included cumulative mortality rate of both challenged and unchallenged fish, plasma antibody titres (Tier 2) and the virus neutralization (Tier 2) capability of survivors. Specific details relevant to each specific challenge are provided in each subsection. Sub-samples of adult and juvenile herring were submitted for bacteria and virology testing at the PBS Fish Health Laboratory at 5-days after capture (see Chapter 2 for details). In both adult and juvenile

experiments, a separate net was dedicated to each tank for the duration of the WSFO and VHSV challenges to ensure no contamination between tanks when removing fish for sampling or removing mortalities. A 10% bleach solution was used to rinse and disinfect nets between uses.

In each WSFO/VHSV experiment, sample day was identified with two numbers, the first representing the time since initiation of WSFO exposure and the second number, in parentheses, referring to duration since VHSV-challenge. Fish size did not vary significantly between treatments at any sample time. Gender of adults was unknown because fish were frozen intact for viral assay with no internal examination done.

3.2.2.1. Preliminary pathogen challenges

Prior to using VHSV or *I. hoferi* as challenge pathogens for evaluating Tier 3, pilot challenges were conducted. These experiments were conducted to assess the effect of VHSV and *I. hoferi* in the absence of experimental exposure to WSFO. Challenges were conducted within a controlled laboratory setting, but attempted to mimic the encounter of the given pathogen within the natural environment to maximize applicability to a field setting.

3.2.2.2. I. hoferi challenge - Response of Tier 1 and 2 measures

Several thousand, wild stock, 0+year class, Pacific herring were obtained from sites in PS and Admiralty Inlet, WA and transported to the MFS (Kocan *et al.*, 2001a). A sub-sample of these fish (n=100) was screened for VHSV within 6 h of capture. No fish tested positive using the *epithelioma papulosum cyprini* (EPC) cell line virus assay (Kocan *et al.*, 2001a). However, within 2 to 3 d following capture, an outbreak of VHSV occurred resulting in 60% mortality and 93% of the dead fish tested positive for VHSV. Virus titres peaked (1×10^8 pfu/g of tissue) at 7 d post-capture. Tissue titres from dead fish declined with time and were below detection limit by 30 d post-capture. Tissues sampled from 30 of the remaining herring at 35 d post-capture were also below the detection limit of the viral assay and by this stage no gross lesions were evident. Concurrently, the background prevalence of *I. hoferi* was approximately 5%, no erythrocytic necrosis virus was detected on blood smears. Nematodes and coccidia seen on histopathologic examination were not considered sufficient to contribute to the mortality level seen (Kocan *et al.*, 1999).

0+year Pacific herring survivors of the above-described VHSV epizootic (n=60) were used to examine the effects of oral *I. hoferi* exposure 42 d after tissue titres for VHSV were below detection limits (< $10^{2.6}$ pfu/g). Water temperature ranged from 9°C to 10°C during the course of the experiment. Body mass ranged from 4.4 to 11.5 g (mean 6.2 ± SE 0.2) and fork length ranged from 8.4 to 11.4 cm (mean 9.1 ± SE 0.1). There were 20 fish per treatment group and 20 control fish (n=10 per tank). Duplicate groups were dosed with a single dose of *I. hoferi* spores of either low (100 spores) concentration or high (1000 spores) concentration *via* their feed (Kocan *et al.*, 1999) and both groups were sampled 21 d and 63 d post-challenge. The Tier 1 variables were CF, Hct, Lct, and WBC differential count. The Tier 2 response assessed was plasma lysozyme activity. Cumulative mortality rate was also determined for each treatment group.

The mortality was not statistically significant at the 95% confidence level (ANOVA, arcsine square root transformed data, $p>0.05$) among controls (5%), low (30%) and high (10%) *I. hoferi*-challenged herring. Changes in Tier 1 variables were evident in both control and *I. hoferi*-challenged juveniles with time between day 21 and 63. CF decreased significantly in both control and *I. hoferi*-challenged fish. The lymphocyte proportion also decreased with time, but only in control and high *I. hoferi*-challenged fish. Additionally, Hct increased with time in all *I. hoferi*-challenged fish and thrombocytes increased in the high-*I. hoferi* challenged fish.

A low dose concentration of *I. hoferi* had no significant effect on Tier 1 and 2 variables compared with controls, however, higher mortality in the low dose group may have masked any effects (power=0.05 to 0.61). High *I. hoferi* challenge was associated with changes in some Tier 1 variables. The WBC differential count demonstrated a lower median percentage of monocytes in the high concentration *I. hoferi* group (0%) compared with control fish (2%) at day 21 ($p=0.0098$). At 63 d post-challenge, Hct was significantly higher in the high concentration *I. hoferi* fish (median of 35.9%, 95^{th} PE= 25.6 to 47.0) compared with controls (median of 23.7%, 95^{th} PE=5.5 to 41.4, $p=0.0330$). However, the general lack of response in measurement variables attributable to *I. hoferi* exposure *via* this exposure model precluded continued use of this pathogen to assess WSFO exposure effects.

3.2.2.3. Pilot VHSV challenge - effects on mortality and Tier 1 and 2 variables

Pre-spawning, adult herring were collected from sites within PS and transported to the MFS (Kocan *et al.*, 2001a). Upon capture no VHSV was detected, but this group demonstrated the same pattern of VHSV progression as did the 0+year PS herring. An epizootic began within the first 7 d post-capture (25% total mortality and a peak of 33% prevalence of VHSV at day 8), and VHSV becoming undetectable by 30 d post-capture (Kocan *et al.*, 2001a). *I. hoferi* prevalence in this group (n=100) was 78% with 4% having skin lesions and 5% having visceral lesions (Kocan *et al.*, 1999). At 90 d post-capture, all 120 survivors of the laboratory VHSV epizootic were used in a VHSV challenge. For this challenge, herring were held in flowing natural seawater at ambient PS temperature (8 to 11°C) in four separate, circular, 265 L, 76 cm diameter tanks and were fed frozen brine shrimp (*Artemia sp.*), and krill each day *ad libitum*. Body mass ranged from 27.7 to 122.5 g (mean 62.5 ± SE 1.7 g), and fork length ranged from 14.1 to 23.5 cm (18.9 ± 0.2 cm).

Herring were challenged in duplicate tanks for 1 h with VHSV, using 5 x 10^3 pfu/ml *via* a static bath exposure in which the water level was lowered to 30 cm and the stock solution of VHSV was added (Kocan *et al.*, 1997). After a 1 h exposure period, water flow was restored and water samples were taken to confirm virus exposure and the viral titre (Kocan *et al.*, 1997). An initial group of 30 unexposed fish were sampled prior to initiation of the VHSV challenge. The Tier 1 variables evaluated included CF, Hct, Lct, and WBC differential counts. The Tier 2 variables assessed were macrophage phagocytosis activity (Appendix 1, No. 5), respiratory burst activity (whole blood, slide assay, Appendix 1, No. 6b), and virus tissue titre (at 10 d). Control and challenged fish were sampled at 10 d (n=30 per group) and 80 d (n=15 per group) post-VHSV challenge for Tier 1 and 2 assays.

No mortality occurred in control or VHSV-challenged herring and none of the control or challenged fish had a detectable VHSV tissue titre when sampled at 10 d post-challenge (n=30 per treatment group). Changes over time occurred in control and VHSV-challenged fish. The control herring were also compared with the baseline fish sampled at day 0 (pre-challenge, n=30) to assess the effects of the 'mock' challenge at the subsequent sample times. CF decreased significantly by day 80 in control fish, while lysozyme, NBT macrophage activation (respiratory burst) and phagocytosis ability were

all significantly lower at both day 10 and 80. Both CF and Lct decreased significantly in VHSV-challenged fish from day 10 to day 80. Lct was increased significantly in the challenged herring at 10 d after VHSV exposure (1.00%) compared with control fish (0.75%) and was the only variable that differed significantly. The modality of Lct distribution changed in both control and challenged herring becoming bimodal by day 80, showing a trend towards more fish with undetectable Lct over time. The fact that VHSV infection or disease could not be induced using standard techniques implied that there was background immunity. However, this pathogen was still used to assess Tier 3 following the WSFO exposures to determine if the WSFO-pathogen combination could "overcome" the background immunity.

3.2.3. Additional calculations and statistics

The crude mortality rate (CMR) as seen in Chapter 2 and cumulative mortality are not classified as part of the tiered immunotoxicological testing scheme, whereas proportional mortality is a Tier 3 measure. In experiments with no pathogen challenge, or where it was not possible to obtain proportional mortality, cumulative mortality was used as a means of assessing overall survival. Proportional mortality was only applicable to the *V. anguillarum* challenge experiment. The calculations used are shown below.

Cumulative mortality (%) = ((No. of dead fish)/ (N at risk))/ 100

$$\text{Proportional mortality (\%)} = \frac{\text{((No. of dead fish due to pathogen/(N at risk))}}{\text{(N at risk previously defined in Chapter 2)}} \times 100$$

The prevalence of lesions and anemia are only reported and discussed for juvenile herring because the presence of lesions or anemia did not significantly affect the reference ranges of measurement variables for adult herring. Therefore, any differences in prevalence of lesions or anemia among adult treatment groups would be unlikely to alter the significance of statistical changes in measurement variables (Chapter 2). Prevalence among treatment groups of juvenile fish was compared using the Likelihood Ratio test with $p<0.05$ as significant.

Table 3.2. Summary of experimental conditions and variables evaluated in Pacific herring from 1996 -1999 at the Bamfield Marine Sciences Centre (BMSC) and the Marrowstone Field Station (MFS). pc=post challenge, cfu=colony forming units, pfu=plaque forming units, ITP= *Ichthyophonus hoferi*, VHSV=viral hemorrhagic septicemia virus, CMR=crude mortality rate, PBS=Pacific Biological Station, Hct=hematocrit, Lct=leucocrit.

Season and Year	**Nov 1995 -Jan 1996**	**July 1996**	**Aug-Nov 1996**	**Jan – June 1997**	**Sept-Oct 1997**	**Nov 1997-Jan 1998**	**July 1998**	**Nov 1998 - Jan 1999**	**May-June 1999**
Source Population	Puget Sound	Barkley Sound	Barkley Sound	Puget Sound	Quadra Island	Quadra Island	Barkley Sound	Barkley Sound	Quadra Island
Field station location	MFS	BMSC	BMSC	MFS	BMSC	BMSC	BMSC	BMSC	BMSC
Water temperature	8.5-10 °C	11-14 °C	8-14.5 °C	8-11 °C	13.6–15.7 °C	8.5 –12.4 °C	11.0-12.8 °C	8.1 –12 °C	8.9-11.1 °C
Water flow rate	2 L	5 L	2 L	5 L	5 L	5 L	5 L	2 L	5 L
Screening bacteriology and virology (location, results)	VHSV survivors	MFS n=18 1 VHSV +	PBS, n=10, negative	Unknown	PBS, n=10, negative	PBS, n=40, negative	PBS, n=30, negative	PBS, n=30, negative	PBS, n=30, negative
Age	0+year	Adult	0+year	Adult	Adult	Adult	Adult	0+year	1+year
Oil concentration; pathogen challenge dose	*I. hoferi*	Oil 40 g, 80 g, 120 g	Oil - 40 g, 80 g, 120 g *Vibrio* (1×10^4 cfu/ml)	VHSV (5×10^3 pfu/ml)	Oil – 120 g	Oil – 120 g VHSV (1×10^4 pfu/ml)	Oil – 120 g	Oil – 120 g VHSV ($1 \times 10^{5.5}$ pfu/ml)	*I. hoferi* status
Sample points (days post initiation of oil exposure or post challenge)	21& 63	6 & 16	21 & 63 / 14 & 42 pc	14 & 84	28 & 70	7 & 21, / 14, 28, 42 & 56 pc	0, 4, 7, 14, 21, 63	4, 7, 14, 21 & 63 / 7 & 14 pc	No exposure
Hct & Lct	√	√	√	√	√	√	√	√	√
Condition indices	√	√	√	√	√	√	√	√	√
WBC differential	√	√	---	√	---	---	---	---	---
Biochemistry	---	√	---	---	√	---	√	√	
Lysozyme	√	√	---	√	√	√	√	√	√
Phagocytosis	---	√	√	√	√	√	√	√	√
Respiratory burst	---	---	---	√ (slides)	√	---	---	---	---
CMR	√	√	√	---	√	√	√	√	---
Antibody titre	---	---	√	---	---	√	---	√	---
Pathogen isolation	---	√	√	---	---	√	---	√	√

3.2.4. Juveniles - Multiple concentration experiment

3.2.4.1. 21-day WSFO exposure and V. anguillarum challenge and recovery

Juvenile BS herring were exposed to three WSFO concentrations for 21 days. Their response was followed for a further seven weeks (70 days total) after termination of the WSFO exposure (49-day recovery). Fish ranged in body mass from 1.3 to 7.4 g (mean 3.8 ± SE 0.1) and in length from 6.0 to 9.3 cm (mean 7.8 ± SE 0.1), and did not vary statistically among treatment groups (Wilcoxon/Kruskal-Wallis, $p<0.05$). After a 6 d acclimation period, 100 fish were distributed into each of ten 265 L tanks. The flow through water was treated by ultraviolet sterilization for 10 minutes before distribution to experimental tanks.

Four tanks of fish served as controls, with no WSFO exposure. The remaining six (3 x 2 replicates) tanks contained one of three WSFO concentrations (Table 3.1). Ten fish were sampled prior to initiating the experiment to serve as a baseline. Ten fish were sampled from each WSFO-exposed tank and two control tanks after a 21-day exposure. The variables evaluated included: CF, Hct, Lct, WBC differential counts, antibody titre, cumulative mortality and gross post mortem.

After cessation of WSFO exposure (day 22), all WSFO-exposed tanks and half of the control tanks were exposed to 1 x 10^4 cfu/mL of *V. anguillarum* (Appendix 1, Protocol No.7). All tanks were dosed simultaneously to prevent differences in bacterial numbers due to the culture changing with time and the concentration was verified using serial dilution plates (Appendix 1, Protocol No. 8). The water temperature during the challenge and observation period ranged from 10.0°C to 13.8°C.

Fish were observed twice daily for mortality and signs of disease for a six-week period following the challenge and a record of cumulative mortality was maintained. Each dead herring was evaluated for body mass and length, and the head kidney was removed to test for the presence or absence of *V. anguillarum* using standard confirmatory microbiological techniques (Appendix 1, Protocols No. 9 and 10; Noga, 1996). These results, in combination with consistent clinical signs, were used to assign a positive or negative *V. anguillarum* infection status to the herring and to determine proportional mortality due to *V. anguillarum.* Challenge survivors were euthanized and their serum tested for the presence and titre of serum antibodies to *V. anguillarum* using the slide

agglutination assay (Appendix 1, Protocol No. 10). *V. ordalii* and *V. salmonicida* are also responsible for some cases of Vibriosis (Anderson and Conroy, 1970) so cross-reactivity for these pathogens was also examined (Appendix 1, Protocol No. 10).

3.2.5. Juveniles - Time course, high concentration experiment

3.2.5.1. 21-day WSFO exposure and VHSV challenge and recovery

The impact of exposure of juvenile herring to the maximum achievable concentration of WSFO (120 g oil at peak) was examined. Sampling occurred at more frequent intervals and used a greater range of Tier 1 and 2 variables to evaluate the response than in the multiple concentration juvenile experiment. Tier 3 was assessed by response to VHSV challenge.

Juvenile BS herring (n=480) ranged in body mass from 1.5 to 8.7 cm (mean 3.4 ± SE 0.1) and in fork length from 6.1 to 10.0 g (mean 7.8 ± SE 0.1), with no significant variation among treatment groups (Wilcoxon/Kruskal-Wallis, $p<0.05$). Fish were divided among eight 265 L experimental tanks. Four tanks served as controls, and the remaining 4 tanks of herring were exposed to WSFO. After 7 days of exposure to WSFO, 2 of the control and 2 of the WSFO-exposed tanks of juvenile herring were challenged with $1 \times 10^{5.5}$ pfu/ml VHSV per tank from a stock suspension of titre 1.05×10^{9} pfu/mL. The bath challenge dose was based on the previous work of Kocan *et al.* (1997) and the preliminary VHSV challenge. Kocan *et al.* (1997) found $1.00 \times 10^{1.5\text{-}2.0}$ pfu/mL to be the minimum dose of VHSV required to initiate infection of juvenile SPF herring. Also, wild pre-spawning adult herring exposed to 5×10^{3} pfu/mL did not develop clinical signs during the 80-day post-challenge period (Kocan *et al.*, 1997). Therefore, the challenge dose used in this experiment was greater than the juvenile SPF minimum dose, and the adult doses used earlier in this study. A sub-sample of fish (n=6) was removed from each tank on days 4 and 7 (i.e., pre-VHSV challenge), on days 14 and 21 (i.e., 7 and 14 d post-VHSV challenge) and at 42 d after termination of exposure (day 63) to assess Tier 1 and 2 variables and pathology. Tanks were checked twice daily for mortalities for the first 14 d post-VHSV challenge. Tanks were checked once daily during the remaining observation period for a total of 56 d post-VHSV challenge. Water samples were taken at 5 min post-viral exposure, then hourly for 3 h, then every 24 h for 3 d to establish the actual exposure and to monitor for virus shedding in the water.

The Tier 1 variables measured included CF, LSI, Hct, Lct, glucose, albumin, total protein and lactate. Plasma lysozyme and macrophage phagocytosis were measured to evaluate Tier 2. Tissue viral titre, an additional Tier 2 measure, was determined from only the dead fish (all), which were frozen whole at -80°C. Tier 3 was evaluated by comparing the cumulative mortality rate following VHSV challenge in control and WSFO exposed fish. The volume of blood from these small fish was too low to allow both white blood cell differentials and plasma biochemistry assays to be performed; so differential counts were not done. Likewise, there was insufficient blood to assess serum VHSV titre in addition to plasma biochemistry. Evaluation of the number of head kidney macrophage cells per fish was determined prior to performing the immunoassays. The small size of the fish and low number of head kidney macrophage cells per fish (<2.0 x 10^6) made it necessary to pool tissue from two fish per sample for the macrophage phagocytosis assay. The distribution pattern (modality) of Hct and Lct was also examined at each time point.

3.2.6. Adults - Multiple concentration experiment

Three concentrations of WSFO were used for exposures of 6 to 16 d to determine the relationship between sublethal responses and WSFO concentration, and to serve as a preliminary range finder for chronic adult exposures.

3.2.6.1. 6-day and 16-day WSFO exposures

A total of 260 adult BS herring were exposed to three WSFO concentrations to assess sublethal responses following a sub-chronic exposure. Body mass ranged from 38.2 to 143.9 g (mean 73.5 ± SE 2.2) and fork length ranged from 18.0 to 31.8 cm (mean 21.8 ± SE 0.2). Size did not differ significantly among the control and treatment groups (Wilcoxon/Kruskal-Wallis, $p<0.05$). Fish were divided among 8 tanks, which duplicated the four treatment groups (control, 40 g oil, 80 g oil, 120 g oil). Four fish per tank (n=8 per treatment) were sampled on day 6, and the remaining fish were sampled on day 16. Differences in mortality resulted in unequal sample numbers per treatment on day 16. Variables evaluated were: CF, Hct, Lct, WBC differential counts, plasma biochemical measurements (glucose, lactate, total protein, albumin, cortisol, chloride, calcium, phosphorus and ALP), lysozyme activity and macrophage phagocytosis activity. The MFS laboratory tested a sub-sample of these fish (n=18) for the presence of VHSV in liver tissue samples (Appendix 1, Protocol No. 13) after the experiment.

3.2.7. Adults - Single concentration exposure - pilot experiment

3.2.7.1. 28-day WSFO exposure and recovery

The purpose of this experiment was to evaluate the effect of a 28-day exposure to the maximum achievable WSFO (Table 3.1) and a subsequent 42 d recovery. Adult QI herring (n=100) were divided equally among four experimental tanks. Two tanks served as controls and two tanks contained WSFO for 28 d. Body mass did not differ among treatment groups and ranged from 14.6 to 41.9 g (mean 29.1 ± SE 0.9), but fork length was significantly greater by 1.5 cm in WSFO-exposed herring on day 70 ($p=0.03$). This size difference was considered in the interpretation of results as several variables were correlated to size (Table 2.10). Fish were sampled on day 28 (24 per treatment) and day 70 (7 per treatment) after the initiation of exposure. All fish sampled on day 70 had recovered for 42 d. The decrease in sample size on day 70 was due to mortality during the experiment (an additional 5 fish in the WSFO-exposed group were not sampled). Tier 1 variables included: Hct, Lct, condition indices (CE, LSI, GSI and SSI), and plasma glucose and lactate. Tier 2 was evaluated with macrophage function assays (Appendix 1, No. 5 and 6).

3.2.8. Adults - Time course experiments

These experiments examined the impact of WSFO exposure on adult herring using the maximum achievable concentration of WSFO. Sampling occurred at frequent intervals over a long period (up to 77 days) and used more variables to evaluate responses than in the earlier adult experiments.

3.2.8.1. 21-day WSFO exposure (Part I) and VHSV challenge and recovery

Adult QI herring (n=480) used in this experiment ranged in body mass from 15.2 to 57.2 g (mean 29.1 ± SE 0.6 g) and in length from 12.2 to 18.8 cm (mean 15.0 ± SE 0.1 cm) and were divided among eight 450 L tanks prior to starting exposures. Fish did not vary significantly in size among treatment groups (Wilcoxon/Kruskal-Wallis test, $p<0.05$). Four control tanks and four tanks receiving water containing WSFO were used. Six fish were sampled from each tank on day 7 and day 21 after initiation of the WSFO exposure. Tier 1 variables included: Hct, Lct, condition indices (CF, LSI, SSI, GSI) and plasma lysozyme. Tier 2 was evaluated using macrophage phagocytosis activity.

Immediately following the 21-day WSFO exposure, adult herring were challenged with 1 x 10^4 pfu/mL VHSV (based on the virulence of the stock solution) in duplicate tanks (see

Chapter 4) *via* the 1 h static bath challenge method (Kocan *et al.*, 1997). In this experiment, the challenge dose of VHSV was higher than that used in the preliminary VHSV challenge, because the previous dose did not induce clinical disease. Duplicate tanks of control, none WSFO-exposed fish were similarly exposed. The original VHSV stock (PWS isolate) was 4.0 x 10^8 pfu/mL and was obtained from National Fisheries Science (MFS), WA (courtesy of Nancy Elder). Exposures were staggered by 5 min to facilitate identical exposure times in all tanks. After a 1 h exposure period, water flow was restored and water samples were taken from all virus-challenged tanks at 5 min, 1 h, 24 h, and 5 d post-exposure (Appendix 1, Protocol No. 14). The water samples were frozen immediately at -80°C and tested later for viral loads in the water (Appendix 1, Protocol No. 13). These tests showed that the effective dose after 5 min was 1.92 x 10^4 pfu/ml, which declined to 4.15 x 10^3 pfu/mL by the end of the 1 h static exposure. VHSV was not detected in the water at 24 h or 5 days post-challenge. The 50% survival time in seawater for VHSV is approximately 10 h (Kocan *et al.*, 2001b).

Mortality rates were recorded daily for 56 d following the challenge. A sub-sample of 20 fish from each treatment group was removed by dip net and euthanized every 14 d for Tier 1 and 2 variable measurement and pathology. Immediately after taking blood, fish were frozen at -80°C until the virus assay was performed at the MFS laboratory. The Tier 1 variables included CF, Hct, and Lct. Tier 2 was evaluated using plasma lysozyme and the virus neutralization assay, which was a means of measuring serum antibody and tissue virus titres. On these sample days , it was not possible to evaluate macrophage phagocytosis activity, SSI, GSI, or gender as the tissues from which these are determined were required for the virus neutralization assay (tissue VHSV load) for which the fish had to be frozen whole.

3.2.8.2. 21-day WSFO exposure (Part II) and recovery

Adult BS herring (n=200) ranged from 36.2 to 90.1 g (mean 61.4 ± SE 1.3), and from 18.0 to 23.5 cm (mean 21.0 ± SE 0.1) in fork length and were divided equally into four 450-L experimental tanks. Size was not significantly different among treatment groups at each sample time during the experiment (Wilcoxon/Kruskal-Wallis test, $p<0.05$). Two tanks served as controls and two contained WSFO. A sample of 5 fish per tank (10 per treatment) was taken as a baseline prior to initiation of the WSFO exposure. On day 4, 7, 14, 21, and 63 after the initiation of exposure, 5 fish per tank (10 per treatment) were euthanized and sampled. CF, Hct, Lct, plasma glucose, lactate, cortisol, albumin,

protein, chloride, calcium, phosphorus, plasma lysozyme activity, *I. hoferi* infection status and macrophage activity (phagocytosis and respiratory burst) were all measured.

3.3. RESULTS

3.3.1. Juveniles - Multiple concentration experiment

3.3.1.1. 21-day WSFO exposure, V. anguillarum challenge, and recovery

TIME EFFECTS

Statistically significant changes in Tier 1 and 2 variables were seen in both control and WSFO-exposed herring over time. The median values for Hct and Lct fell below the 95th PE reference ranges established for captive juvenile herring in controls and in most treatment groups (Chapter 2). There was a significant reduction in the median value of both Hct and Lct in control fish at day 70 compared with baseline (day 0) fish (Figure 3.2). Control fish that were not *Vibrio*-challenged also showed significant decreases in Hct and Lct, but no change in CF from day 21 to day 70.

WSFO-exposed fish had similar Hct and Lct at day 21 compared with control fish and also showed the same changes as control fish in Hct and Lct after *Vibrio* challenge in the day 70 recovery samples except for Lct in the 80 g oil/*Vibrio* group (Figure 3.3 A, B). However, CF was significantly increased in all WSFO-exposed and/or *Vibrio*-challenged herring at the day 70 recovery sample compared with the day 21 (pre-*Vibrio*) sample for each treatment group (Figure 3.3 C). However, there was no significant difference from control fish at day 70. These changes are attributed to a captivity effect and were not latent effects of either WSFO exposure or *Vibrio* challenge with the exception of the increase in median Lct in the 80 g oil/*Vibrio* group seen at day 70.

Lct was detectable in all fish sampled at the start of the experiment, but over time the proportion of fish with no detectable Lct (0%) increased. At day 21, 30 to 40% of fish in all treatment groups had 0% Lct and this increased to 52 to 87% by day 70, except in the 80 g WSFO/*Vibrio* group (Table 3.4). This increase in the proportion of fish with no detectable Lct resulted in a change in the Lct distribution pattern over time. At day 21, Lct was bimodally distributed in the control and 40 g oil group (Figure 3.4). At day 70, these distribution plots showed a loss of the upper mode in the control and 40 g WSFO group concurrent with the increase in undetectable Lct (Figure 3.3). Given that the decrease in Lct seen in the 40 g and 120 g WSFO exposure groups was the same at that seen in the control fish it was likely a captivity effect. In the 80 g oil/*Vibrio* group of

fish there was clearly an opposite effect of elevated Lct with some values at 6.8 and 8.4%, some of the highest recorded during this study. The distribution of Hct at day 70 was altered in all groups compared with at day 21 following the WSFO exposure (Figure 3.5).

Table 3.3. Comparison of the prevalence of undetectable Lct between treatment groups of 0+year BS herring using the Likelihood Ratio test at the 95% confidence level. ** Varies significantly from non-*Vibrio* exposed controls. *Sample lost during processing was not counted as an undetectable value. Lct=leucocrit

Prevalence of undetectable Lct after 21-day WSFO exposure			Prevalence of undetectable Lct in recovery sample (42 days after ending WSFO exposure & *V. anguillarum* challenge, day 70)		
Treatment	Lct Undetectable	P-value	Treatment	Lct undetectable	P-value
40 g oil	8 / 20 (40%)	0.90	Oil (40 g oil/*Vibrio*)	9 / 17 (52.9%)	0.02
80 g oil	8 / 21 (38%)		Oil (80 g oil/*Vibrio*)	4 / 11 (36.4%)**	
120 g oil	8 / 20 (40%)		Oil (120 g oil/*Vibrio*)	20 / 23* (87.0%)	
Control	6 / 20 (30%)		Control	7 / 9 (77.8%)	
			Vibrio only	27 / 38* (71.1%)	

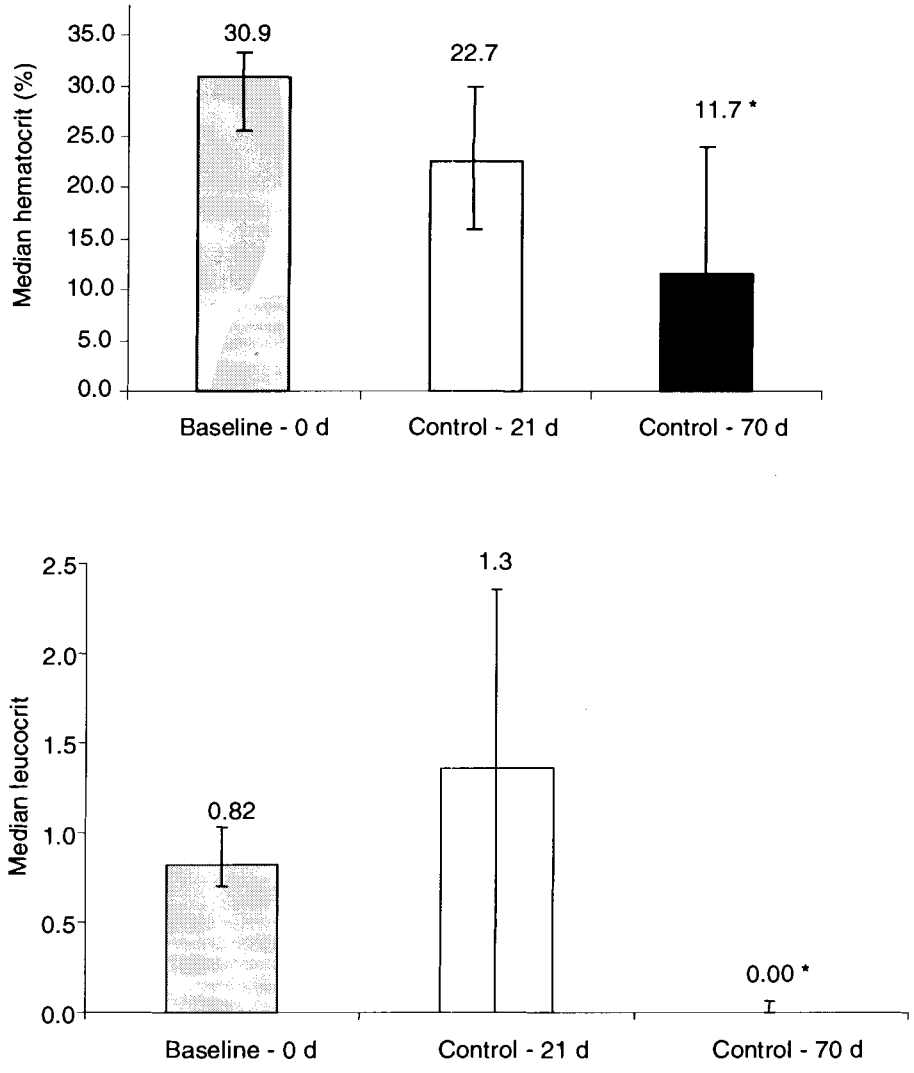


Figure 3.2. The median Hct and Lct values of non-*Vibrio* exposed control juvenile herring at day 21 and day 70 compared with baseline (day 0) juvenile herring. The vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles). The Wilcoxon/Kruskal Wallis test was used with $p<0.05$ significant and the asterisk (*) shows a significant change compared to the baseline value.

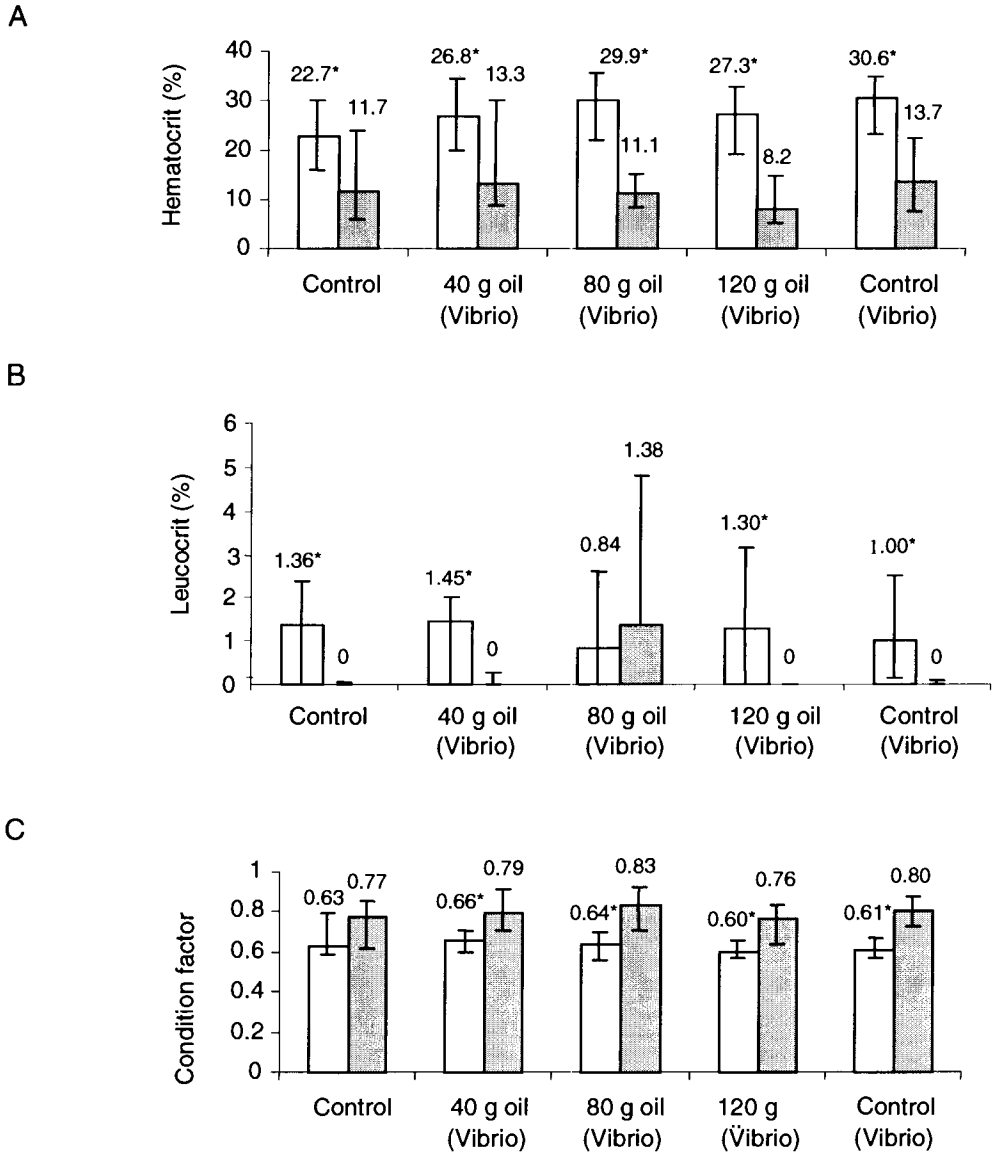


Figure 3.3 A-C. The median value of Hct, Lct, and condition factor at day 21 *vs.* day 70 within each exposure group of juvenile herring. Vertical bars are the inter-quartile range (25th and 75th percentiles). Wilcoxon/Kruskal-Wallis test used with $p<0.05$ significant and denoted by an asterisk (*). (□) 21 days (■) 70 days

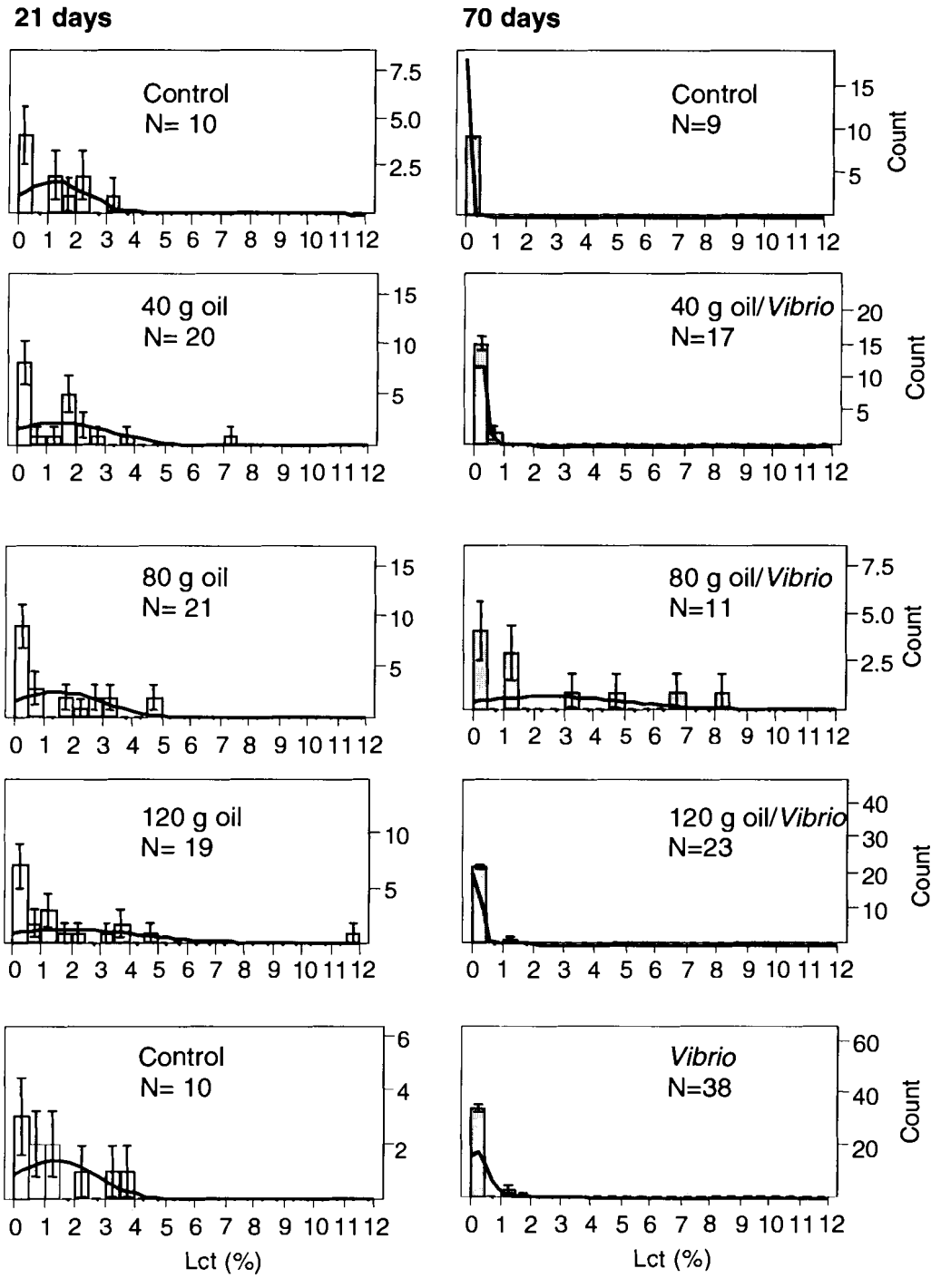


Figure 3.4. The distribution of Lct for juvenile herring at 21 and 70 days after initiation of WSFO exposure. WSFO exposure ended after 21 days. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

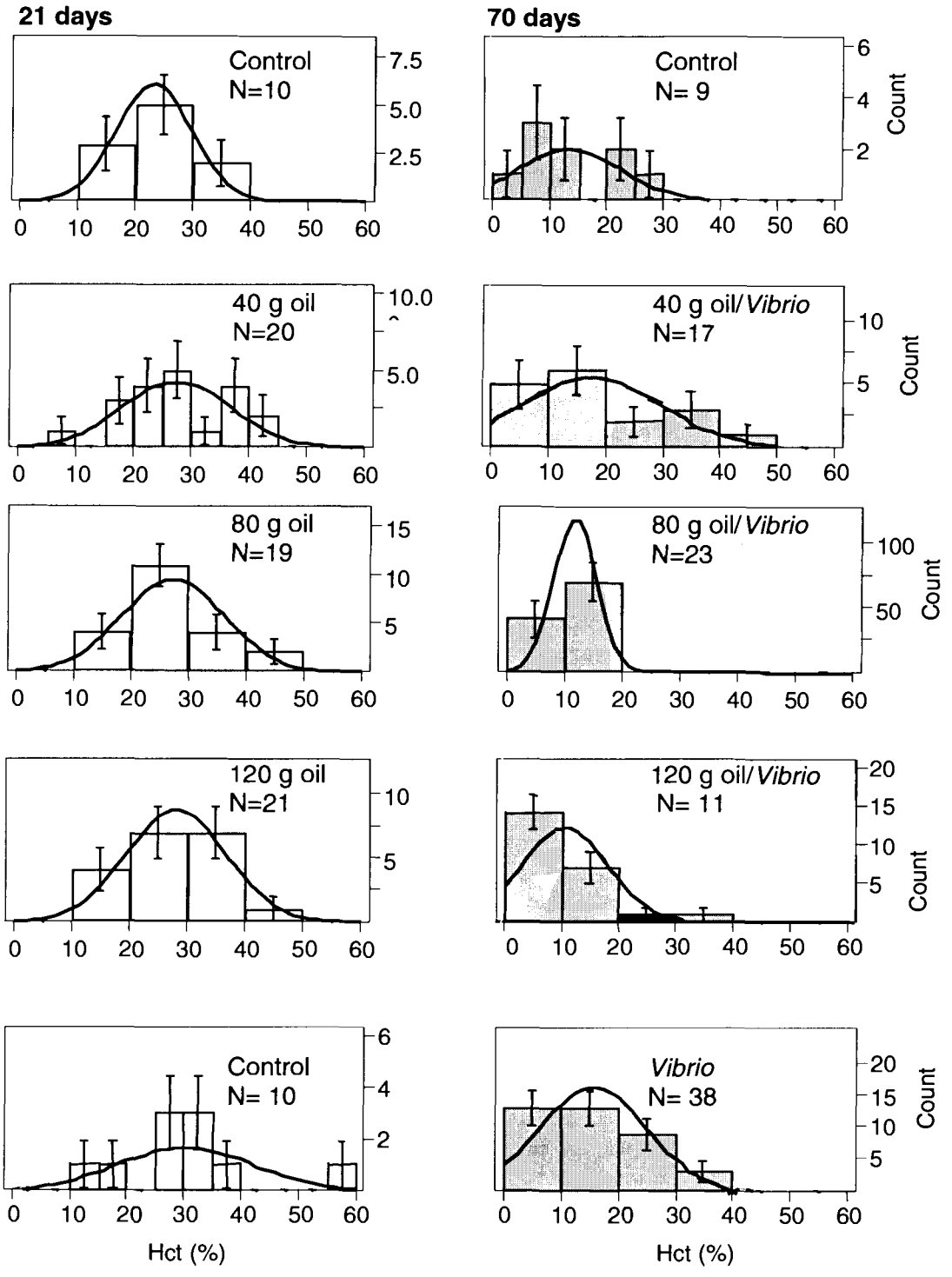


Figure 3.5. Comparison of Hct distribution between treatment groups of 0+year BS herring at 21 and 70 days after initiation of WSFO exposure. WSFO exposure was terminated after 21 days. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

EXPOSURE EFFECTS

In 0+year herring there was no statistically significant difference in cumulative mortality among treatment groups during the 21-day exposure period (ANOVA). Mortality reached 15 to 35%, which was somewhat lower than the adult 16-day WSFO exposure (Section 3.3.3). Mortality continued during the recovery period that followed the pathogen challenge (day 22 to 77), but there were no statistical differences among treatment groups in either cumulative or proportional mortality (Figure 3.6, 3.7.A). Nonetheless, the proportion of mortality attributable to V. *anguillarum* was higher in all WSFO-exposed groups compared with the control fish (Figure 3.7B). Mortality due to *Vibrio* infection was confirmed in 75 to 91% of mortalities of WSFO plus *V. anguillarum*-challenged herring, in 78% of herring challenged with *V. anguillarum* only and in 25% of dead control mortalities (4% of all controls tested) (Table 3.4). The control mortalities positive for *V. anguillarum* infection identified a background level of this pathogen as care was taken to avoid laboratory cross-contamination. The prevalence of *Vibrio*-positive dead fish was significantly greater in all *Vibrio*-challenged groups compared with the control fish. Of the dead herring previously exposed to the WSFO, the group exposed to the lowest concentration had the highest proportion of culture positive individuals.

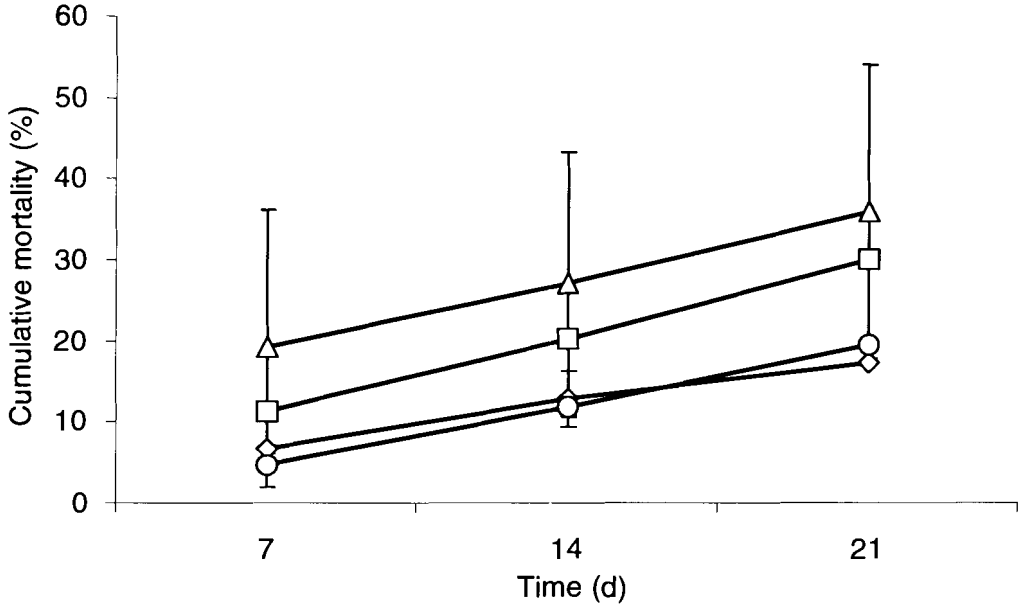


Figure 3.6. Cumulative mortality in control and WSFO-exposed juvenile herring. Values are means ± SE. There were 4 replicates of controls and duplicates for each WSFO exposed group. ANOVA was used to evaluate normalized (arcsine square root transformed) percent data using tank as the experimental unit with $p<0.05$ as significant.
(-□-)Control (-◇-)40 g oil (-○-) 80 g oil (-△-) 120 g oil

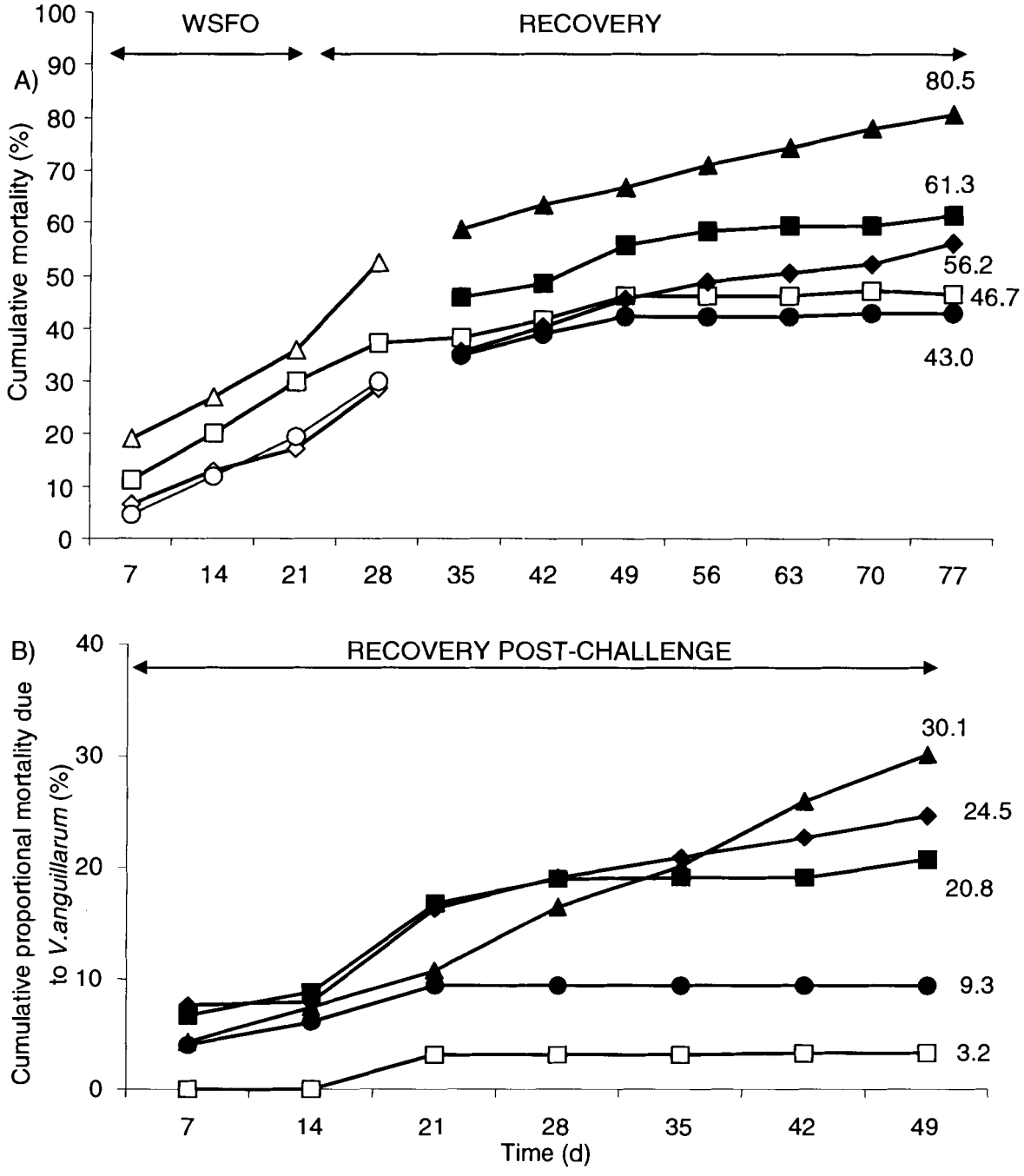


Figure 3.7. A) Cumulative percentage mortality among treatment groups of juvenile herring during WSFO exposure (7 to 21 d) and during the post-*V. anguillarum* observation period (35 to 77 d). *V. anguillarum* challenge occurred on day 28; B) Cumulative proportional mortality attributable to *V. anguillarum* during the post-challenge observation period among treatment groups of juvenile herring. ANOVA was used to evaluate normalized (arcsine square root transformed) percent data with $p<0.05$ considered significant. Values are tank means (2 tanks per treatment group). Cumulative proportional mortality = (No. of dead fish due to pathogen/N at risk) x 100

(-□-)Control (-◇-)40 g oil (-○-) 80 g oil (-△-) 120 g oil

(-▲-) 120 g oil/*Vibrio* (-◆-)40 g oil/*Vibrio* (-●-) 80 g oil/*Vibrio* (-■-)*Vibrio*

Table 3.4. The proportion of dead herring that were culture and agglutination test positive for *V. anguillarum* in each treatment group. The Likelihood Ratio test was used with p-value of <0.05 considered significant.

Treatment group	Positive for *V. anguillarum*	Number of dead fish during observation period	Proportion of dead fish that were *V. anguillarum* positive	p-value (LR test)
Control	4	16	25.0%	0.0003
40 g oil/*Vibrio*	30	33	90.9%	
80 g oil/*Vibrio*	12	16	75.0%	
120 g oil/*Vibrio*	20	26	76.9%	
Vibrio only	18	23	78.3%	

A 21-day exposure to WSFO alone did not significantly alter any Tier 1 (CF, Hct, Lct, WBC differential counts) immune system variables and was not associated with a difference in the prevalence of anemia or lesions (Tables 3.5 and 3.6). Herring challenged only with *V. anguillarum* demonstrated a 3- to 20-fold higher antibody titre than WSFO-exposed fish (Figure 3.8). Therefore, WSFO exposure suppressed *Vibrio*-induced antibody titre (Tier 2), but antibody titre did not vary with WSFO concentration. The prevalence of lesions also did not vary significantly among treatment groups when assessed after the recovery period (Table 3.7). Most of the lesions, including those in control fish, were internal and involved the liver, with green discolouration of the liver being the most common abnormality observed.

Table 3.5. The median value for the variables measured at day 21 following WSFO exposure among the treatment groups of juvenile herring. The Wilcoxon-Kruskal/Wallis test was used to test for significant differences between groups at the 95% confidence level. CF= condition factor, Hct=hematocrit, Lct=leucocrit.

Variable	Control	40 g oil	80 g oil	120 g oil	p-value
CF	0.63 (20)	0.66 (20)	0.64 (21)	0.60 (20)	0.69
Hct (%)	27.3 (20)	28.3 (20)	26.8 (21)	29.9 (19)	0.85
Lct (%)	1.25 (20)	1.45 (20)	0.84 (21)	1.30 (19)	0.99
Lymphocytes (%)	72.2 (14)	67.1 (16)	53.6 (15)	62.5 (19)	0.42
Neutrophils (%)	12.2 (14)	12.1 (16)	22.1 (15)	13.7 (19)	0.65
Thrombocytes (%)	15.7 (14)	11.4 (16)	11.3 (15)	18.6 (19)	0.24

Table 3.6. The prevalence of any gross lesion and Hct <13% with respect to treatment group in 0+year Pacific herring after 21 days of WSFO exposure using the Likelihood Ratio test at the 95% confidence level to compare among treatment groups. *Hct unknown for one 120 g oil-exposed herring due to lost sample. Hct=hematocrit.

Treatment	Hct <13%	p-value	Treatment	Lesions	p-value
40 g oil	1 / 20 (5.0%)	0.92	40 g oil	0 / 20 (0%)	0.21
80 g oil	2 / 21 (9.5%)		80 g oil	2 / 21 (9.5%)	
120 g oil	1 / 19* (5.3%)		120 g oil	1 / 20 (5.0%)	
Control	1 / 20 (5.0%)		Control	0 / 20 (0%)	

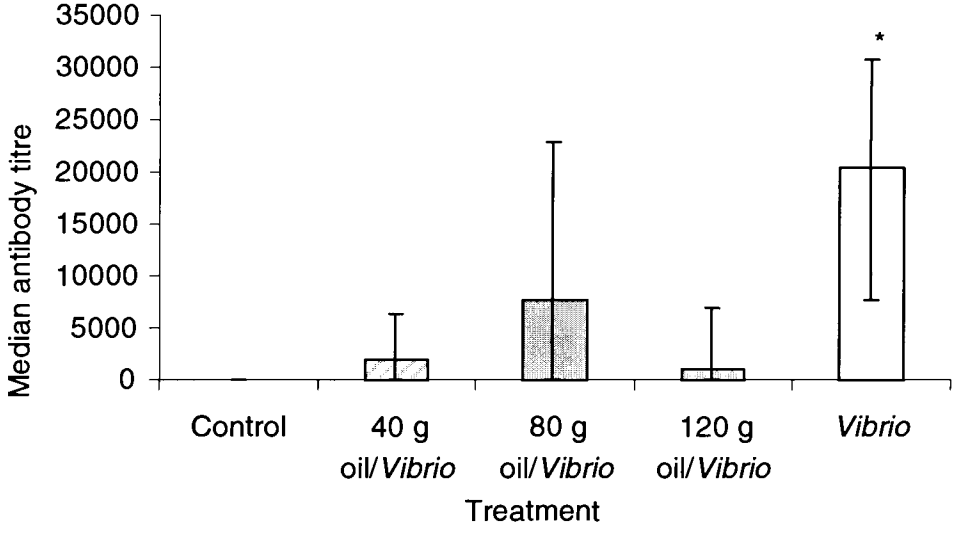


Figure 3.8. The median *V. anguillarum* antibody titre of control, WSFO/*Vibrio* challenged, and *Vibrio*-only challenged juvenile herring at day 70 (42 d after WSFO exposure and challenge with *V. anguillarum*). Vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles). Wilcoxon/Kruskal-Wallis test, $p<0.05$ significant and denoted by an asterisk (*).

Table 3.7. The prevalence of Hct <13% and lesions among treatment groups of 0+year BS herring using the Likelihood Ratio test at the 95% confidence level after the recovery period. *Indicates reduction in denominator due to loss of sample during processing. Hct=hematocrit.

Prevalence of Hct <13%			Prevalence of lesions		
Count	Yes	p-value	Count	Present	p-value
Control	4/ 9 (44.4%)	0.24	Control	5 / 9 (55.6%)	0.45
Oil (40 g oil/*Vibrio*)	5/17 (29.4%)		Oil (40 g oil/*Vibrio*)	10 / 17 (58.8%)	
Oil (80 g oil/*Vibrio*)	4/11 (36.4%)		Oil (80 g oil/*Vibrio*)	6 / 11 (54.5%)	
Oil (120 g oil/*Vibrio*)	14/23*(60.9%)		Oil (120 g oil/*Vibrio*)	12 / 24 (50.0%)	
Vibrio only	13/38*(34.2%)		*Vibrio* only	14 / 39 (35.9%)	

3.3.2. Juveniles - Time course experiment

3.2.2.1. 21-day WSFO exposure and recovery and VHSV challenge

TIME EFFECTS

Over the course of the experiment, liver-somatic index decreased in control fish (Figure 3.9). However, the controls showed only minor fluctuations in Hct and Lct over time, which is in contrast to the marked changes in Hct and Lct in the controls from the first BS juvenile experiment. With respect to biochemical parameters in control fish, plasma lactate increased and plasma glucose was elevated at day 14 (Figure 3.9). **Phagocytosis activity showed a peak at day 21 in control fish (Figure 3.9).** In WSFO-exposed juvenile herring, phagocytosis was the only variable to change with time with a peak occurring at day 21 (Figure 3.10).

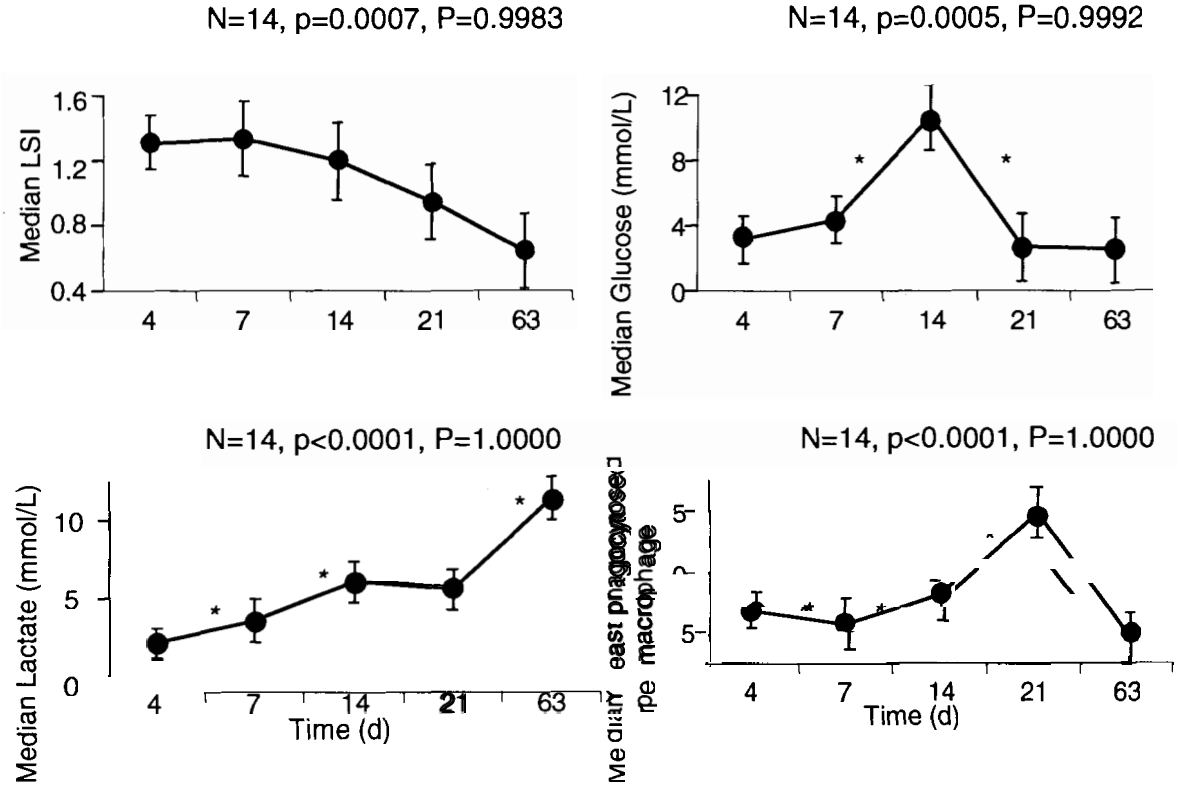


Figure 3.9. Variables that differed significantly over time in control, 0+year BS herring using the tank as the experimental unit and comparing median values using the GLM (least square means) at the 95% confidence level. There were 4 replicate tanks for day 4 and 7 and 2 replicate tanks for the remainder of the sample days. An asterisk indicates which time intervals were significant.

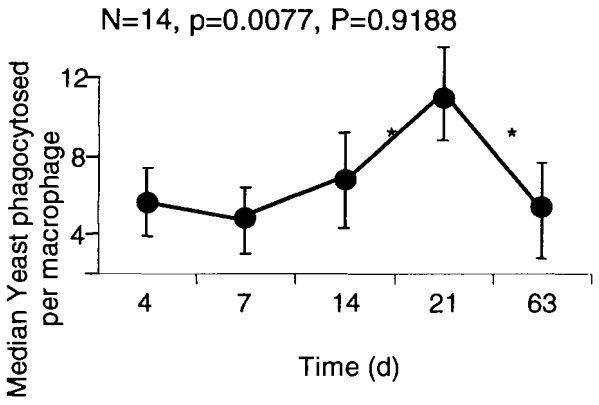


Figure 3.10. Variation in phagocytosis activity in juvenile herring exposed to the WSFO for 21 days using the tank as the experimental unit and comparing median values using the GLM (least square means) at the 95% confidence level. There were 4 replicates for day 4 and 2 replicates for the remainder of the sample days. An asterisk indicates which time intervals were significant.

EXPOSURE EFFECTS

Cumulative mortality in control fish in this second juvenile experiment was lower, being 15% at 21 d and 26% at 70 d and did not reach the 47% observed in the previous experiment by 77 d. Cumulative mortality was significantly higher in WSFO-exposed fish by day 14 and was almost 60% by 21 d (Figure 3.11, ANOVA, $p<0.05$). However, the daily mortality rate reached a plateau in WSFO-exposed and control fish during the latter half of the 42-day recovery period. Therefore, while cumulative mortality was significantly different between day 14 and day 42, the majority of the increase in mortality occurred during the WSFO exposure period and not during recovery. This was the only experiment in this study with statistically significant differences in mortality rate.

During the pathogen challenge component of this experiment, the highest cumulative mortality in juvenile herring occurred in the WSFO (68.9%) and the WSFO-dosed/VHSV-challenged (64.8%) fish, with no significant difference between these two treatments. Mortality was significantly greater starting at 35 d post-WSFO exposure (28 d post-VHSV challenge) in the WSFO/VHSV-challenged fish only compared with control fish (ANOVA on arcsine square transformed data, $p=0.04$). During the remainder of the post-challenge period, both the WSFO/VHSV-challenged and WSFO-exposed herring had significantly higher cumulative mortality than control fish. There was no statistical difference in the cumulative mortality between VHSV-only challenged (51.0%) and control fish (26.3%) or between VHSV-only challenged and WSFO-exposed or WSFO/VHSV-challenged fish (Figure 3.12).

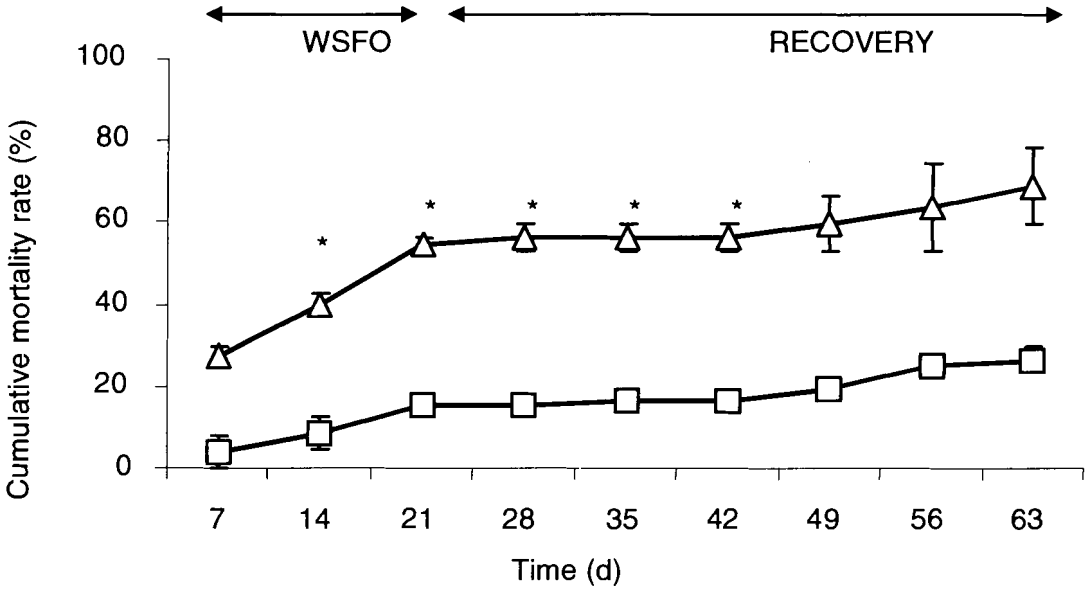


Figure 3.11. Cumulative mortality among control and WSFO-exposed juvenile BS herring. Values are means ± SE of averaged mortality rate of replicate tanks (2 per treatment). ANOVA was used -to evaluate normalized (arcsine square root transformed) percent data with tanks as the experimental unit. $p<0.05$ considered significant and denoted by an asterisk (*). (—□—) Control (—△—) Oil

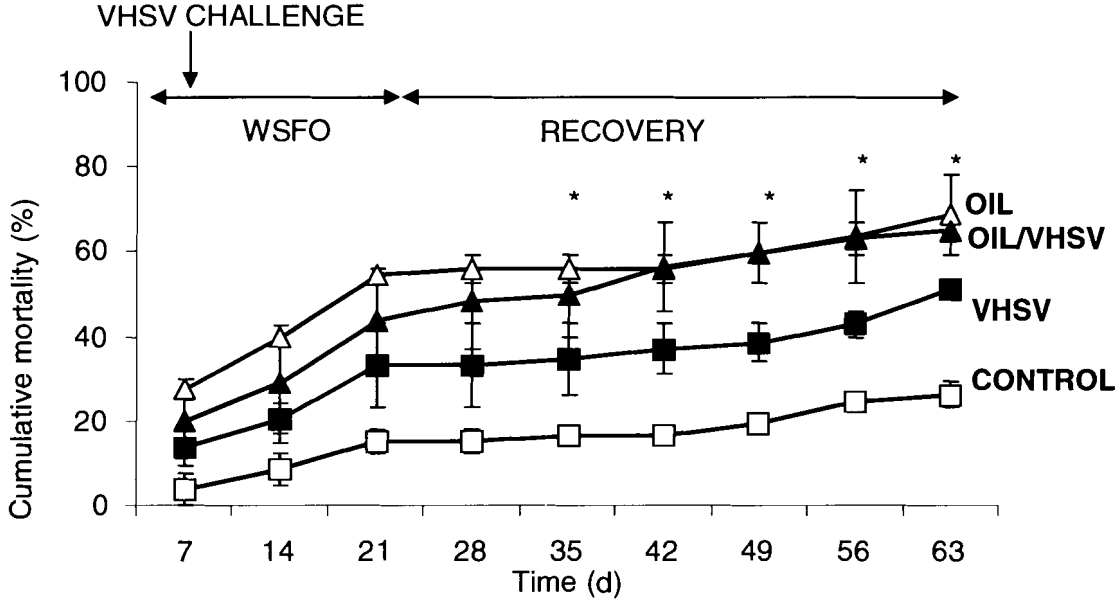


Figure 3.12 Comparison of cumulative mortality between treatment groups of juvenile BS herring during the 56-day post VHSV challenge period. Values are mean ± SE, based on the mean of replicate tanks, with tanks as the experimental unit. ANOVA on arcsine square root transformed percentage data was used to test for differences between groups at each time point, $p<0.05$ considered significant. An asterisk (*) denotes a significant difference from controls.

(—□—)Control (—△—)Oil (—■—)VHSV (—▲—)Oil/VHSV

VHSV was isolated from 9.5% (12/126) of juvenile fish (Table 3.8). During week 1, the proportion of dead fish testing positive (42.9%, 3/7) in the VHSV-only challenged group was significant compared to the proportion in all other groups, p=0.03 (Likelihood Ratio test, p<0.05 significant). There was no significant difference in the tissue titre among control, WSFO-exposed, VHSV-challenged, or WSFO/VHSV-challenged fish overall or at any specific time. The majority of the dead fish testing positive occurred during the first 21 d post-challenge, with none testing positive after 49 d. Proportional mortality attributable to VHSV (isolated from tissues) was only significantly different at 7 d post-challenge and only for the VHSV-challenged group. There were no other significant differences in proportional mortality throughout the remainder of the observation period (Figure 3.13).

Table 3.8. Comparison of the overall proportion of mortalities among treatment groups of juvenile herring with a positive viral tissue titre using the Likelihood Ratio test at the 95% significance level.

Treatment	Proportion VHSV positive	p-value
Control	2 / 20 = 10.0%	0.11
Oil	0 / 26 = 0.0%	
Oil/VHSV	5 / 43 = 11.6%	
VHSV	5 / 37 = 13.5%	

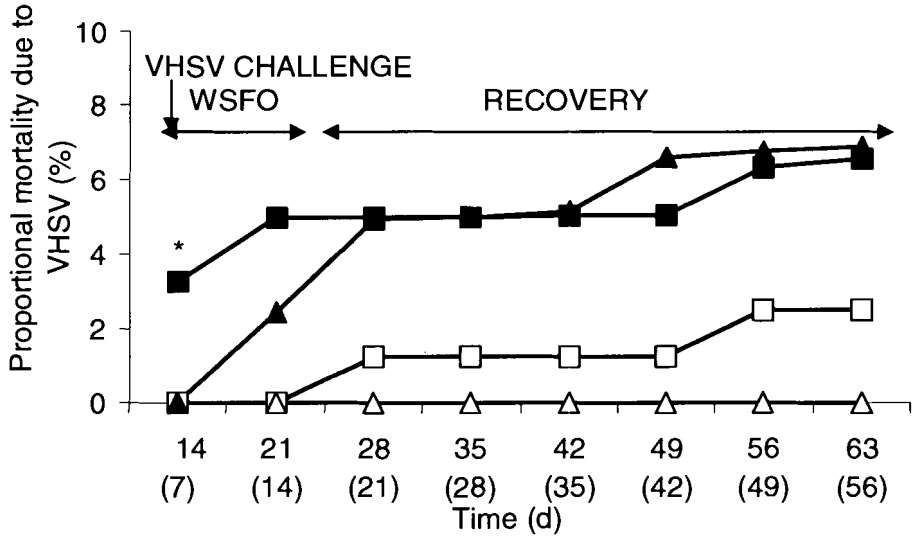


Figure 3.13 Comparison of proportional mortality attributable to VHSV between treatment groups of juvenile BS herring during the 56-day post VHSV challenge period. Values are mean ± SE, based on the mean of replicate tanks, with tanks as the experimental unit. ANOVA on arcsine square root transformed percentage data was used to test for differences between groups at each time point, p<0.05 considered significant. An asterisk (*) denotes a significant difference from controls.

(-□-)Control (-△-)Oil (-■-)VHSV (-▲-)Oil/VHSV

In this second juvenile experiment, herring were exposed to a single (maximum possible) concentration of WSFO. This resulted in significant changes in Tier 1 and 2 measurements (CF, LSI, glucose, lactate, total protein and lysozyme) during exposure and recovery that were not observed in control fish (Figures 3.14 and 3.15). However, no single variable remained altered for the entire period and different variables responded at different times during the exposure period.

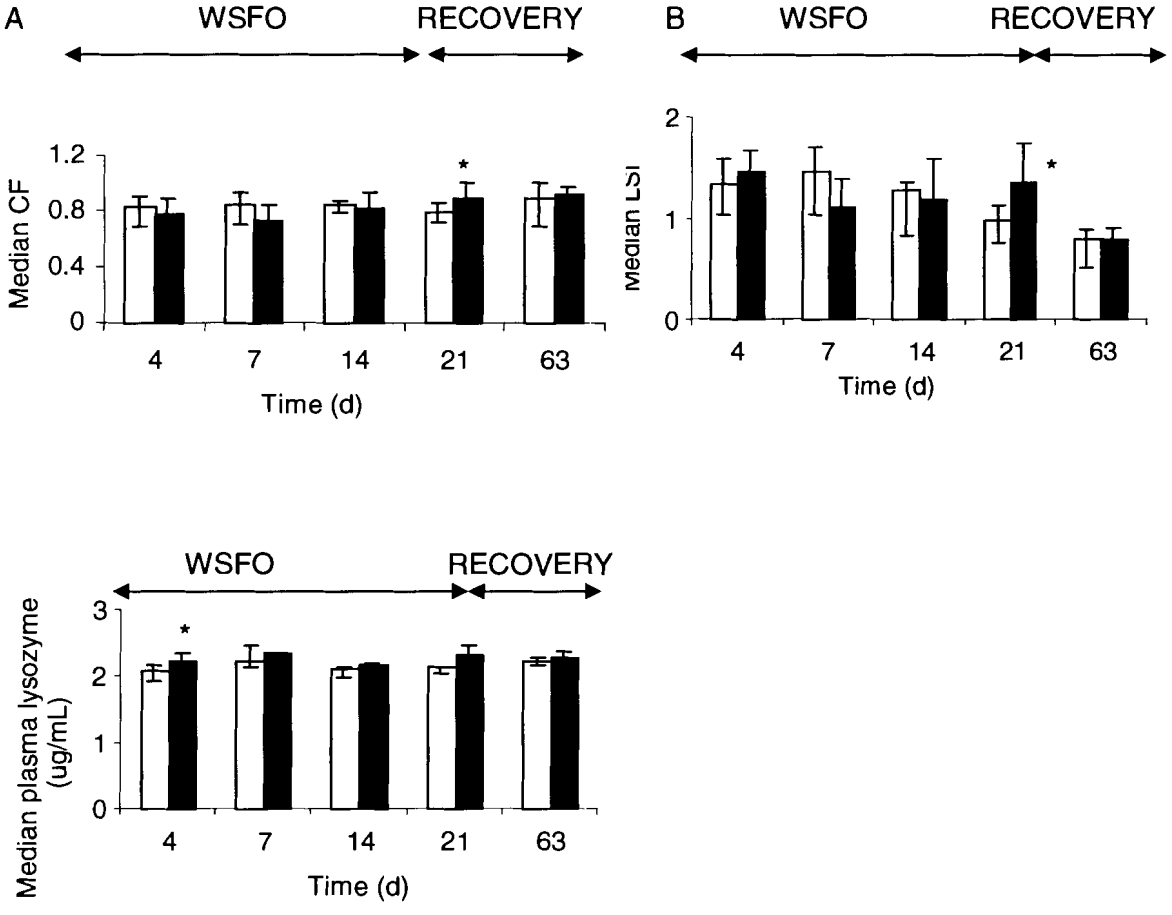


Figure 3.14 A-C. The median value for variables that changed significantly with WSFO exposure at varying times post initiation of WSFO exposure: A) CF, B) LSI, and C) plasma lysozyme. Significant differences from controls at a specific time are shown with an asterisk (*), with $p<0.05$ considered significant. Comparisons were made with the Wilcoxon/Kruskal-Wallis test at each point. The vertical bars represent the inter-quartile range (25^{th} and 75^{th} percentiles). (□) Control (■) Oil

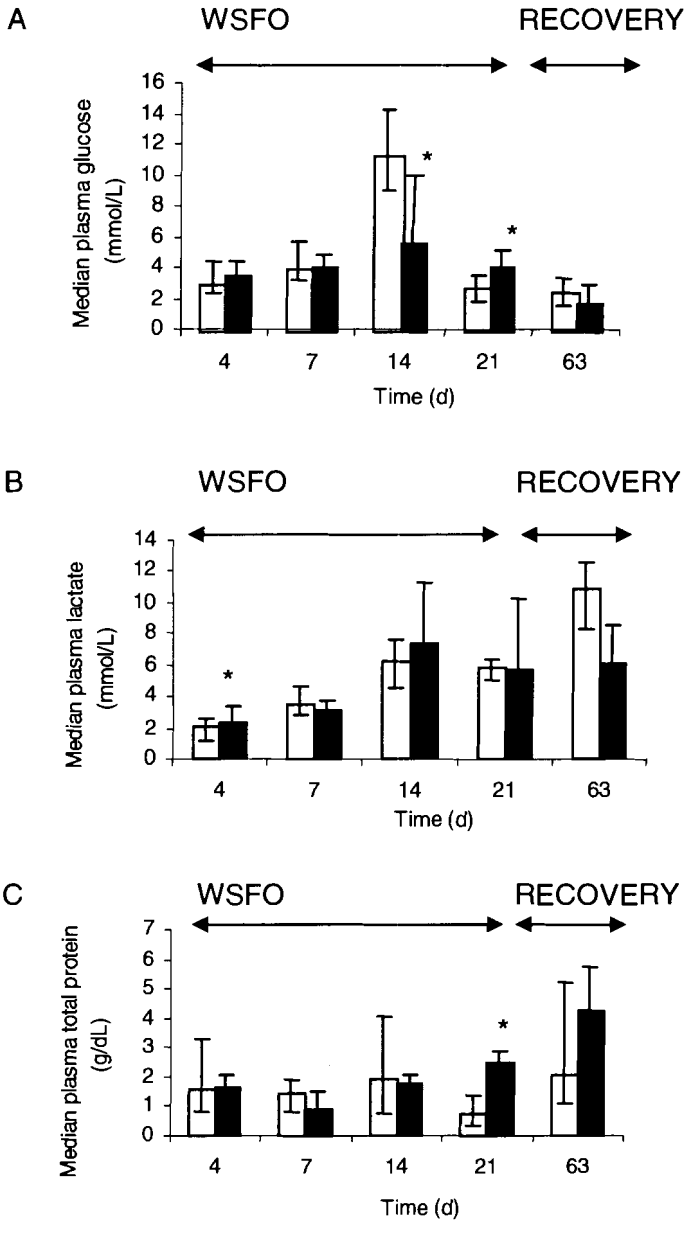


Figure 3.15 A-C. The median value for the Tier 1 variables of plasma glucose, lactate, and protein among treatment groups of juvenile BS herring following exposure to the WSFO. Significant differences from controls at a specific time are shown with an asterisk (*), $p<0.05$ considered significant. Comparisons were made with the Wilcoxon/Kruskal-Wallis test at each point. The vertical bars represent the inter-quartile range (25^{th} and 75^{th} percentiles). (□) Control (■) Oil

An increase in LSI was evident in WSFO-exposed and VHSV-challenged fish after the 21-day WSFO exposure (14-days concurrent VHSV exposure) (Figure 3.16 A). Plasma glucose was significantly lower in WSFO-exposed; VHSV-challenged, and WSFO/VHSV-challenged herring compared with the control fish at the first sample, day 14 (7) (Figure 3.16 B). Phagocytotic activity was much greater in the WSFO/VHSV-challenged fish compared with all other groups including control fish at the first sample point (Figure 3.16 C). The concurrent WSFO exposure/VHSV challenge had no significant impact on the other Tier 1 and 2 variables (CF, Hct, Lct, plasma lactate, albumin, and lysozyme).

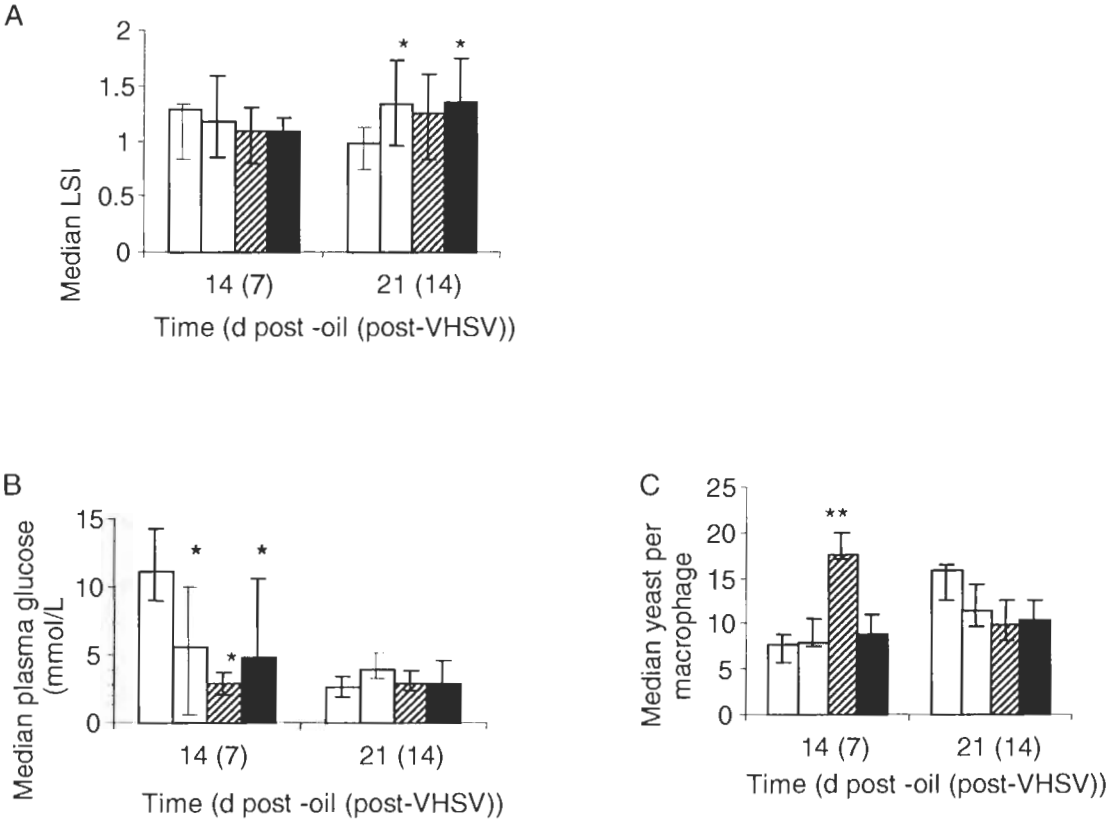


Figure 3.16 A-D. The median A) LSI, B) plasma total protein, C) plasma glucose, D) yeast phagocytosed per macrophage in juvenile BS herring at 14 and 21 d post WSFO exposure/7 and 14 d post-VHSV challenge. Comparisons were performed separately at each time point with the Wilcoxon/Kruskal-Wallis test at the 95% confidence level. Vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles). An asterisk (*) indicates exposure differs significantly from controls. The double asterisk indicates a significant difference from all other groups.

(□) Control (□) Oil (▨) Oil/VHSV (▮) VHSV

Differences in the distribution pattern of Lct and Hct between control and WSFO-exposed fish were evident. Lct was bimodal in only WSFO-exposed fish at day 14. At day 21, this bimodality was no longer present, but the controls were bimodal (Figure 3.17). These differences in distribution pattern may reflect changes in the proportion of herring with no detectable Lct (counted as zero) over time (Table 3.9). This absence of detectable Lct was very apparent in the first juvenile experiment with the same source population (BS) in which the prevalence of Lct equal to zero increased significantly with time in all but one treatment group. In this experiment, there was no significant increase in the prevalence of undetectable Lct with time or among treatment groups ($p>0.05$).

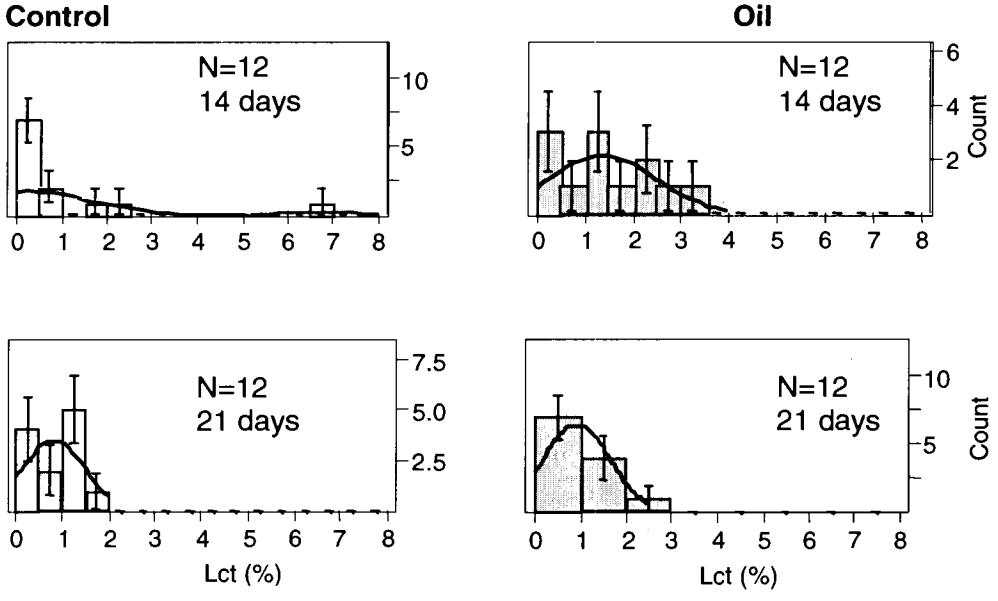


Figure 3.17. The distribution of Lct (%) among treatment groups at 14 and 21 d after initiation of exposure to WSFO in 0+year BS herring. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

The modality of the Hct distribution varied over time and between treatment groups (Figure 3.18). There was an initial loss of the upper mode with WSFO exposure, while on days 7 and 14 bimodality was present in WSFO-exposed fish compared with control fish. On the two later sample days, Hct was bimodal in both WSFO-exposed and control fish.

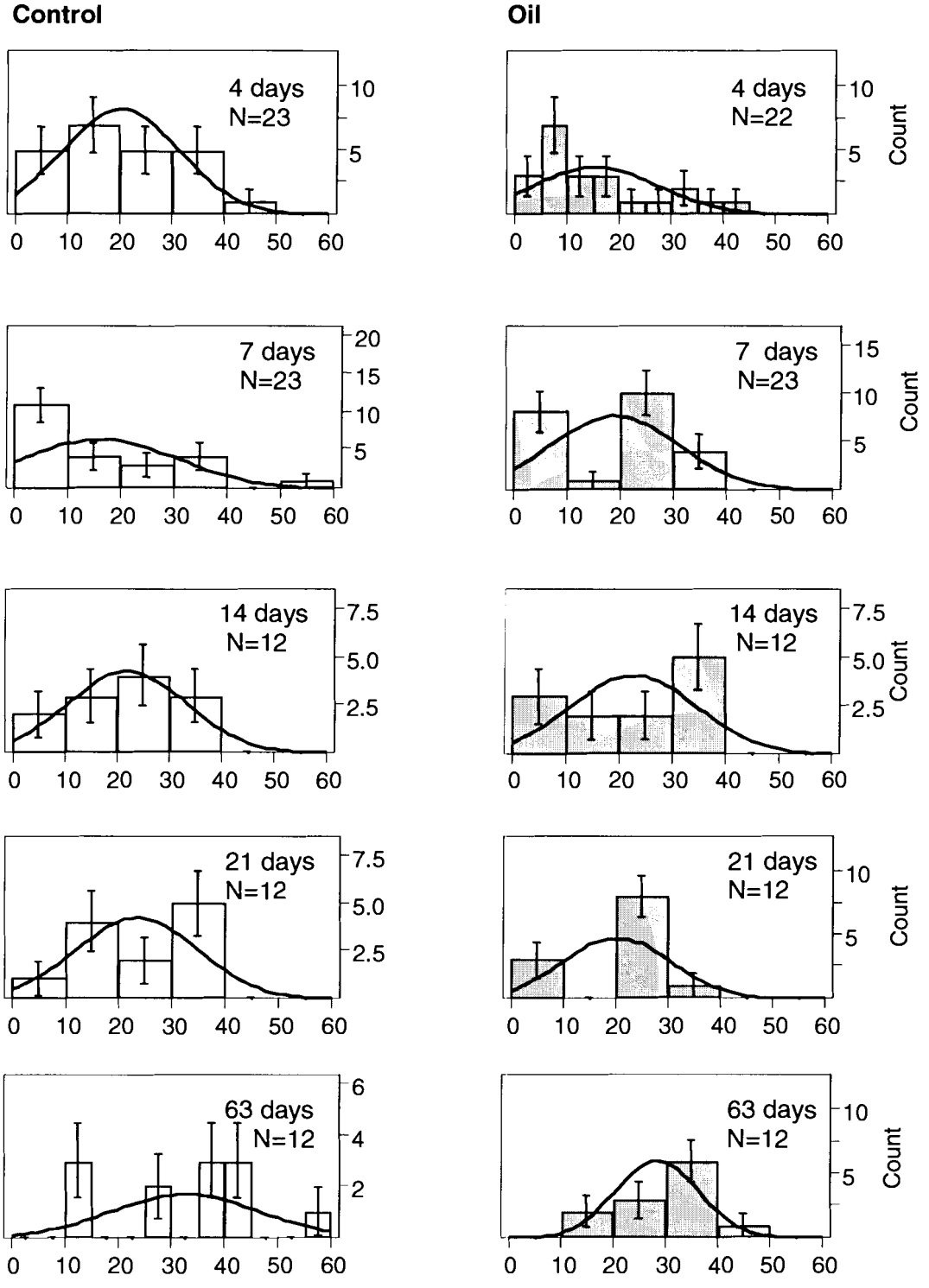


Figure 3.18. The distribution of Hct (%) among control and WSFO-exposed juvenile herring at 4, 7, 14, 21, and 63 d after initiation of exposure. The normal curve is superimposed. Bars are the mean for each level of the histogram and the error bars are the corresponding SE for each level.

Lesions were more prevalent in WSFO-exposed fish on day 4 with liver lesions predominating (Table 3.9). In addition, the prevalence of lesions was significantly greater in fish exposed to WSFO and concurrently challenged with VHSV after a 21-day WSFO exposure (14-day VHSV exposure) than in the control fish and the other two exposure groups (Table 3.9). These lesions were all classified as hemorrhagic skin lesions. The prevalence of anemia or the absence of measurable Lct in juvenile herring did not differ significantly among treatment groups throughout this experiment.

Table 3.9. The prevalence of lesions, anemia, and the proportion of immeasurable Lct among treatment groups of juvenile BS herring. The Likelihood Ratio test was used at the 95% confidence level to compare treatment groups. The first sample day number signifies the days since initiation of WSFO exposure and the number in parentheses represents the time post-VHSV challenge, which began one week after the start of the WSFO exposure.

Lesions		p	Anemic		p	Undetectable Lct		p
Day 4			**Day 4**			**Day 4**		
Control	1 / 24 (4.2%)	0.03	Control	8 / 23 (34.8%)	0.18	Control	4 / 23 (17.4%)	0.15
Oil	6 / 24 (25.0%)		Oil	12/ 22 (54.5%)		Oil	8 / 22 (36.4%)	
Day 7			**Day 7**			**Day 7**		
Control	3 / 24 (12.5%)	0.29	Control	11/ 23 (47.8%)	0.37	Control	5 / 23 (21.7%)	0.73
Oil	1 / 24 (4.2%)		Oil	9 / 23 (39.1%)		Oil	6 / 23(26.1%)	
Day 14 (7)			**Day 14** (7)			**Day 14** (7)		
Control	1 / 12 (8%)	0.19	Control	2 / 12 (17%)	0.82	Control	6 / 12 (50%)	0.38
Oil	0 / 12		Oil	3 / 12 (25%)		Oil	3 / 12 (25%)	
Oil/ VHSV	2 / 12 (17%)		Oil/ VHSV	3 / 12 (25%)		Oil/ VHSV	5 / 12 (42%)	
VHSV	0 / 12		VHSV	4 / 12 (33%)		VHSV	7 / 12 (58%)	
Day 21 (14)			**Day 21** (14)			**Day 21** (14)		
Control	0 / 12	0.01	Control	1 / 12 (8%)	0.45	Control	4 / 12 (33%)	0.80
Oil	0 / 12		Oil	3 / 12 (25%)		Oil	3 / 12 (25%)	
Oil/ VHSV	5 / 12 (42%)		Oil/ VHSV	2 / 12 (17%)		Oil/ VHSV	5 / 12 (42%)	
VHSV	1 / 12 (8%)		VHSV	4 / 12 (33%)		VHSV	5 / 12 (42%)	
Day 63			**Day 63**			**Day 63**		
Control	0 / 12	n/a	Control	2 / 12 (16.7%)	0.09	Control	3 / 12 (25.0%)	0.65
Oil	0 / 12		Oil	0 / 12		Oil	4/ 12 (33.3%)	

3.3.3. Adults - Multiple concentration experiment

3.3.3.1. 6-day and 16-day WSFO exposures

TIME EFFECTS

Several hematological and immunological variables changed significantly during the course of the experiment in control and WSFO-exposed fish (Table 3.10). In control fish, neutrophils increased and lysozyme decreased. The decrease in lysozyme seen in control fish was present to a similar degree in all WSFO-exposed groups, while the increase in neutrophils seen in controls occurred only in the 40 g WSFO-exposed group (Table 3.10). Unlike controls, the proportion of thrombocytes decreased in both the 40 g and 120 g WSFO fish with time, while Lct increased only in the 80 g WSFO group (Table 3.10). None of these changes occurred in a concentration-dependent manner. Of these statistically significant changes, the median lysozyme values measured at both day 6 and 16, for controls and all WSFO-exposed fish alike, were outside the reference range for lysozyme established in Chapter 2 using time zero fish (2.12 to 2.71 µg/mL). In addition, the percentage of thrombocytes in the 40 g and 120 g WSFO-exposed groups were greater than the 95th PE for thrombocytes on day 6.

Table 3.10. Hematological and immunological variables, which differed significantly with time in at least one treatment group (specified by YES/NO). Multivariate correlation among sample times and Spearman's non-parametric measures of association were used to compare each group over time. Differences were considered significant at $p<0.05$. S-R value is the Spearman Rho correlation coefficient. The percentage value is the magnitude of change, and the arrow indicates the direction of change. Lct=leucocrit.

Variable	Control (N=25)	40 g (N=16)	80 g (N=30)	120 g (N=21)
Neutrophils	YES ↑ (104.3%)	YES ↑ (216.3%)	NO	NO
S-R value	0.4000	0.5966		
p-value	0.0429	0.0147		
			NO	YES ↓ (41.7%)
		(52.3%)		
S-R value		-0.7462		-0.8177
p-value		0.0009		<0.0001
Lct	NO	NO	YES ↑ (204.8%)	NO
S-R value			0.4651	
p-value			0.0049	
Lysozyme	YES ↓ (69.3%)	YES ↓ (71.4%)	YES ↓ (75.3%)	YES ↓ (70.9%)
S-R value	-0.8086	-0.8677	-0.7247	-0.4618
p-value	0.0429	<0.0001	<0.0001	0.0305

EXPOSURE EFFECTS

When adult BS herring were exposed to several concentrations of WSFO, mortality did not differ significantly among exposure and control groups at any time. Cumulative mortality reached 35 to 60% by day 16 of exposure, and 35% in control fish (Figure 3.19).

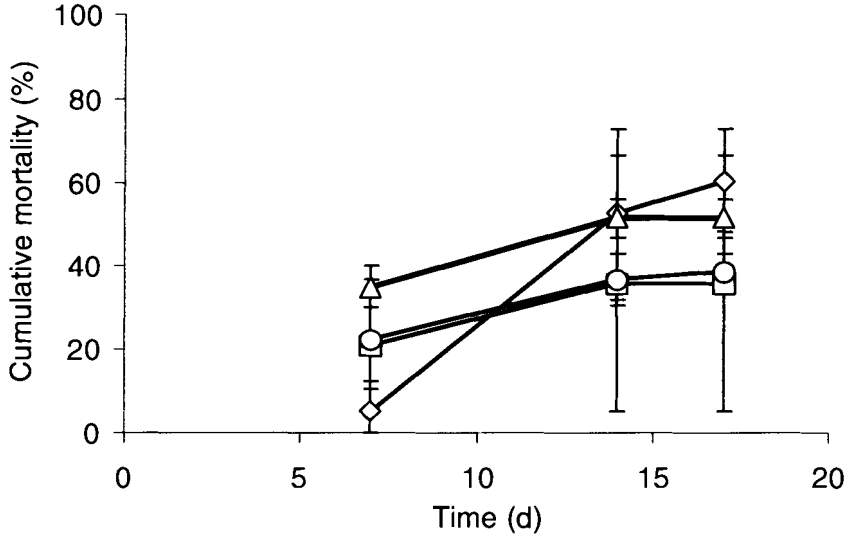


Figure 3.19. Cumulative mortality in control and WSFO-exposed adult herring. Values are tank means ± SE (2 replicate tanks per treatment). ANOVA was used to evaluate normalized (arcsine square root transformed) percent data with $p<0.05$ considered significant using the tank as the experimental unit.
(-□-)Control (-◇-)40 g oil (-○-) 80 g oil (-△-) 120 g oil

A significant difference between control fish and WSFO-exposed fish occurred only in the proportion of thrombocytes, but only at the lowest concentration of WSFO. The median percentage of thrombocytes was higher in the 40 g WSFO group (27.7%) compared to the controls (17.2%) on day 6 (Figure 3.20). Variables that did not vary significantly in either control or WSFO-exposed fish over time, or among control fish and those exposed to the three WSFO concentrations included: CF, Hct, % lymphocytes, plasma glucose, lactate, total protein, albumin, cortisol, chloride, calcium, phosphorus, alkaline phosphatase and phagocytosis.

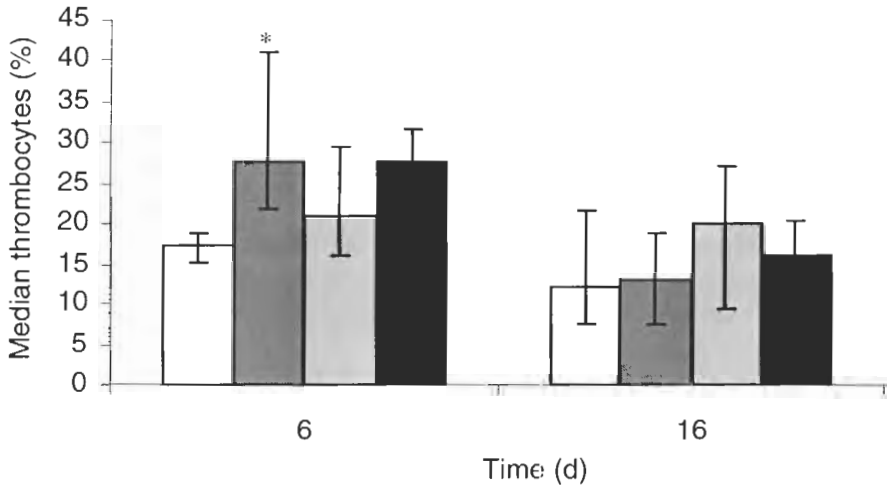


Figure 3.20. The median percentage of thrombocytes in each group of herring exposed to WSFO at 6 and 16 d. A p-value of <0.05 is considered significant (Wilcoxon Kruskal/Wallis test) and an asterisk (*) denotes a significant difference from controls. Vertical bars represent the inter-quartile range (25^{th} and 75^{th} percentiles).

(□) Control (■) 40 g oil (□) 80 g oil (■) 120 g oil

3.3.4. Adults - Single concentration exposure - pilot experiment

3.3.4.1. 28-day WSFO exposure and recovery

TIME EFFECTS

Unlike juveniles, only phagocytosis increased significantly in the control adult fish between day 28 and day 70 (Table 3.11). This statistically significant increase in phagocytosis with time also occurred in the WSFO-exposed fish and to a similar degree. Unlike control fish, GSI, plasma lactate and respiratory burst activity also changed significantly in the WSFO-exposed herring with time (Table 3.11).

Table 3.11. Variables shown to be significantly correlated with the duration of the experiment (day 28 *vs.* day 70) in adult QI herring using multivariate correlation and Spearman's nonparametric measures of association to compare each treatment group between the two sample points, $p<0.05$ significant. S-R value designates the Spearman Rho correlation coefficient. The magnitude of change is shown as a percentage, and the arrow indicates direction of change with time or exposure.

Variable	Control (N=26)	Oil (N=27)	Oil-exposed fish significantly different from control fish
Lactate	NO	YES ↓ 46.9%	YES ↓ 57.0% day 70
S-R value		-0.6146	
p-value		0.01	
GSI	NO	YES ↑ 63.0%	NO
S-R value		0.4360	
p-value		0.01	
Lysozyme	NO	NO	YES ↓ 20.0% day 28
S-R value			↑ 25.0% day 70
p-value			
Phagocytosis	YES ↑ 125.7%	YES ↑ 165.8%	YES ↓ 32.1% day 28
S-R value	0.7248	0.6901	↓ 20.1% day 70
p-value	<0.0001	<0.0001	
Respiratory burst	NO	YES ↑ 86.7%	YES ↑ 47.4% day 70
S-R value		0.3709	
p-value		0.04	

In adult herring exposed to the maximum concentration of WSFO and in control fish, the cumulative mortality reached 20 to 40% by day 28 of exposure, i.e., similar to the juveniles, and increased to 45 to 60% by day 70. There was no statistically significant difference in mortality between WSFO-exposed and control fish during the exposure or recovery period, which was also similar to the first juvenile experiment (Figure 3.21).

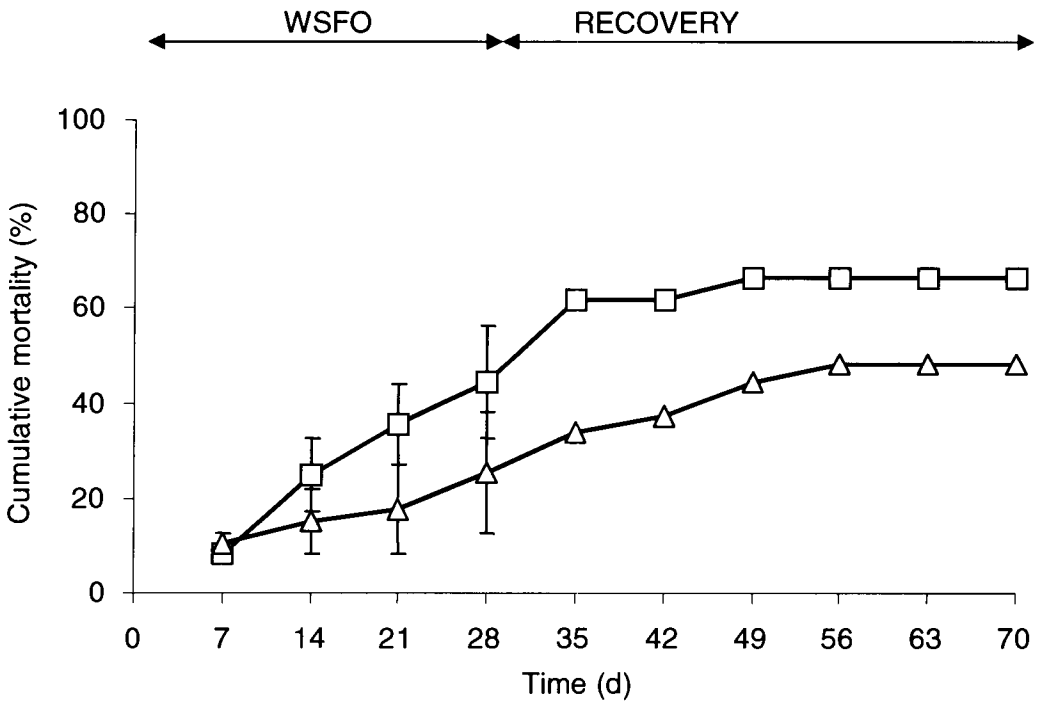


Figure 3.21. Cumulative mortality in control and WSFO-exposed adult QI Pacific herring compared at weekly intervals. Values are tank means ±SE from 2 replicate tanks per treatment from day 7 to 28 and are a single tank value from day 35 to 70. ANOVA was used to evaluate normalized (arcsine square root transformed) percent data for replicated time points only with $p<0.05$ considered significant.
(—□—) Control (—△—) Oil

The 28-day WSFO exposure significantly decreased plasma lysozyme and phagocytosis compared with control fish (Figure 3.22 B, C). Following a 42-day recovery period (day 70), both phagocytosis and plasma lactate remained significantly decreased compared with controls, while plasma lysozyme and respiratory burst activity were significantly elevated (Figure 3.22). However, the changes in plasma lysozyme and lactate at day 70 may be due to the significant increase in fork length in WSFO-exposed compared with control fish at this sample point. Plasma lysozyme was shown to be positively correlated, and plasma lactate negatively correlated, to fork length (Table 2.10).

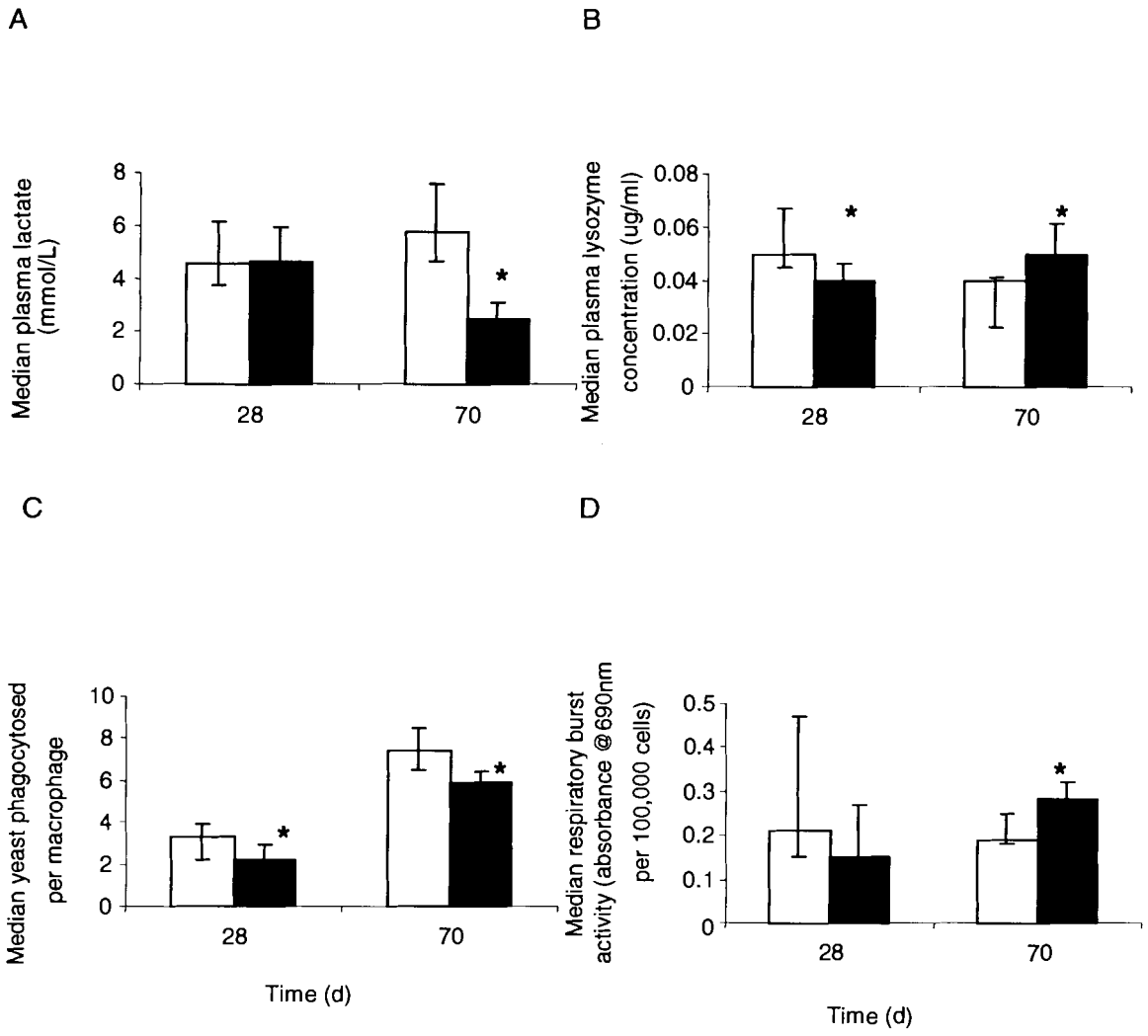


Figure 3.22 A-D. Median values for variables differing significantly among control and WSFO-exposed adult herring (plasma lactate and lysozyme, yeast phagocytosed per macrophage and respiratory burst activity). Vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles). The Wilcoxon/Kruskal-Wallis test was used with $p<0.05$ considered significant. An asterisk (*) denotes a significant difference from the control group. (□) Control (■) Oil

3.3.5. Adults - Time course experiments

3.3.5.1. 21-day WSFO exposure (Part 1) and recovery

TIME EFFECTS

Unlike in the 28-day adult exposure, phagocytosis did not increase significantly in control fish over time. Instead, CF, Hct and lysozyme changed significantly in controls at various times (Figure 3.23 A-C). WSFO-exposed herring showed similar changes in CF and lysozyme over time, whereas the changes in Hct were timed differently, remaining lower until day 35, then increasing and remaining elevated (Figures 3.24 A-C). Therefore, the changes in CF, Hct and lysozyme were unlikely due to WSFO exposure or latent effects of WSFO exposure. In particular, lysozyme increased 10 times after 35 days and remained elevated. Lct also varied over time in the WSFO-exposed fish with a significant decrease seen at day 63 unlike control fish (Figure 3.24 D). However, compared with the persistent decreases and increased proportion of fish with undetectable Lct in juveniles, these were minor changes over time.

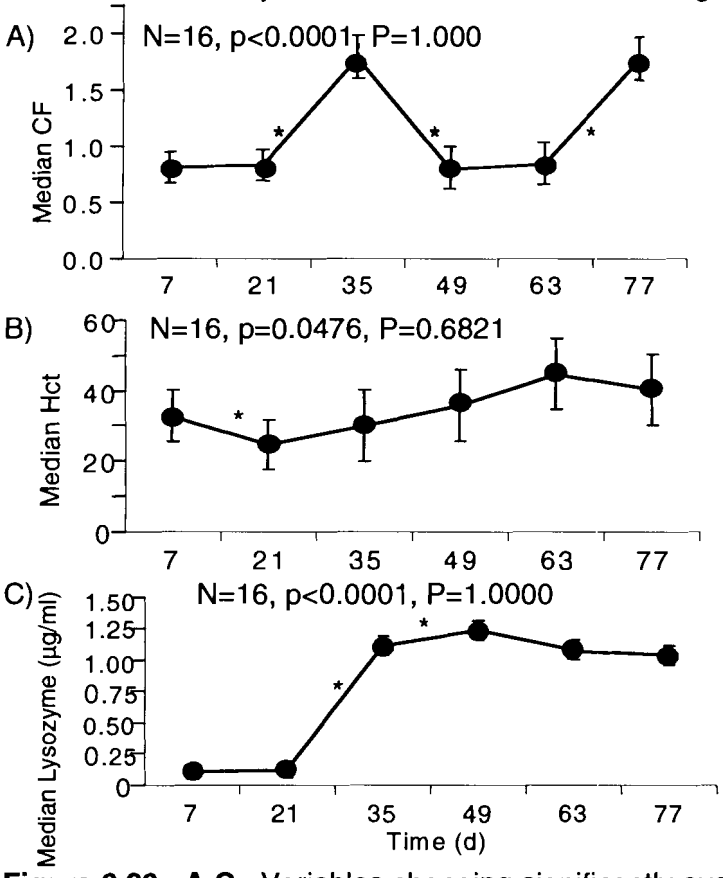


Figure 3.23. A-C. Variables changing significantly over time in control, adult QI herring using tank as the experimental unit and comparing median values using the general linear model (GLM, least square means) at the 95% confidence level. There were replicates for day 7 and 21 and 2 replicates for the remainder of the sample days. An asterisk indicates which time intervals were significant.

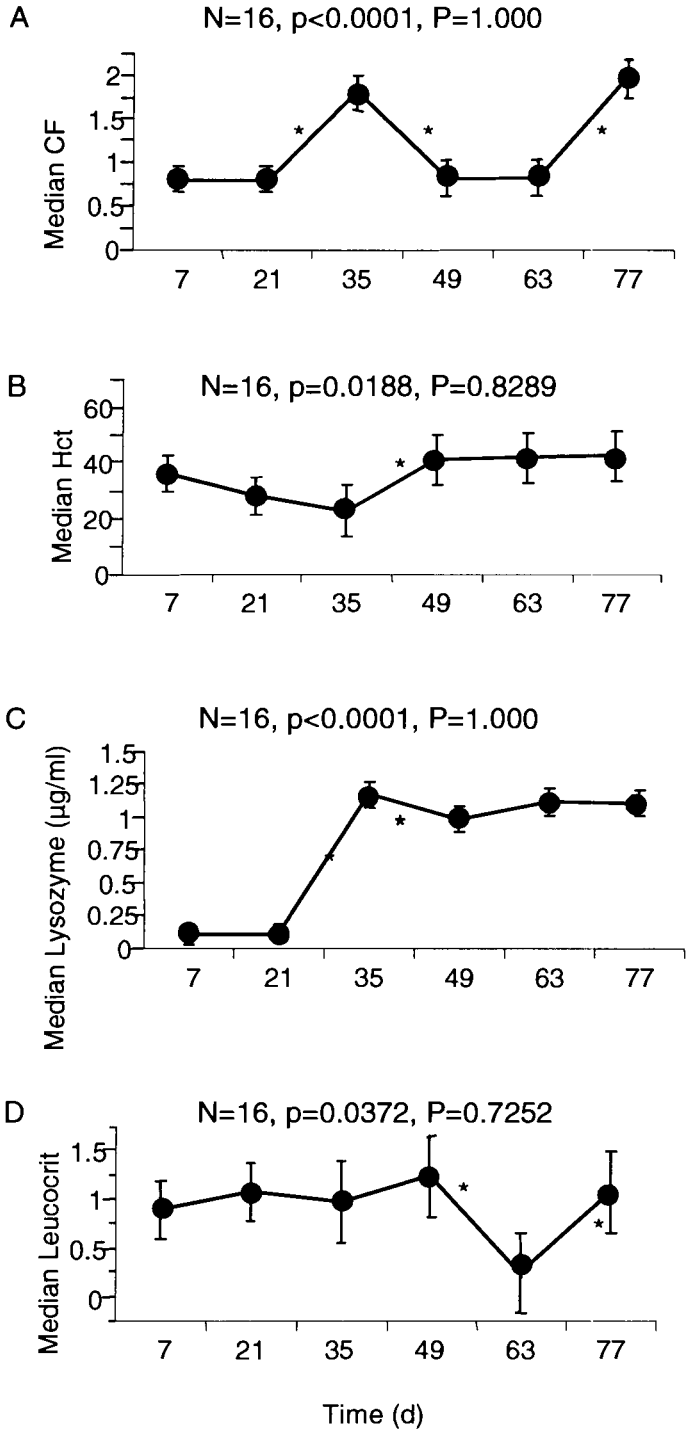


Figure 3.24. A-D. Variables that changed significantly over time in WSFO-exposed, adult QI herring using the tank as the experimental unit and comparing median values using the general linear model (GLM, least square means) at the 95% confidence level. There were 4 replicates for day 7 and 21 and 2 replicates for the remainder of the sample days. An asterisk indicates which time intervals were significant.

EXPOSURE EFFECTS

Cumulative mortality did not vary between the control and WSFO-exposed herring during the 21-day WSFO exposure to the maximum WSFO concentration or during the recovery period (Figure 3.25). Unlike the juvenile and adult pilot experiments, and the 28-day WSFO exposure, cumulative mortality reached only 5% at the end of the 21-day WSFO exposure, and was only 10 to 15% at the end of the 77-day experiment.

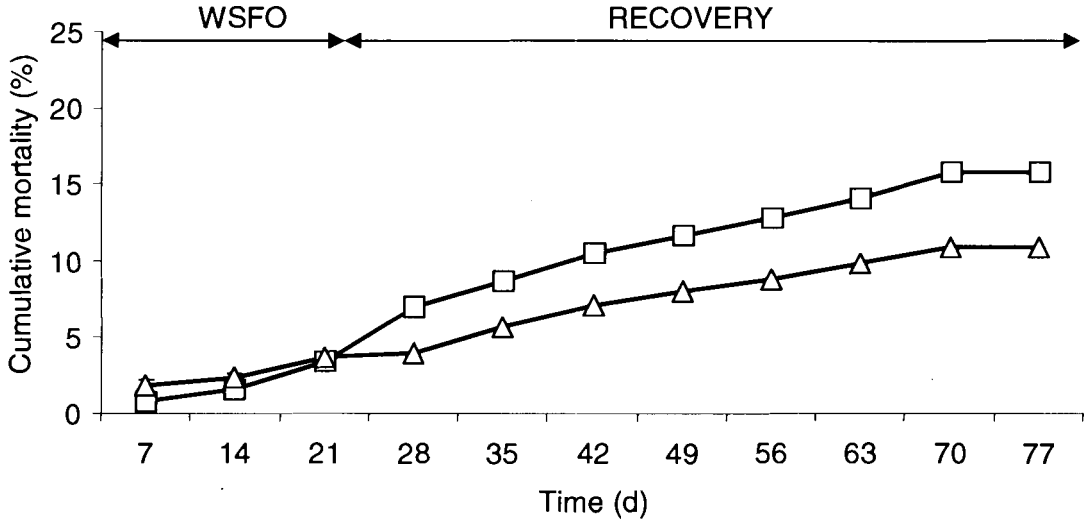


Figure 3.25. Cumulative mortality in control and WSFO-exposed adult QI herring. ANOVA was used to evaluate normalized (arcsine square root transformed) percent data with $p<0.05$ considered significant. There were 4 replicate tanks per treatment during the 21-day exposure and 2 replicate tanks per treatment for the remainder of the experiment. (Error bars not shown for clarity). (—□—) Control (—△—) Oil

There was no significant difference in the cumulative mortality between control (15.9%), WSFO-exposed (10.8%), VHSV-challenged (11.6%) and WSFO/VHSV-challenged (17.6%) groups during the 56-day post-challenge observation period (Figure 3.26). Proportional mortality attributable to VHSV was not specifically determined due to experimental constraints that did not permit evaluating dead fish for tissue VHSV titre.

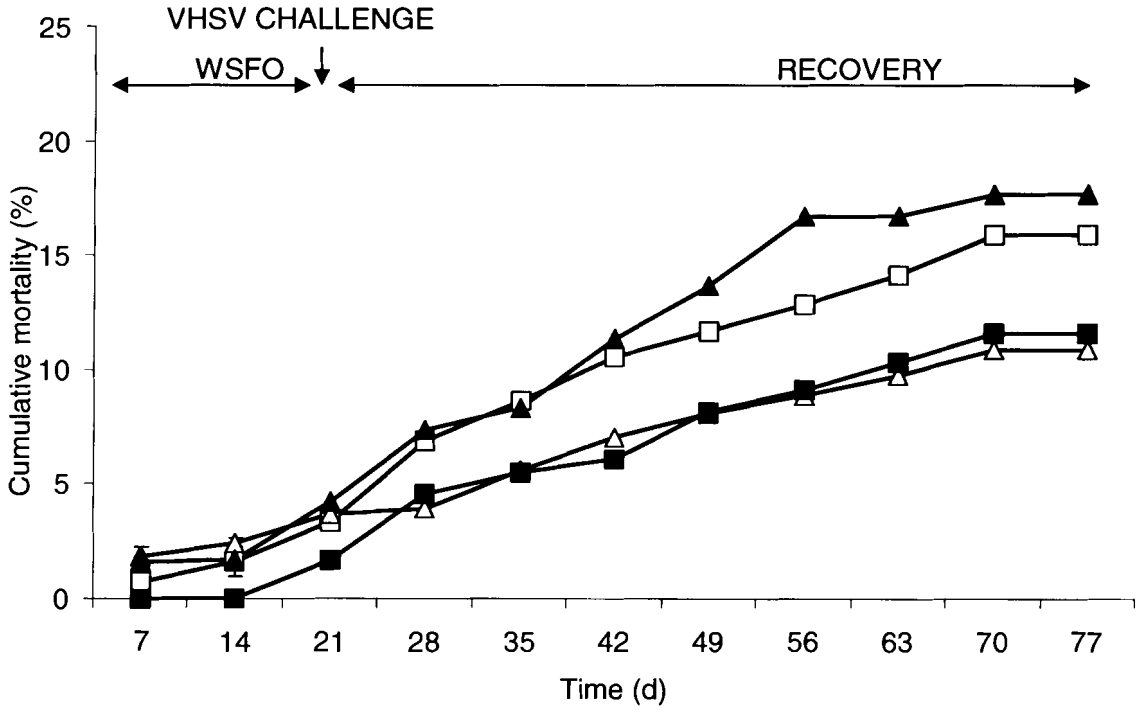


Figure 3.26. Cumulative mortality rate among treatment groups of adult herring during the 56-day observation period following VHSV-challenge. Values are mean cumulative mortality rate, based on the mean of replicate tanks, with tanks as the experimental unit. ANOVA on arcsine square root transformed percentage data was used to test for differences between groups at each time point, with $p<0.05$ considered significant. (Error bars not shown for clarity).
(-□-)Control (-△-)Oil (-■-)VHSV (-▲-)Oil/VHSV

The majority of herring tested for tissue viral titre were below the detection limit of the plaque assay ($<10^{2.6}$ pfu/g). Tissue VHSV titre was detected in only 2/319 herring (0.6%). One herring in the VHSV-exposed group had a tissue titre of 2.64×10^6 pfu/g at 28 d post challenge (day 42 (28)). The other positive herring was a non-challenged, WSFO-exposed herring with a tissue titre of 1.16×10^5 pfu/g at day 63 (42). In addition, serum antibody titres (35/319, 11%) were shown to be at very low levels (1:40) in all groups (including non-challenged fish) at 14 d post-challenge and no pattern of elevation of titre in WSFO-exposed or VHSV-challenged fish was apparent. (Figure 3.27). The prevalence of VHSV titre did not vary among treatment groups at any time point (Figure 3.28).

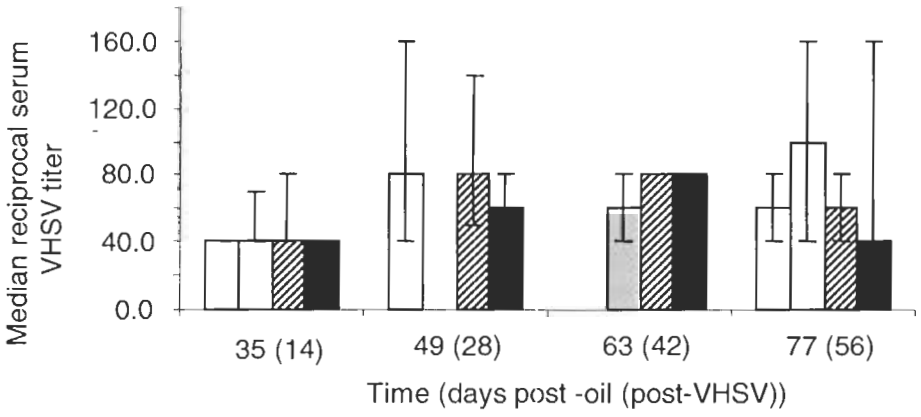


Figure 3.27. The median reciprocal serum VHSV titre for all herring with a positive serum titre (total n=35) among treatment groups of adult Quadra Island herring exposed to the WSFO and challenged with VHSV. Comparisons were performed separately at each time point using the Wilcoxon/Kruskal-Wallis test at the 95% confidence level. Error bars are the inter-quartile range (25th and 75th percentiles). An absence of error bars signifies n=1 for a given treatment group and sample day.
(□) Control (■) Oil (▨) Oil/VHSV (▮) VHSV

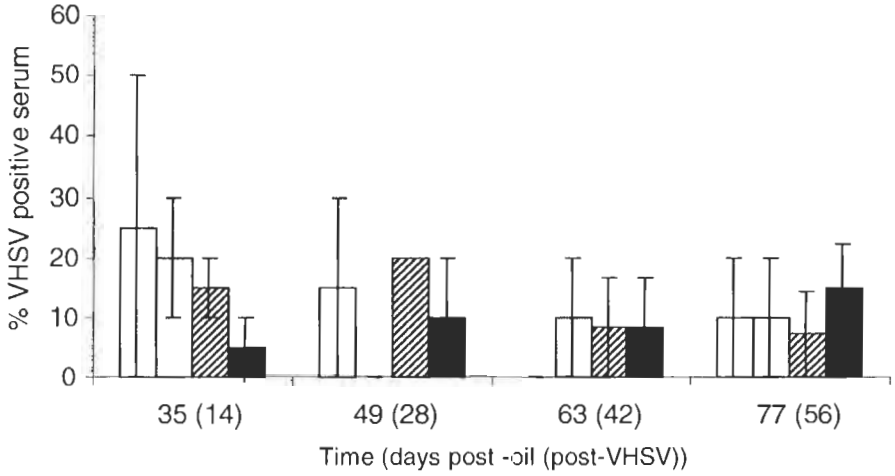


Figure 3.28. The prevalence of VHSV-antibody positive plasma among treatment groups during the 56-day post-VHSV challenge observation period. Values are the mean of 2 replicate tanks per treatment group and error bars are the SE. Comparisons were performed separately at each sample time using ANOVA on arcsine square root transformed percentage data at the 95% confidence level.
(□) Control (□) Oil (▨) Oil/VHSV (▮) VHSV

Exposure to 120 g of the WSFO for 21 d resulted in significant differences in Tier 1 and 2 variables. However, these changes occurred episodically post-exposure. For example, on day 21 GSI was 46.9% lower (Figure 3.29A), phagocytosis was 25.4% increased (Figure 3.29 C) and on day 49 plasma lysozyme was 16.5% lower (Figure 3.29B).

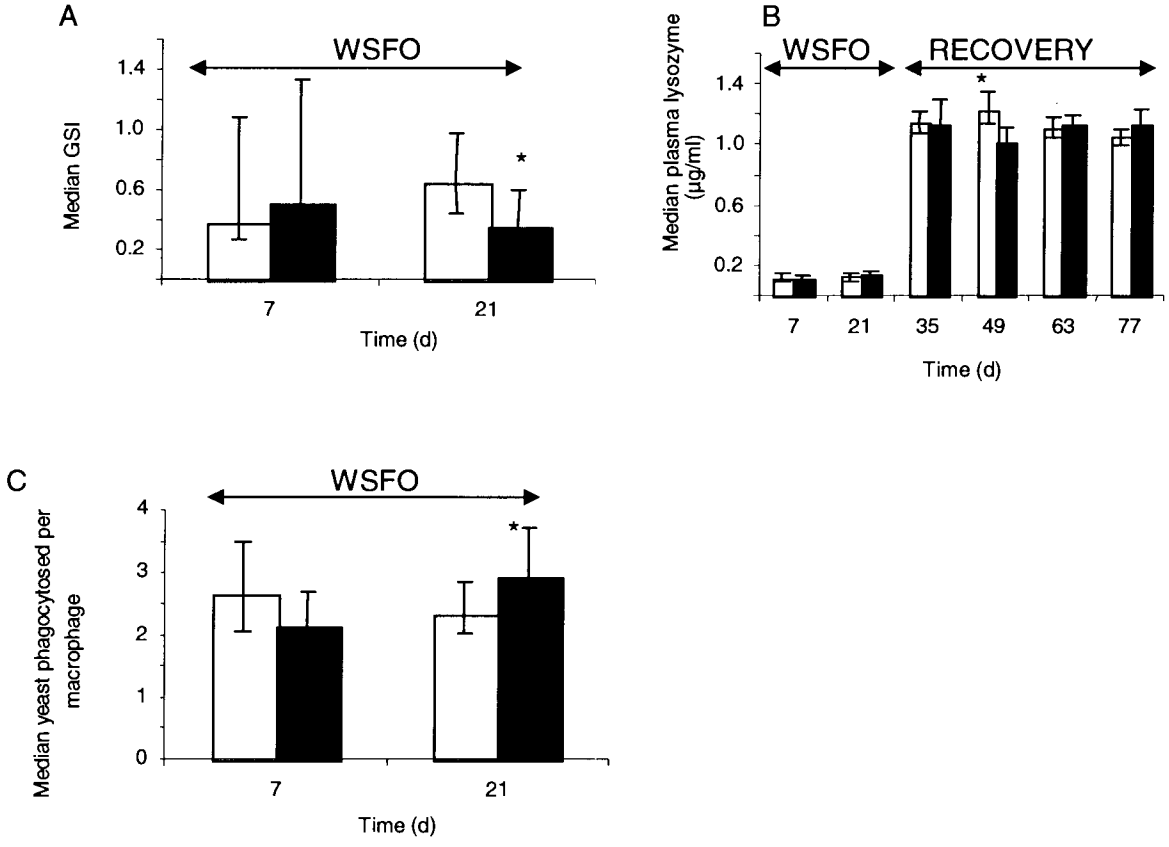


Figure 3.29. A-C. Variables that differed significantly among groups of adult QI herring following exposure to WSFO: A) GSI, B) plasma lysozyme, C) phagocytosis per macrophage. The Wilcoxon/ Kruskal-Wallis test was used for each comparison with $p<0.05$ considered significant. Vertical bars represent the inter-quartile range (25^{th} and 75^{th} percentiles). An asterisk (*) denotes a time point at which WSFO -exposed fish differed significantly from the control group. (□) Control (■) Oil

Significant differences among control, VHSV-challenged and WSFO/VHSV-challenged adult herring were seen for both Tier 1 and 2 variables. CF, plasma lysozyme Lct and Hct all changed significantly but episodically and were not persistent at all sample points (Figure 3.30). WSFO exposure alone did not alter CF, but VHSV (with or without

WSFO) was associated with a significant change in the median CF at 14 days post-VHSV challenge (Figure 3.30 A).

A significant decrease in plasma lysozyme concentration was evident at day 49(28) in WSFO-exposed, VHSV-challenged and the WSFO/VHSV-challenged herring compared with controls only (Figure 3.30 B). At day 63(42), the VHSV-challenged fish had an increased median Lct (1.49%) compared with WSFO-exposed fish (0.72%) (Figure 3.30 C). Fourteen days later, the VHSV-challenged herring had a significantly lower Lct compared with control fish, with a median of zero. Hct increased significantly at day 77(56) in all WSFO-exposed, VHSV-challenged, and WSFO/VHSV-challenged herring compared with control fish (Figure 3.30 D).

Most of the statistically significant results could not be assessed against the adult 95th PE reference ranges as there was no time zero data for this specific experiment. However, the decrease in Lct and increase in Hct on day 77(56) occurred concurrently in the VHSV-challenged fish. The prevalence of undetectable Lct was significantly greater in the control and WSFO-exposed herring at day 63 (42) compared with both VHSV-challenged and WSFO/VHSV challenged herring. On day 77 (56), the reverse was true, with a higher proportion of both VHSV-challenged groups of fish with no detectable Lct.

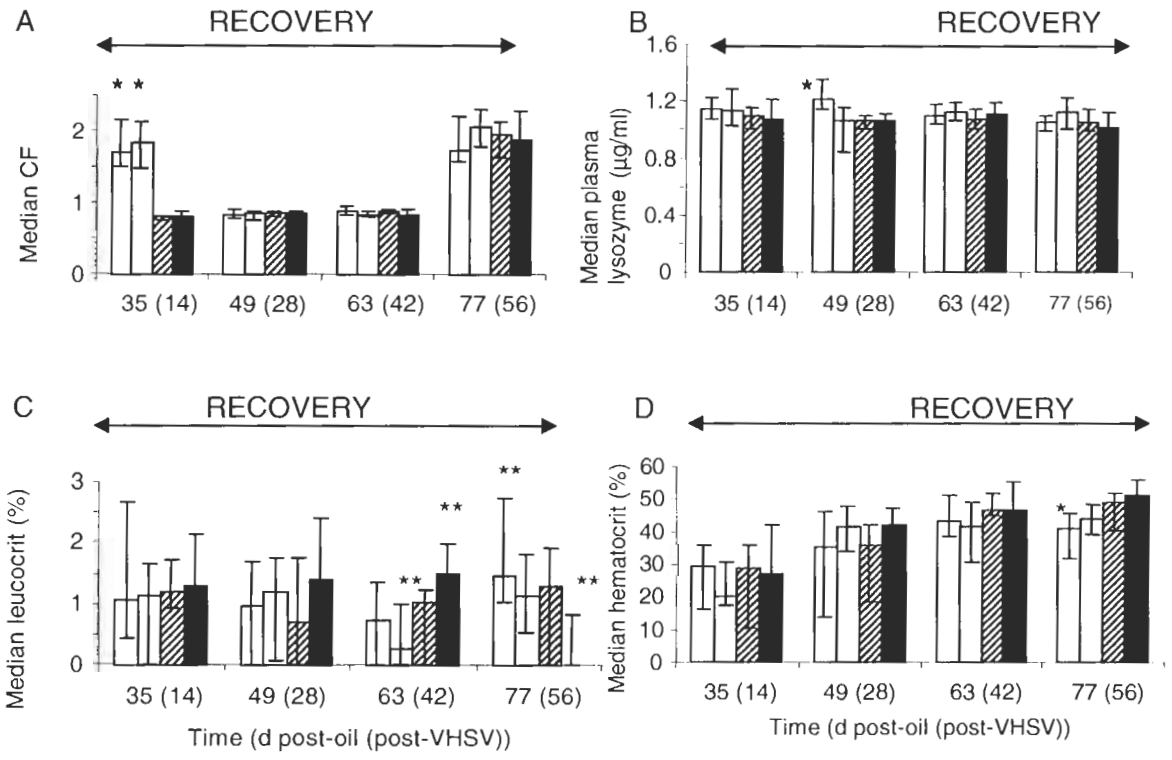


Figure 3.30 A-D. Variables that changed significantly among control, WSFO-exposed, VHSV-challenged and WSFO/VHSV-challenged adult herring. Comparisons are between groups at each time using the Wilcoxon/Kruskal-Wallis test at the 95% confidence level. Vertical lines are the inter-quartile range (25th and 75th percentiles). Asterisk (*) indicates group differs significantly from non-asterisked groups at the specific sample time ($p<0.05$). Double asterisk (**) indicates a significant difference between the two groups at each time that have a double asterisk. WSFO exposure was terminated after 21 days. (□) Control (□) Oil (▨) Oil/VHSV (■) VHSV

3.3.5.2. 21-day exposure WSFO (Part II) and recovery

TIME EFFECTS

In contrast with the QI adults, phagocytosis activity in BS adults increased significantly in control fish from the outset of the exposure. In addition, plasma glucose, lactate, and albumin also increased in the control fish during the experiment and were significantly greater on day 63 than on day 0 (Figure 3.31). WSFO-exposed herring showed the same changes as control fish over time for plasma glucose, lactate and phagocytosis activity, but not for albumin. Albumin differed in WSFO-exposed fish compared with control fish by decreasing between day 4 and 21 and then increasing on day 63 after the 42-day recovery period (Figure 3.31).

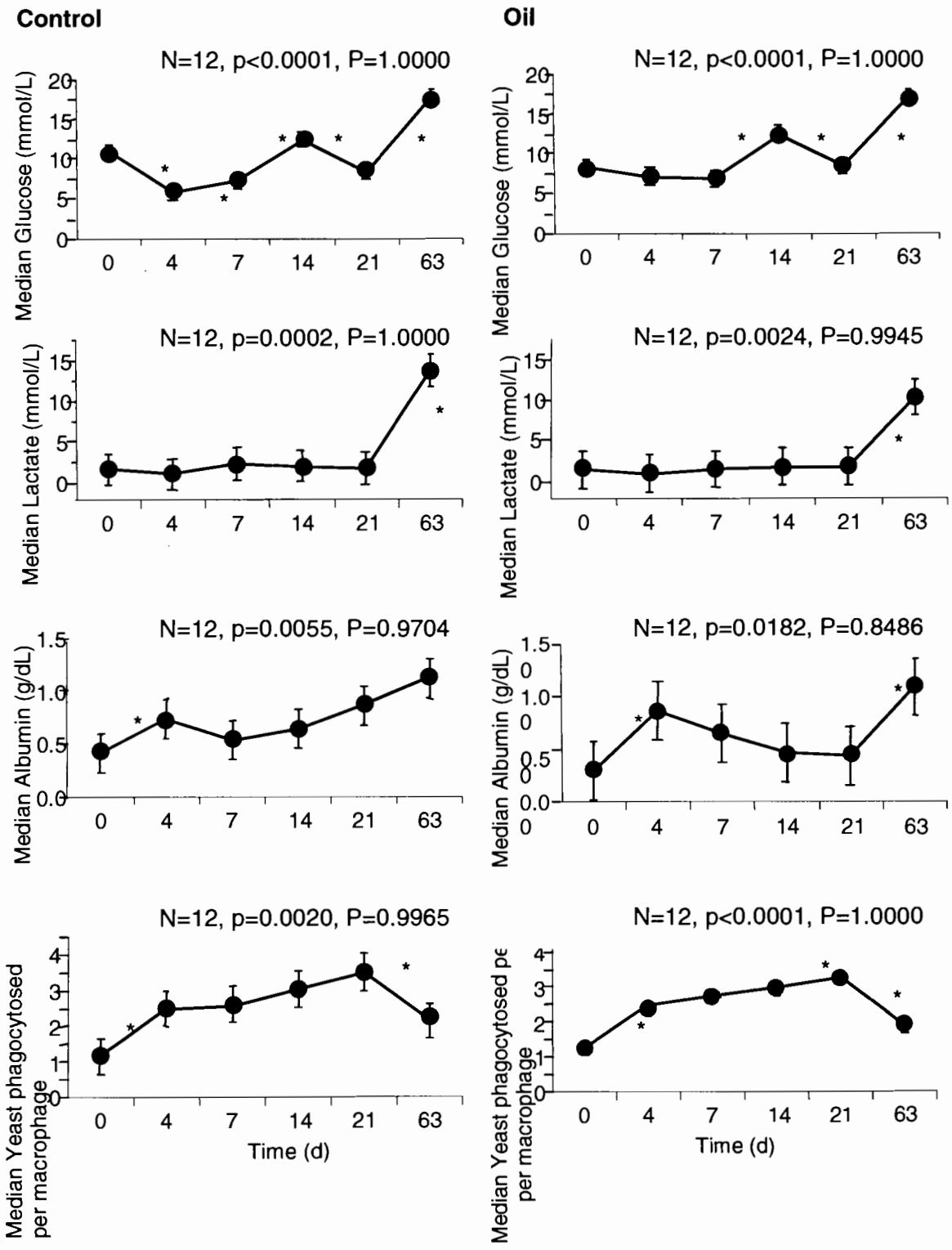


Figure 3.31. Variables that changed significantly over time in control and WSFO-exposed adult BS herring using the tank as the experimental unit and comparing median values using the GLM at the 95% confidence level. There were 2 replicates per sample day. An asterisk indicates which time intervals were significant.

Unlike control fish, Hct and plasma lysozyme also changed significantly in WSFO-exposed herring with time. Hct showed a significant peak at day 4 while plasma lysozyme was decreased at day 21 (Figure 3.32).

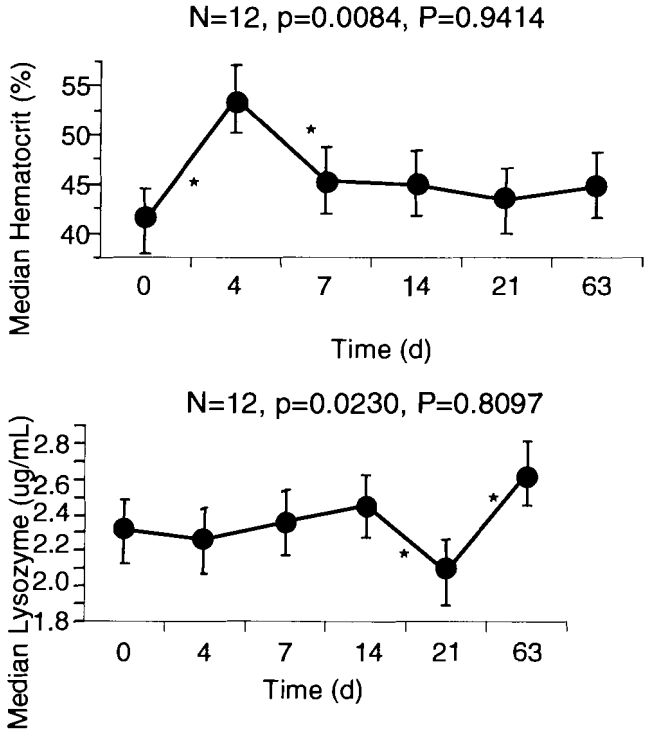


Figure 3.32. Variables that changed significantly over time in WSFO-exposed, adult BS herring using the tank as the experimental unit and comparing median values using the GLM at the 95% confidence level. There were 2 replicates per sample day. An asterisk indicates which time intervals were significant.

EXPOSURE EFFECTS

Cumulative mortality did not vary significantly with WSFO exposure of adult fish throughout the course of the experiment (Figure 3.33). When 21-day WSFO exposure and recovery experiments were repeated using BS adults, cumulative mortality again was low (8.4 to 11.2% at 21 d; 12.0 to 12.2% at 63 d). This was much lower than the mortality in the initial BS experiment (35.9 to 60.5% at 16 d)

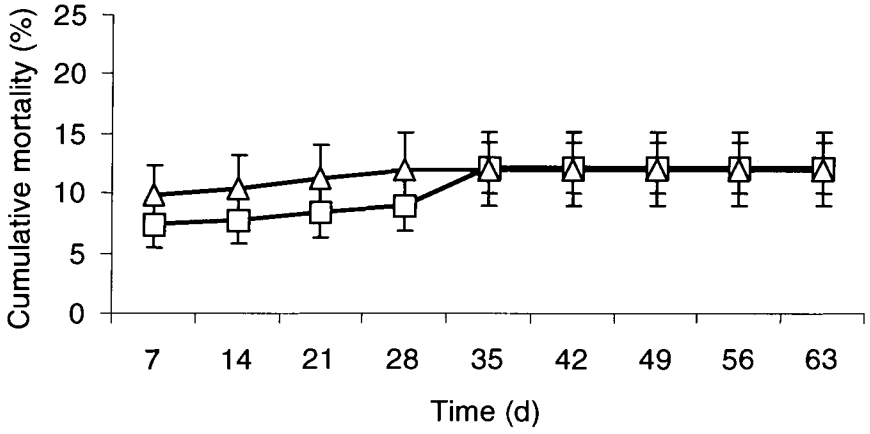


Figure 3.33. Cumulative mortality in control and WSFO-exposed adult BS herring. ANOVA was used to evaluate normalized (arcsine square root transformed) percent data with $p<0.05$ considered significant. There were 2 replicate tanks per treatment.
(—□—) Control (—△—) Oil

When compared with control fish, the 21-day WSFO exposure of adult herring resulted in changes in Tier 1 variables only. On day 4, the median Hct was 26.5% higher than controls (Figure 3.34A). SSI was 53.8% lower than controls on day 7, but was elevated on day 21 by 50.0% along with a 29.5% increase in plasma chloride (Figure 3.34 B, D). On day 21, Hct, plasma phosphorus, calcium, albumin and the A/G ratio were all lower than controls by 16.1%, 31.5%, 16.0%, 50.6% and 54.5%, respectively (Figure 3.34 A; Figure 3.35). After the recovery period (day 63), GSI was increased by 43.8% whereas plasma chloride was decreased by 35.0% compared with control fish (Figure 3.34 C; Figure 3.34 D). There was no significant difference in plasma lysozyme or phagocytosis activity with WSFO-exposure.

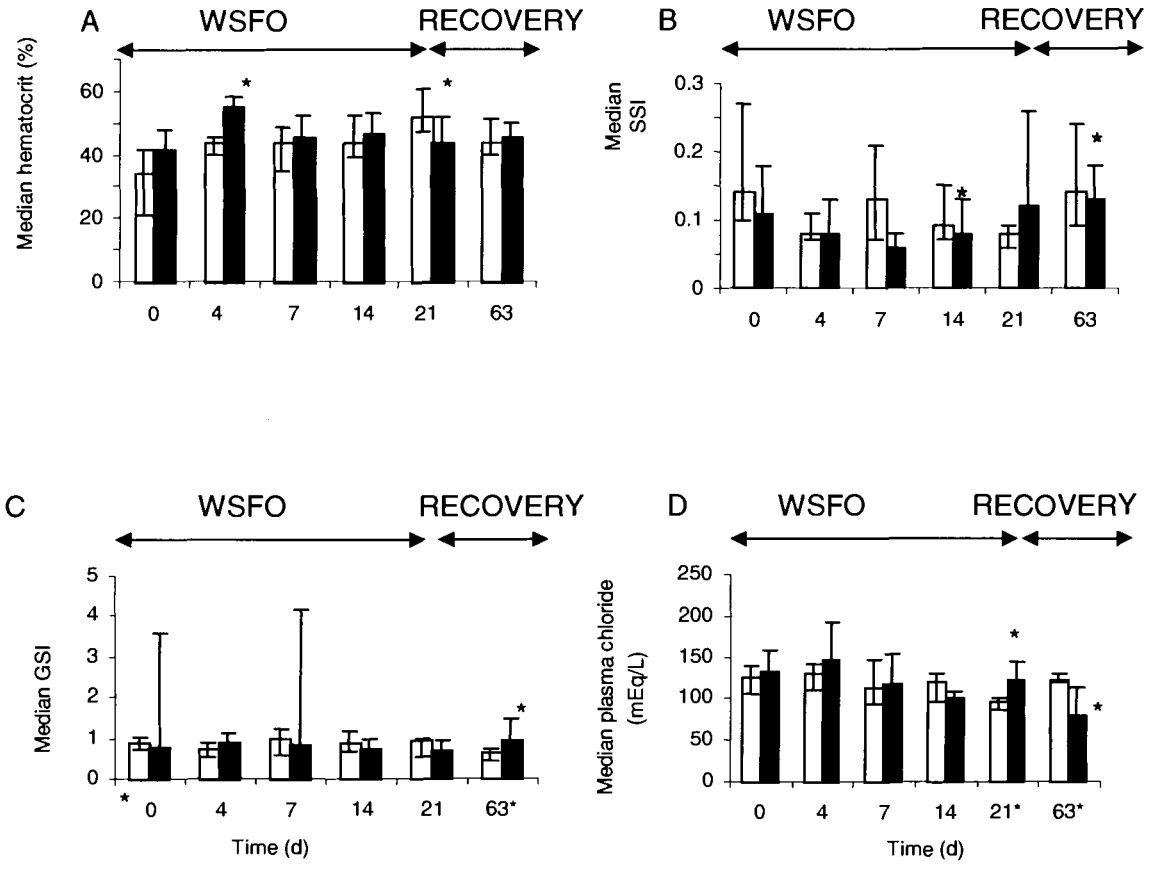


Figure 3.34 A-D. The median Hct, SSI, GSI, and plasma chloride in control and WSFO-exposed adult Pacific herring at specific times after initiation of exposure. Comparisons among treatment groups used the Wilcoxon/Kruskal-Wallis test at each time point. The asterisk marks the significant sample day(s) for each variable. Vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles); $p<0.05$ significant. (□) Control (■) Oil

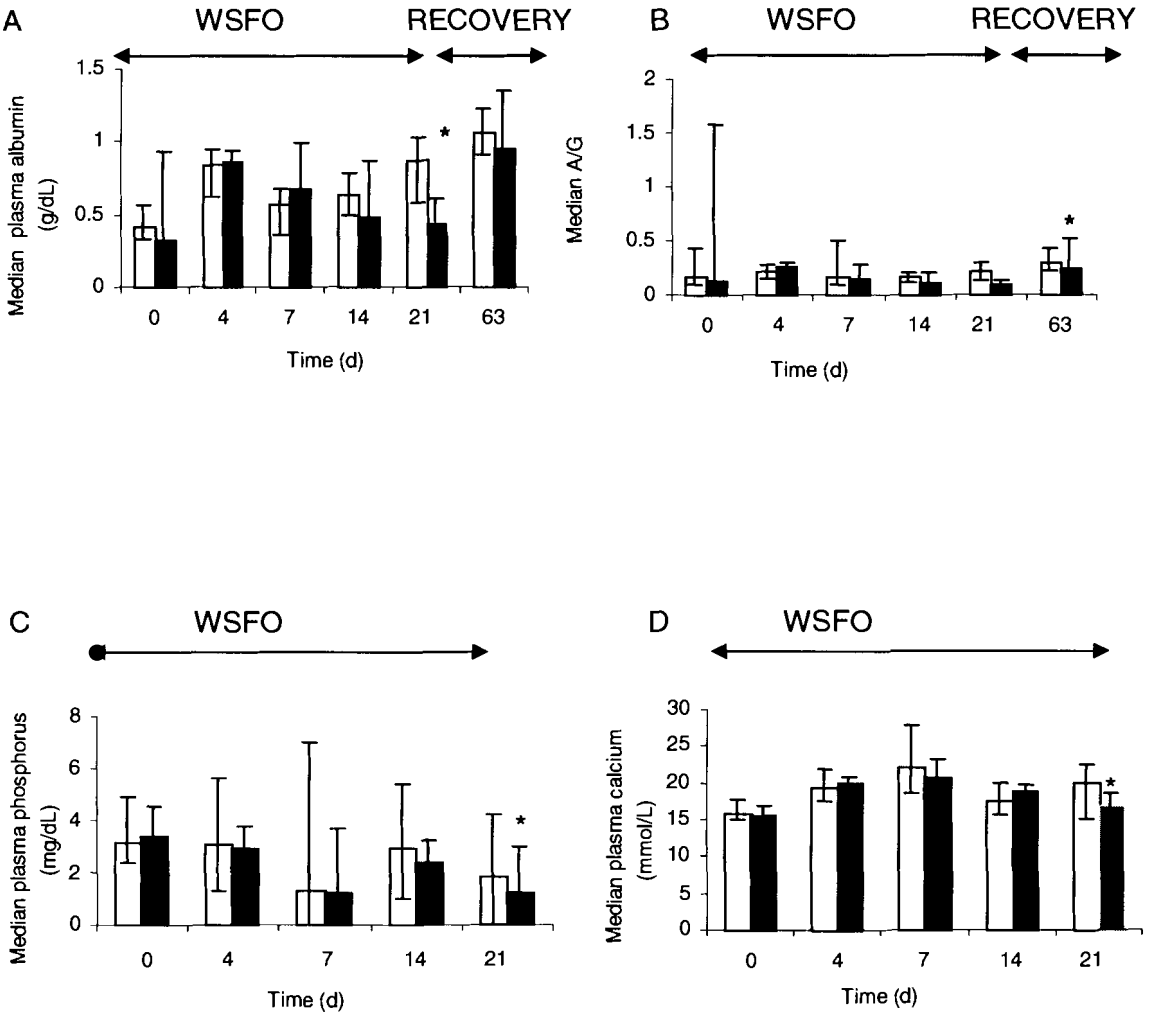


Figure 3.35 A-D. The median plasma albumin, A/G, phosphorus, and calcium in control and WSFO-exposed adult herring at specific times after initiation of exposure. Comparisons among treatment groups used the Wilcoxon/Kruskal-Wallis test at each time point. The asterisk marks the significant sample day(s) for each variable. Vertical bars are the inter-quartile range (25^{th} and 75^{th} percentiles); $p<0.05$ significant.

(□) Control (■) Oil

3.4. DISCUSSION

The purpose of these experiments was four fold: to determine (a) the effect of captivity time on control and exposed/challenged fish; (b) the effect of exposure to WSFO; (c) the effect of a recovery period following WSFO exposure; and (d) the effect of VHSV challenge plus exposure to WSFO on the Tier 1, 2, and 3 variables measured in both juvenile and adult herring. Consequently, this discussion is accordingly organized into four separate sections, with each discussing juveniles and adults separately because they were previously shown to have differences in their range of measured variables (Chapter 2). Similarities and differences between juveniles and adults in general trends are summarized at the end of each section. For juveniles there is an additional discussion of the impact of WSFO exposure plus challenge with *V. anguillarum*.

Within each section and each age class, the statistically significant changes in variables are listed and those that fulfilled the criteria described below for potential biological significance are discussed further. Statistically significant changes were considered potentially biologically important if any of the following criteria were fulfilled: 1) the change fell outside the 95th percentile reference range (from Chapter 2 or from time-matched controls); 2) there were changes in more than a single variable at a given sample point that indicated a consistent change in immune function or the variable changed in more than one experiment within an age class; 3) changes in the variable have been observed in other fish studies involving toxicant exposures and these changes have been associated with altered biological function; and 4) a biologically plausible mechanism for the change exists. Statistical changes that fulfilled combinations of these criteria were considered most significant.

An alternate reference range, the inter-quartile range (IQR) has been reported in other studies for defining normal ranges (Harms *et al.*, 2002). Therefore, statistical changes were also evaluated against the IQR although overall there were few additional changes in variables that exceeded the IQR but not the 95th PE. However, given the concerns about variability, statistical power and small sample size the IQR reference ranges are still wide. Use of the IQR is less conservative than the 95th PE. By definition, any statistically significant change that exceeds the 95th PE range will also exceed the corresponding IQR. Therefore, values that exceeded the 95th PE reference range were considered of greater significance than values that only exceeded the IQR. The

reference range or IQR profile that was used as a benchmark (T_0 or T_P or time-matched only) depended on whether or not the variable in question was stable over time, both during the specific experiment and as assessed in Chapter 2 (Table 2.33).

3.4.1. Time effects –responses in control fish

It was important to examine control time series to determine the effect of captivity during experimental sampling on the variables being measured. This proved to be especially true for Pacific herring, which were difficult to hold in captivity at BMSC, had high pre-experimental mortality and showed wide variation in most variables examined. In addition, variation in holding time prior to initiation of experiments was shown to impact reference ranges (Section 2.4.8). The results from control fish showed varying responses among several of my experiments. Control juveniles and adults clearly responded differently over time, as did the QI and BS adults. BS herring showed greater experimental mortality than QI herring. Several variables differed significantly in controls over time, with relatively large changes occurring over the course of experiments. LSI, Lct and Hct all decreased in juvenile fish with time. Plasma glucose, lactate and phagocytosis increased in both juvenile and adult fish. In addition, CF, Hct, neutrophils, and albumin also increased in adult fish. These changes may indicate captivity-induced anemia and leucopenia in juvenile fish, and a generalized secondary stress response (changes in plasma biochemistry) in both age classes. While plasma lysozyme was unaffected in control juvenile fish, it decreased in one adult trial and increased in another, thus showing it can be quite variable which brings its value as a consistent immune indicator into question.

In control juvenile herring, statistically significant changes occurred over time for LSI, Hct, Lct, plasma glucose, lactate and phagocytosis. Of these changes, Hct and Lct decreased below the corresponding T_0 95^{th} PE reference range. The reduction in Lct with time, which was largely attributable to a tendency towards increasing prevalence of undetectable Lct with time, was of particular potential concern and was frequently recorded in both control and WSFO-exposed juvenile herring. This may have been indicative of a captivity-induced stress leucopenia (Torres *et al.*, 1986), which is a result of induction of the primary stress response and an increase in circulating plasma cortisol, which can directly inhibit fish leucocytes (Anderson, 1993). The primary stress response can result in a very low Lct and is influenced by other factors such as temperature (Hille, 1982). Cortisol was not measured in juveniles so it was not possible

to be certain that the leucopenia was due to a primary stress effect alone. Conversely, Lct was not detected in up to 30% of captive, juvenile herring (Chapter 2, Table 2.13). Therefore, the prevalence of undetectable Lct below or at 30% could be considered normal for a population of captive juvenile herring, in particular for the group with a 25% prevalence of *V. anguillarum* infection in the dead control fish (4/16), whilst prevalence above 30% may be abnormal. The prevalence of absent, measurable Lct in juvenile herring warrants further study into the possible causes.

It was feasible that the small blood volume obtained from the youngest, and therefore smallest, of the juvenile fish, could also explain the prevalence of undetectable Lct. However, Lct was also undetectable in a small percentage (8 to 12%) of adult herring, from which a larger volume of blood was collected. Therefore, this implied that adults with no Lct were leucopenic, that a proportion of juveniles with no detectable Lct were truly leucopenic and that a prevalence of undetectable Lct in excess of 30% in juveniles likely represented substantial leucopenia within the population and was not solely attributable to a detection limit of the measurement technique. In general, exposure to toxicants and stress is reportedly associated with a reduction in Lct (leucopenia), as was evident in the 40 g oil and 120 g WSFO exposure groups and controls over the course of the experiment (Ellsaesser and Clem, 1986; Murad and Houston, 1988). Both leucopenia and anemia, indicated by decreased Lct and Hct, have been previously observed as a captivity-stress effect in other marine fish (Torres *et al.*, 1986). Stress due to a 4-day confinement was shown to decrease both Hct and Lct in the dogfish (Torres *et al.*, 1986). The changes in other variables in juvenile control fish over time could not be assessed against a T_0 95th PE or IQR range due to a lack of T_0 data for these particular variables.

In control, adult herring statistically significant changes were observed in CF, Hct, neutrophils, plasma glucose, lactate, albumin, lysozyme and phagocytosis over time. Of these variables, the changes in lysozyme, glucose, lactate, and phagocytosis extended beyond the T_0 95th PE reference ranges developed from T_0 control fish only. Using the T_0 IQR as an alternate reference range, the changes observed in Hct and albumin were additionally outside of their respective T_0 IQRs. The changes in neutrophils and CF could not be assessed, as there was no T_0 data for these variables within their specific holding time grouping.

A common response pattern over time for control herring in captivity for both age classes was increased plasma glucose, lactate and phagocytosis. Responses unique to control juvenile fish were decreased LSI, Lct and Hct, whereas those unique to adults were increased CF, neutrophils, and albumin, as well as small fluctuations in both Hct and lysozyme. In addition, phagocytosis was increased in both the BS and QI populations of adult control fish. These changes in control fish were indicative of captivity-induced leucopenia and anemia in juveniles, neutrophilia, hyperalbuminemia, hemoconcentration, and reduced lysozyme production in adults, and a generalized stress response (elevated glucose, lactate, and phagocytosis) in both age classes.

In this study, the captivity-induced leucopenia in juveniles was not related to the prevalence of gross lesions at the time of sampling, but it may have been associated with a sub-clinical infection. Ransom *et al.* (1984) found decreases in WBC differential counts that correlated with the presence of bacteria in the blood of diseased fish. Wedemeyer *et al.* (1983) challenged fish with furunculosis *(A. salmonicida)* and found that Lct declined significantly in moribund fish with active infections once mortalities began. Iwama *et al.* (1986) also reported an initial leucopenia followed by leucocytosis in juvenile Chinook salmon exposed to a toxicant and challenged with bacteria. Leucopenia can also be associated with overwhelming infection in moribund fish and can reflect an inability of the immune system to respond adequately to challenge (Wedemeyer *et al.*, 1983; Evenberg *et al.*, 1986). High cumulative mortality was observed in some experiments, including the juvenile multiple concentration experiment, in which the increased prevalence of undetectable Lct over time was particularly apparent.

A comparison among the six experimental series, which varied in exposure duration from 16 to 77 d, showed a substantial difference in the cumulative experimental mortality of control herring. For BS juveniles, the 16-day BS adults, and the 28-day QI adults, the control cumulative mortality rates approached 50%. However, in the three time series experiments, mortality rates were only 12% (BS) and 16% (QI) for adults, and 26% (BS) for juveniles. Assessment of pre-experimental mortality showed no statistical significance between age classes or between experiments within age class when the standardized mortality per day was compared (Section 2.4.1). Yet, crude cumulative mortality varied from 10.7 to 27.1% in juvenile fish and from 18.5 to 81.6% in adult fish

(Table A2.3). While the standardized mortality rate per day accounts statistically for the differences in holding period (from capture to initiation of an experiment) it may be that differences in the crude cumulative mortality over time between experiments has a substantial impact on the health of surviving fish. Regardless of the differences in cumulative experimental mortality, it appears that background mortality was greater in the BS multiple-concentration experiments and the 28-day QI experiment than in the BS and QI time series experiments. Therefore, the time series WSFO exposure experiments might be considered more reliable than the others.

The changes over time in surviving control fish had the potential to mask WSFO effects and variation in time-related changes between experiments was potentially associated with the differences in cumulative mortality over time. For example, the pre-experimental mortality of 27.1% in WSFO/VHSV-challenged juvenile fish may have resulted in a substantially stronger subset of herring remaining for the WSFO exposure and pathogen challenge that had already survived a natural VHSV epizootic and that had acquired immunity. In the second QI adult experimental (WSFO exposure plus VHSV challenge) mortality rate was lower than in all other experiments. This group did not experience a high mortality rate prior to experiments (18.5%, Table A2.3), therefore selection for healthier fish entering the experiment was probably not an issue in that experiment. It is possible however that the lower mortality was a function of improvement of all aspects of capture, holding and performing experiments over time.

This differential mortality observed in control fish is a concern for three reasons: (1) it implies a questionable health status of fish that may mask responses to WSFO, (2) it reduces statistical power for long-term comparisons by decreasing sample size, and (3) it affects the distribution of variables, central tendency and subsequent 95th PE and IQR of reference ranges.

3.4.2. Time effects – responses in WSFO-exposed fish

In juvenile herring exposed solely to WSFO, phagocytosis was the only variable that changed significantly with time and in a parallel manner to control fish. However, as there was no corresponding T_0 reference range the significance of this change could not be further interpreted. Unlike control fish, Hct and Lct did not change with time.

Adult, WSFO-exposed fish demonstrated statistically significant changes over time that paralleled the response of control fish, in terms of both magnitude and direction, in CF, neutrophils, plasma lactate, glucose, lysozyme, and phagocytosis. Of these variables, plasma glucose, lactate, lysozyme and phagocytosis all exceeded the T_0 95th PE reference range. Additionally, GSI and respiratory burst activity increased; thrombocytes decreased; Hct and albumin fluctuated with time with some shift in the degree of change and timing compared with their matched controls; and Lct varied in response between experiments over time in WSFO-exposed fish. Of these additional changes with time, only percentage thrombocytes exceeded the T_0 95th PE reference range. Relative to the respective T_0 IQRs, only the statistically significant changes in Hct and Lct exceeded these ranges. The response of adult WSFO-exposed fish over time included a captivity-induced leucopenia, a general stress response (alteration of plasma biochemistry), and stimulation of Tier 2 non-specific immune responses. These responses paralleled those of control fish with regard to which changes exceeded the respective T_0 95th PE and T_0 IQRs ranges. To summarize the difference in response pattern with age class of WSFO-exposed herring over time, only phagocytosis changed in WSFO-only exposed juvenile fish, and CF, GSI, Hct, Lct, neutrophils, thrombocytes, plasma lactate, glucose, lysozyme, respiratory burst activity and phagocytosis all changed in the adults.

Phagocytosis was the only variable that responded in the same manner in both age classes following exposure to WSFO alone. Significant changes in Lct, plasma lactate, lysozyme and phagocytosis were observed in both adult populations with time, although for plasma lactate and lysozyme the direction of change was opposite between populations. The changes over time in WSFO-exposed fish that paralleled control fish can be attributed to a time (captivity) effect.

3.4.3. Time effects - responses in WSFO plus *V. anguillarum* exposed fish

Juvenile herring exposed to WSFO and then challenged with *V. anguillarum* showed statistically significant changes in CF, Hct and Lct over time that were similar to control fish negative for *V. anguillarum* infection. The decrease in Hct for all exposure groups and in Lct for all but the 80 g oil/*Vibrio* group, fell below the T_0 95th PE reference ranges. It is important to note that because *V. anguillarum* was isolated from 4/16 dead control fish that this particular trial was not an absolutely controlled exposure with respect to *V. anguillarum*. Therefore, although the sampled control fish were not positive for *V.*

*anguilla*rum they may have been naturally exposed to the pathogen. Nonetheless, as the decreases in Hct and Lct occurred in control and WSFO/experimentally challenged fish, and to the same extent, any latent WSFO/pathogen effect could not be distinguished from a general captivity effect. Therefore, the decrease in Hct in all exposure groups of juvenile fish (including controls) in the 21-day WSFO exposure and *V. anguillarum* challenge after the recovery period suggested a captivity-induced anemia, independent of the WSFO and pathogen exposures. Interestingly, the changes in CF and Lct in juveniles exposed to WSFO and *V. anguillarum* were of a similar nature to changes in these same variables in adults exposed only to WSFO.

3.4.4. Time effects – responses in WSFO-exposed/VHSV-challenged fish

Phagocytosis decreased significantly in juvenile WSFO/VHSV-challenged fish with time (same as 3.4.2), while LSI increased in the VHSV-challenged fish. These changes could not be assessed using the reference range profiles due to a lack of T_0 data. A significant decrease in CF occurred in all exposure groups of adult fish but was evident earlier post-challenge in the VHSV-challenged and WSFO/VHSV-challenged fish. This was the only variable that changed with VHSV exposure and WSFO/VHSV exposure. A lack of T_0 data in this experiment also precluded assessment against an appropriate reference range but this change in CF was not associated with any other exposure effects.

Again, the response of the two age classes with time differed. In the juvenile exposure groups, LSI varied in the VHSV-challenged fish and phagocytosis varied, in an opposite manner to the controls, in the WSFO/VHSV-challenged fish. Among all the adult exposure groups, only CF changed with time and in a similar manner to the control fish.

3.4.5. WSFO exposure effects

The effect of WSFO exposure was evaluated by comparing control and exposed fish at each specific time point. With the exception of the juvenile time series experiment, there was no statistically significant difference in the cumulative mortality rate with WSFO exposure despite initial higher mortalities during the first two weeks of exposure in several cases. It can be concluded that exposure to WSFO, at a starting concentration of 40 g, 80 g and 120 g, did not affect the mortality of adult Pacific herring, but that juveniles exposed to 120 g had higher cumulative mortality after two weeks exposure.

Overall, exposure to WSFO showed considerable variability in the Tier 1 and 2 variables that were altered. Statistically significant changes were not consistent either within or between experiments, and no single measure stood out as a consistent indicator of exposure. As acute (<6 days) or chronic (>21 days) effects may have been missed in the initial, multiple-concentration experiments, exposures were repeated with sampling from 4 to 77 days in other experiments. As there was no concentration-dependent response observed in the multiple-concentration experiments, only the maximum concentration (120 g oil) was used in later time series experiments. It was hypothesized that if WSFO was immunosuppressive, then exposure to the highest concentration possible would most likely identify significant differences in measurement variables.

In juvenile herring exposed to WSFO, statistically significant changes compared with control fish occurred in CF, LSI, plasma lactate, glucose, total protein and lysozyme. None of the statistically significant changes exceeded the appropriate 95th PE comparison reference range (criterion 1), but the change in total protein and lysozyme did exceed the T_P IQR. However, changes did occur in more than one variable at the same time (plasma lactate and lysozyme, CF and LSI, and glucose and total protein) compared with controls at three specific sample points (day 4, 7 and 21 respectively) during the exposure. Thus, the second criterion for potential biological importance was fulfilled and all were supported by similar findings in other studies (Lee *et al.*, 1983; Martin and Black, 1996; Demers and Bayne, 1997; Griffin *et al.*, 1999; Gerwick *et al.*, 2002).

Both plasma lactate and lysozyme were increased significantly in WSFO-exposed fish compared with controls after 4 days of exposure. However, the elevation in plasma lactate (2.38 mmol/L) compared with control fish (2.04 mmol/L) was minor in terms of a physiological difference. Plasma lactate increases in fish may reflect internal hypoxemia from gill dysfunction (McDonald and Milligan, 1992). However, a greater magnitude of increase would be necessary in order to be of physiological consequence. Jain and Farrell (2003) found a threshold plasma lactate level of 12.2 mmol/L (95% confidence interval 7.9 to 16.4) beyond which rainbow trout were unable to sustain swimming and plasma lactate levels in pre-smolt Atlantic salmon following exposure to infectious anemia showed a great deal of individual variation with levels varying from 0.3 to 2.5 mmol/L in control fish and from 0.9 to 15.1 mmol/L in exposed fish (Olsen *et al.*, 1992).

Significant increases in lactate by 54 h post-exposure from 10.0 to 15.3 mmol/L in juvenile channel catfish exposed to copper sulphate for 24 h (Griffin *et al.*, 1999) are more likely to represent physiological consequences compared with the increase seen here in juvenile herring. In the current study, the simultaneous elevation in plasma lysozyme could be a beneficial immunostimulatory response to WSFO exposure although overall it was an inconsistent response. Demers & Bayne (1997) found that the classic fight-or-flight response was responsible for rapid increases in lysozyme of 0.27 ± 0.73 µg/mL after acute stress in rainbow trout as these changes were associated with corresponding elevations in cortisol and adrenaline. Demers and Bayne (1997) state that enhanced innate defenses, such as lysozyme concentration, should increase an individual's ability to neutralize potential pathogens and increase survival.

Both CF and LSI were significantly lower in WSFO-exposed juveniles relatively early into the exposure (day 7). These concurrent statistical changes in condition indices may indicate potential biological significance, and may have represented an initial loss of energy stores (Heath, 1995). Starvation also decreases liver glycogen (Goede and Barton, 1990). Although the fish were not starved in the current study, (fed daily and observed feeding) a decrease in CF was evident in some control fish over time. However, in juvenile rainbow trout, a 105-day fasting period resulted in no significant change in LSI although relative weight did decrease (Simpkins *et al.*, 2003). Decreases in LSI have been seen in other fish populations stressed by acidity (Lee *et al.*, 1983) and other chemicals (Larsson *et al.*, 1984). Asian green catfish (*Mystus nemurus*) exposed to hydrogen sulfide had significantly lower LSI values relative to controls although no change in CF was evident in that case (Hoque *et al.*, 1997). In contrast with the present study, liver hypertrophy was reported in winter flounder exposed to petroleum hydrocarbon-contaminated sediments (Payne *et al.*, 1988), in slimy sculpin (*Cottus cognatus*) exposed to pulp mill effluent and sewage discharges (Galloway *et al.*, 2003) and in flatfish from petroleum and PCB-contaminated areas (Khan, 2003).

The concurrent changes seen in plasma glucose and protein after 21 days of WSFO exposure were suggestive of a secondary stress (elevated glucose) and inflammatory response that differed from controls. The significant difference in plasma glucose at day 21 among treatment groups may reflect a difference in response to captivity. In this study, both control and WSFO-exposed juvenile herring were hyperglycemic at day 14,

but the WSFO-exposed fish had a less prominent peak in glucose and their levels fell more slowly. This persistence of elevation in glucose in the WSFO-exposed herring only at day 21 (4.01 mmol/L) suggested a combined captivity/WSFO effect with impairment of their ability to lower glucose to baseline levels and possible chronic stress. Increases in glucose are well documented in fish under stress (Hill and Fromm, 1968; Silbergeld, 1974; van Vuren *et al.*, 1994; Yin *et al.*, 1995; Martin and Black, 1996; Knoph and Thorud; 1996) with the severity of the stress governing the extent and the duration of the hyperglycemic state (McDonald and Milligan, 1992). Others have also reported elevation in blood glucose following exposure to toxicants. Oikari *et al.*, (1976) showed a slight increase in plasma glucose in pike (*Esox lucius*) from heavily polluted waters. Increased plasma glucose (6.6 mmol/L) to twice the control level was also seen in channel catfish exposed to copper sulfate (Griffin *et al.*, 1999). Catfish exposed to heavy metals (lead, cadmium and zinc) showed a transient increase in glucose (12.4 mmol/L) above reference values (Martin and Black, 1998). Therefore, it is feasible that exposure to WSFO could also result in hyperglycemia.

In conjunction with the persistent increase in plasma glucose, a three-fold increase in total protein was also observed in WSFO-exposed juvenile herring on day 21 (2.50 g/dL). Hct was not concurrently elevated above control levels, as might be expected if this increase in total protein was due to dehydration (assuming no underlying anemia). In addition, there were no skin lesions or other supporting evidence to support the possibility of dehydration. True hyperproteinemia is rare and so it is possible that this was occurring as a response to WSFO exposure. Hyperproteinemia has previously been associated with stimulation of the non-specific immune system as a response to inflammatory stimuli (Gerwick *et al.*, 2002). Hyperproteinemia has also been reported in rosy barb (*Puntius conchonius)* exposed to an organochlorine insecticide, endosulfan (Gill *et al.*, 1991); in rosy barb exposed to copper (Gill *et al.*, 1992); in catfish (*Heteropneustes fossilis*) exposed to a carbamate pesticide, propoxur, (Singh *et al.*, 1997). It was possible that WSFO-exposed juveniles were dehydrated but this would need to be repeated with larger sample sizes to be confident of such a conclusion.

During the exposure periods, the median varied significantly for GSI, Hct, thrombocytes, SSI, plasma chloride, phosphorus, calcium, albumin, A/G, lysozyme and phagocytosis WSFO-exposed adult fish when compared with controls. Phagocytosis was the only

variable to change significantly during WSFO exposure in more than one experiment. None of these changes resulted in the median value exceeding the corresponding T_P or time-matched control 95th PE range but the changes in thrombocytes and albumin/globulin did exceed their respective IQRs. However, most of these statistical changes occurred simultaneously at either day 21 or 28 of exposure. After 21 days of exposure, Hct, SSI, albumin, phosphorus, calcium, plasma chloride and A/G were all significantly different in WSFO-exposed BS adults, while GSI and phagocytosis were altered in WSFO-exposed QI adults. In addition, significant changes in plasma lysozyme and phagocytosis occurred after a 28-day exposure in the QI population. Therefore, criterion 2 (two or more significant changes occurring concurrently) for biological significance was fulfilled for most of the statistical changes in adult WSFO-exposed fish. The potential biological importance of these changes was considered in conjunction with other literature evidence discussed below.

The elevation in Hct after 4 days of WSFO exposure in adult herring occurred in isolation of other significant changes and did not exceed the 95th PE reference range (8.5 to 62.3%). However, Hct has been shown in the literature to increase (criterion 3) following exposure to a variety of stressors. For example, Hct was increased in adult sockeye salmon after exposure to long-term (48 h) hypoxia (Korstrom *et al.*, 1996); in carp exposed to pesticide preparations (Svobodova and Pecena, 1988); in post-smolt Atlantic salmon fed high (below the known toxic concentration) or low (below minimum requirement) levels of dietary iron for 14 days (Lygren *et al.*, 1999) and in carp after short-term (96-hours or less) exposure to zinc (Witeska and Kosciuk, 2003). During capture and handling, elevation in Hct is due to the release of RBCs from the spleen and from fluid movement between tissue compartments (Gallaugher and Farrell, 1998). Therefore, the observed increase in Hct suggested that the splenic contraction or hemoconcentration response to handling (the two main mechanisms to increase Hct) were being accentuated by WSFO exposure on day 4.

While an increase in thrombocytes in adult WSFO-exposed fish was not substantiated by a change in any other variable, or observed in any other experiment, it has been reported in other studies (criterion 3) and, therefore may be important. An elevation in thrombocytes (10.5% greater than controls) after a short-term (6-day) WSFO exposure, as seen in the adult 40 g oil group, suggested an increased capacity for responding to

injury as the thrombocyte is thought to be equivalent to the mammalian platelet and to be involved in clotting (Fänge, 1992). An increase in thrombocytes may also increase phagocytosis of xenobiotic molecules such as crude oil. Ferguson (1976) saw uptake of carbon microparticles following intravenous administration and speculated that this cell may also have some phagocytic capability. Therefore, this suggested that at a low concentration, WSFO could be acting as an immunostimulant in herring. In contrast, Murad and Houston (1988), working with goldfish (*Carassius auratus*), found that sublethal concentrations of cadmium resulted in a decrease in the thrombocyte count from 35.0 ± 0.66% to 31.0 ± 0.04%, which they concluded might decrease clotting ability and increase susceptibility to injury. While the magnitude of the change in the median percentage thrombocytes in herring was almost 2-fold (17.2% vs. 27.7%) the 95th PE ranges of control (12.1 to 24.5%) and WSFO-exposed (20.7 to 76.9%) fish did overlap slightly. This means that although thrombocytes were elevated well above control values in the majority of the 40 g WSFO-exposed fish a few were still within the range of normal for captive, non-exposed herring.

After 21 days of WSFO exposure, adult BS fish were hypoalbuminemic compared with control fish. This could reflect intestinal vasculitis and loss of albumin following ingestion of WSFO or may reflect decreased albumin production secondary to PAH-induced liver necrosis (Woodward *et al.*, 1983; Fatima *et al.*, 2000; Rudolph *et al.*, 2002). In support of the liver necrosis hypothesis, there was a positive association between the elevation in albumin and reduction in LSI ($p=0.0034$, $r^2=0.93$). Plasma protein levels in teleosts can be affected by several factors including season (Srivastava and Srivastava, 1994); gender (Poston, 1971); steroid hormones (Sunny *et al.*, 2002); environmental temperature (Langston *et al.*, 2002) and disease status (Cardwell and Smith, 1971; Simko *et al.*, 2001; Gerwick *et al.*, 2002) and are thought to be important in immune response processes (Houston, 1973; Holland and Lambris, 2002). Decreases in plasma albumin have been seen in fish exposed to other toxicants. Pfeifer and Weber (1979) found a decrease to 0.8 g/dL after injecting rainbow trout with 2.0 mL/kg carbon tetrachloride. The concurrent reduction in plasma P_i and Ca^{2+} in the current study was most likely a consequence of the decreased albumin in these fish. A large proportion of these two ions in plasma is protein-bound, so changes in protein levels and plasma proteins (such as albumin) can be expected to affect the levels of these ions (McDonald and Milligan, 1992).

Plasma chloride was also significantly elevated at day 21 of exposure, which may have signified a stress-induced osmoregulatory change as marine fish under stress have shown elevation in chloride due to the effect of catecholamine-mediated changes in osmoregulation (Pickering and Pottinger, 1995). Osmoregulation is known to be sensitive to the effects of toxicants causing disruption of homeostasis of the ions, Na^+, Cl^-, and Ca^{2+} (Wendelaar Bonga and Lock, 1992). However, the magnitude of the change in herring was 28 mEq/L and it is questionable if this would have a physiological effect as neither the control nor WSFO-exposed fish exceeded the T_P 95th PE reference range (75 to 171 mEq/L). This wide degree of variability in chloride in adult herring made it difficult to be confident of a biologically relevant treatment effect. In comparison, the reported range in other fish is from 95 ± 20 mEq/L in roach (Jeney *et al.*, 1996) to 167 ± 1 mEq/L in flounder (Alkindi *et al.*, 1996)

Again, on day 21, there was an increase in phagocytosis in adult WSFO-exposed QI herring. Phagocytosis has been shown to increase/decrease in conjunction with plasma lysozyme (Kokoshis and Di Luzio, 1979), but this was not the case in the present experiment as lysozyme was unaffected. It appeared that a 21-day exposure to WSFO was sufficient to stimulate the phagocytosis ability of macrophages, but insufficient to suppress plasma lysozyme significantly. In contrast, after a 28-day WSFO exposure, statistically significant, concurrent decreases were evident in both plasma lysozyme and phagocytosis in adult WSFO-exposed QI herring.

The reduction of plasma lysozyme in adult herring following WSFO exposure had the potential to increase disease susceptibility as low lysozyme levels are thought to contribute to an increase of susceptibility to pathogens, in particular to Gram-negative bacteria (Caruso and Lazard, 1999). Suppression of lysozyme has also been reported in rainbow trout exposed to 5 and 56 µl/L creosote for 28 days where the mean lysozyme was 2.0 ± 0.2 µg/mL in controls (Karrow *et al.*, 1999). Lysozyme activity has been seen to decrease in Atlantic salmon during smoltification, which is generally accepted as a time of immunosuppression, due to an increase in inter-renal activity and elevated cortisol levels (Anderson *et al.*, 1982; Ellsaesser and Clem, 1987; Maule *et al.*, 1989; Muona and Soivo, 1992; Coutant, 1998).

Other investigators have also documented a decrease in phagocytosis following toxicant exposure such as that observed in the 28-day WSFO QI adult exposure in this study. Karrow *et al.* (2003) reported a decrease in macrophage phagocytosis in rainbow trout exposed to PAH-contaminated water. Spot (*Leiostomus xanthurus*) and hogchoker (*Trinectes maculates*) that were chronically exposed to PAHs within a polluted river system (Weeks and Warinner, 1984) and European seabass exposed to B(a)P (Lemaire-Gony *et al.*, 1995) also demonstrated a reduction in phagocytosis to 20.0 ± 3.0% and to 31.5 ± 1.6% respectively. However, the increase in phagocytosis in QI adults in my study after a 21-day exposure, which may indicate immunostimulation, was somewhat unusual and has not been reported in other PAH exposure studies.

Although the Tier 1 and 2 changes observed with WSFO exposure did not occur in a consistent manner between studies or age class, plasma lysozyme did change significantly in both juvenile and adult (QI only) fish during the exposure period. Interestingly, in juveniles an increase occurred early into the exposure period (day 4), whereas in adults the decrease in lysozyme occurred at the end of the exposure period (day 28). However, because plasma lysozyme was only checked at intervals during the time course of the experiments additional fluctuations in lysozyme, in either direction, might have occurred. The variability in the direction of plasma lysozyme response observed in this study has been seen before. Stress can have a variable effect upon plasma lysozyme activity, both between and within studies, with some researchers observing an increase (Fevolden *et al.*, 1991, Røed *et al.*, 1993; Demers and Bayne, 1997; Price *et al.*, 1997) and others seeing a decrease (Yin *et al.*, 1995; Tahir and Secombes, 1995; Jeney *et al.*, 1997; Karrow *et al.*, 1999). The direction of response of lysozyme may also be related to the degree of stress experienced by the fish. It is suggested that the strength of the stressor and the fish's condition will determine how plasma lysozyme responds (Möck and Peters, 1990). When rainbow trout were handled for 30-minutes (considered a mild stressor) lysozyme levels were either stimulated in some fish by 5 to 10 U/mL or suppressed in others by up to 20 U/mL ($p<0.05$) (Möck and Peters, 1990). Stressors such as transportation for 2 hours or acute water pollution resulted in a consistent significant decrease in lysozyme levels compared with controls (Möck and Peters, 1990).

The immune response is dynamic and it is possible that plasma lysozyme concentration fluctuates over the course of exposure to toxicants and pathogens. Therefore, the time of sampling with respect to the duration of exposure to a specific toxicant or event, in addition with the direction of change, was of great interest. The time series experiments attempted to evaluate this by sampling more frequently during the WSFO exposure. An increase in plasma lysozyme likely reflects a beneficial non-specific immune system response while a decrease may indicate an inability to respond (immunosuppression). Therefore, it would appear that any effect associated with exposure to WSFO resulted in an immunostimulatory response in juvenile herring compared with an immunosuppressive response in the adults. This could be a function of aging and the effectiveness of the immune system and deserves further evaluation of age-specific differences in lysozyme production in herring.

There were some additional notable differences between age classes and between adult source populations. The condition indices, CF and LSI were affected by exposure to WSFO in the juvenile herring only. Biochemical indicators of secondary stress effects (i.e., plasma glucose and lactate) were also altered in juvenile herring but not usually in adults with respect to WSFO exposure. However, the background median plasma glucose level in adults was high at 9.22 mmol/L which could mask any acute increases in glucose if WSFO exposure were eliciting a stress response. This level of plasma glucose is suggestive of a chronic captivity stress response in adult herring. Concurrently, cortisol was relatively low with a median of 14.8 ng/mL in females and 11.2 ng/mL in males (Table 2.12) compared with 22.4 ± 4.8 in control rainbow trout (Milligan, 1996) and 23.4 ± 2.3 in control dab (Hutchinson and Manning, 1996) suggesting that a classical primary, acute stress effect due to captivity was unlikely. The significant difference in background plasma glucose concentration among the two age classes, and the differences in the response of stress variables (glucose and lactate) with WSFO exposure suggests juvenile and adult herring respond differently to captivity stress. Adults appeared to be a more chronic stress state such that WSFO exposure did not elicit a classical secondary stress response. In future studies it may be prudent to measure adrenocorticotropic hormone (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH) to aid in evaluating for a stress response and to see if changes in ACTH and MSH correspond to acute and chronic stress as is the case for other teleosts (Weyts *et al*, 1999).

Hct was affected only in adults. In addition, SSI, GSI and an array of biochemical measures that could not be evaluated in the juvenile fish were affected in the adult fish. Comparison between the variables measured in both adult populations also identified differences in response to WSFO exposure. An increase in Hct and a decrease in SSI occurred in the adult BS population, whereas a reduction in GSI and lysozyme and a variable phagocytosis response, depending on the duration of WSFO exposure, was observed in the adult QI population.

3.4.6. WSFO exposure and recovery effects

In juvenile herring, plasma lactate was the only variable to show a significant latent change following the 42-day recovery period in the absence of pathogen challenge. The reduction in plasma lactate in WSFO-exposed herring relative to controls after the post-WSFO recovery period suggested that either the effect of captivity was lessened in WSFO-exposed fish or that their ability to mount a secondary stress response was impaired. The fact that no other variables changed significantly after the recovery period indicates that, while the statistically significant changes in Tier 1 and 2 variables during the WSFO exposure may have had some biological importance, any impact on function was only of a short-term nature.

In contrast with juveniles, statistically significant latent effects were evident in several Tier 1 and 2 variables (GSI, plasma lactate, chloride, lysozyme, respiratory burst and phagocytosis) in adult WSFO-exposed fish after the 42-day recovery period compared with control fish. Again, none of these changes exceeded the corresponding T_P (chloride only) or time-matched control 95th PE range while the changes in both plasma chloride and lactate fell below their respective IQRs (100 to 131 mEq/L; 4.64 to 7.57 mmol/L). The latent decrease in plasma chloride and increase in GSI occurred in the BS adults only. Similar to juveniles, plasma lactate exhibited a latent decrease in the QI adult fish after the recovery period. Concurrently, phagocytosis ability was also significantly decreased. However, plasma lysozyme and respiratory burst activity were increased compared with control fish in the QI adult population. Therefore, although phagocytosis ability was reduced with WSFO exposure, it appeared that the macrophages could still respond in other ways as lysozyme is thought to be an indicator of macrophage activity (Kokoshis and Di Luzio, 1979; Siwicki and Studnicka, 1987). This demonstrated the multi-functional nature of the macrophage and suggested that, despite one mechanism being impaired, another was able to function at a higher rate.

Similar findings of decreased phagocytosis but increased respiratory burst activity were reported in seabream (*Sparus aurata* L.) following exposure to retinal acetate (Cuesta *et al.*, 2002).

The magnitude of changes observed in plasma lysozyme were of a 10-fold nature depending upon the time post-exposure. The decrease in lysozyme of 0.20 µg/mL at 28 d into the recovery period in WSFO-exposed QI adults (day 49) was of considerably greater magnitude than the decrease after 28-days of WSFO exposure (0.01 µg/mL). Therefore, it appeared that the duration of time post-WSFO exposure was a factor in the degree of lysozyme suppression. This implied that WSFO was unlikely to be impairing immune function at the level of lysozyme expression after a 28-day exposure, but that at 28 d after termination of the 21-day exposure (day 49) that some impairment may have occurred although there was no concurrent significant difference in cumulative mortality among treatment groups. This reduction in lysozyme during the recovery period was not accompanied by a decrease in phagocytosis ability.

In summary, plasma lactate was the only variable that demonstrated a significant latent change in both age classes with a decrease occurring in both cases after the post-WSFO recovery period. Within the adult age class, there were no variables common to both source populations (BS and QI) that were significantly altered either during the exposures or after the 42-day recovery period. It is possible that the responses observed in the 28-day adult QI exposure (primarily Tier 2) may be associated with a higher TPAH concentration in this experiment at 28 d compared with the 21 d exposures if the higher water TPAH (13.64 ± 0.90 ppb) value is accurate and not a technical or analytical error. Nonetheless, there was still no significant difference in mortality compared with the control fish in this experiment.

3.4.7. WSFO exposure and *V. anguillarum* challenge effects

The multiple concentration exposure to WSFO in juveniles was followed by a pathogen challenge that used *V. anguillarum.* Significant latent changes in Lct and antibody titre compared with control fish were observed in juveniles exposed to WSFO and then challenged with *V. anguillarum* as well as in *Vibrio*-only challenged fish. The increase in Lct in the 80 g oil/*Vibrio* group exceeded the time-matched control 95th PE range (0.00 to 0.22%). The changes in antibody titre also exceeded the control 95th PE range (0 to 1600) in all but the 120 g oil/*Vibrio* group.

The increase in Lct in the 80 g oil/*Vibrio* fish is consistent with a leucocytosis and was likely a response to inflammation and various stages of sub-clinical or clinical infection (Wedemeyer *et al.*, 1990). It suggested that while there was no significant difference in Lct after WSFO exposure alone, or *Vibrio* exposure alone, compared with controls, that this dose of WSFO, in conjunction with the *V. anguillarum* challenge, produced an immunostimulatory effect.

Antibody titre was reduced in juveniles exposed to WSFO and challenged with *V. anguillarum*. The lower titre against *V. anguillarum* in WSFO-exposed juvenile herring may indicate a suppression of response to bacterial challenge. Thuvander and Carlstein (1991) reported a reduction in antibody titre in rainbow trout exposed to sublethal doses of Clophen A50 (a PCB-containing compound) and later immunized with *V. anguillarum* vaccine. Antibody levels were significantly lower in the Clophen A50 treated groups. Passive immunization studies with rainbow trout exposed to *V. anguillarum* demonstrated a protective ability of fish antibodies (Harrell *et al.*, 1976; Viele *et al.*, 1980) and Bricknell *et al.* (2000) found a significant correlation between antibody titre and level of protection against *V. anguillarum* in Atlantic halibut (*Hippoglossus hippoglossus* L.). However, protective immunity against Gram-negative bacteria (such as *V. anguillarum*) has also been seen in fish with very low levels of specific humoral antibodies (Croy and Amend, 1977; Thuvander *et al.*, 1987). This suggests that a reduction in antibody titre cannot always be directly associated with a decreased disease resistance during a pathogen challenge. Still, the suppression in the antibody level seen in the present study may represent an alteration of immune function (Thuvander & Carlstein, 1991). However, because repeated titre measurements were not possible with herring it is difficult to draw immune response conclusions based on a single titre, therefore the significance is unknown.

V. anguillarum was isolated from dead controls in addition to those that were challenged with the pathogen. The isolation of *V. anguillarum* from 25% (4/16) of non-exposed control mortalities may indicate false positives or could reflect the background level of this pathogen in wild juvenile BS herring. It may be indicative of the endemic nature of *V. anguillarum* in wild herring populations or could reflect captivity-associated immunosuppression. It is probable that this level of background *V. anguillarum* infection was also present in the other treatment groups. The challenge dose may have

increased the proportion of herring within the challenged groups that became infected and subsequently succumbed to the pathogen compared to the control fish.

Antibody titre against *V. anguillarum* was present at a low level in non-challenged control fish at the final sample. Therefore, this could indicate a population that was protected to some degree against *V. anguillarum* from prior exposure. The isolation of *V. anguillarum* from the head kidney of 25% of the control mortalities (4/16) may represent fish within that group that were chronically infected and that were immunocompromised. However, cumulative proportional mortality was low in the controls (3.2%) and there were no gross lesions, suggesting that there was no widespread infection throughout the tank. In addition, *V. anguillarum* was not isolated from any sampled control fish. It was not possible to assess the antibody titre of the dead fish that were tested for *V. anguillarum* by culturing head kidney tissue. Therefore, the relationship between titre and infection status in herring was not determined.

The low level of *Vibrio* infection that was present following the pathogen challenge may have been a function of either water temperature, the presence of a strong cohort of fish, or WSFO bactericidal activity. Water temperature is an important environmental factor governing the severity of bacterial disease outbreaks and this can impact the experimental induction of vibriosis (Fryer and Rohovec, 1993). The number of rainbow trout showing septicemia and the number of carriers are significantly higher between 13°C to 15°C than at lower temperatures (9°C to 12°C) or higher temperatures (16 to 18°C), (Baudin-Laurencin and Germon, 1987). The water temperature during the experimental challenge period was 10°C to 14°C, which was somewhat below the optimal range as stated above. Therefore, it was possible that this temperature range influenced the level of *Vibrio* infection that was induced and hence the relatively low cumulative proportional mortality in all challenged groups. Toxicity from exposure to petroleum hydrocarbons in the C_{10} to C_{19} range in particular has been demonstrated on several other marine bacteria (Brils *et al.*, 2002). Thus, it is feasible that WSFO had a toxic effect on *V. anguillarum*, which could have reduced the exposure dose and virulence of the challenge organisms.

3.4.8. WSFO exposure and VHSV challenge effects

In the juvenile challenge, the highest cumulative mortality during the post-challenge period occurred in the WSFO/VHSV challenged fish (60%) compared with control fish.

However, there was no statistically significant difference in the proportion of dead juveniles testing VHSV-positive compared with control, WSFO-exposed, or VHSV-challenged fish. Therefore, an increased susceptibility to mortality from VHSV infection with concurrent WSFO exposure was not evident during the experiment. There was a significantly greater prevalence of lesions consistent with VHSV in sampled juveniles exposed to WSFO and VHSV concurrently but serum viral evaluation could not be done. Therefore, their viral titre status was unknown and if they had not been euthanized for sampling they may have died from VHSV at a later date.

In juvenile fish, there was a low prevalence of VHSV isolated from dead fish (12/126) and sub-clinical infection may have been present. This low prevalence of viral tissue titre in dead juvenile herring in all treatment groups may have been due to either a loss of titre from freeze/thaw cycles to below detection limit levels ($<10^{2.6}$ pfu/g), or due to a lack of widespread infection because of acquired immunity from prior exposure. The isolation of VHSV from two dead control juvenile fish again supported the hypothesis that the fish had previously encountered VHSV (prior to capture or during acclimation). If this occurred, then many fish may have acquired immunity by the time of the experimental challenge, which would have lessened the likelihood of inducing clinical VHSV *via* the challenge model used.

Mortality was low in all adult fish treatment groups (< 20%) and mortality in fish exposed to WSFO and a VHSV challenge did not differ significantly from the VHSV-only group. This could be interpreted as exposure to WSFO failing to increase susceptibility to VHSV. However, it was not possible to assay the tissue of adult dead fish for viral titre so the proportion of dead fish with VHSV tissue titre was not known. However, the VHSV challenge of adult fish did not appear to induce clinical disease (no clinical signs). The prevalence of neutralizing antibodies against VHSV in the sera of adult fish at the various sample times was low (35/319), did not vary in magnitude between treatment groups and was detected in control fish. This again suggested that there had been natural exposure of all fish to VHSV prior to the challenge. The relatively low mortality, lack of clinical signs and the lack of moribund herring all supported an inability to induce clinical VHSV *via* the experimental challenge. However, the low prevalence of detectable serum antibody titre or viral tissue titre and the similarity between control and VHSV-challenged fish does not necessarily mean there was no effect from VHSV

exposure. It is now known that peak viral titre occurs at 7 to 14 d after the start of a laboratory outbreak of VHSV and is generally not found by 30 d after the start of the outbreak (Kocan *et al.*, 2001a). Therefore, as plasma antibody titre sampling did not commence until 14 days post-challenge, it is possible that viral titres peaked prior to sampling in challenged fish and that viral titre is not a reliable indicator of exposure unless measured shortly after exposure.

The isolation of virus from a small percentage of sampled adult fish that had no clinical signs suggested that these fish may have been sub-clinically infected (VHSV-infected but no clinical signs). One VHSV-positive individual was only exposed to WSFO, which again supported a prior exposure of adults to VHSV before the challenge with subsequent immunity of some of the fish. The presence of neutralizing antibodies in the sera of 11% of the adult herring may also have been more indicative of a sub-clinical infection than immunity as antibodies could not be detected in the sera of other herring shown to be soundly immune to VHSV (Kocan *et al.*, 2001a). The source of sub-clinical infection may have been attributable to the challenge, may represent the effects of exposure prior to challenge as some non-challenged herring also had detectable serum antibodies, or may have been due to a combination of both factors. A similar virus-specific mortality of 12% was seen following exposure of rainbow trout to North American VHSV at 10^5 pfu/mL (Follett *et al.*, 1997). However, in that study, the fish had clinical signs consistent with VHSV.

Cumulative mortality in juvenile fish was considerably greater in both control and challenged fish (26.3 to 68.9%) compared with adult fish (10.8 to 17.6%). Similarly, the overall pre-experimental mortality for juveniles was higher at 27.1% compared with 18.5% for adults (Appendix 2, Table A.2.1). The juvenile VHSV challenge was concurrent with the WSFO exposure, unlike the sequential exposure to WSFO and VHSV challenge for adults. Combined exposure to WSFO and VHSV was thought to be more likely to induce clinical VHSV if the WSFO were acting as an immunosuppressive agent. Indeed, it appeared that the different time interval between initiation of WSFO exposure and pathogen challenge might be a factor in the development of clinical expression of VHSV. Adult herring were resistant to the induction of clinical disease. However, 42% of juvenile fish exposed to WSFO and VHSV sampled at 14 d post-

challenge had hemorrhagic skin lesions consistent with VHSV infection compared to 8% of VHSV-challenged fish and none of the WSFO-exposed and control fish (Table 3.9).

It was possible that herring were immune, naïve, or in a sub-clinical state of VHSV infection prior to the challenge. As such, challenge of any naïve herring had the potential to cause infection, especially if WSFO were immunosuppressive. However, the results of both challenges suggested that it was not possible to induce clinical VHSV infection *via* experimental challenge in the majority of adult and juvenile herring. Tissue viral titre was below detectable limits in the majority of the adult herring (317/319) and there was no difference in serum antibody titre between treatments in the adults sampled. The presence of tissue viral titre, and serum antibody titre in some of the adult herring may have been due to a combination of the effects of the VHSV challenge in conjunction with prior VHSV exposure in the challenged groups.

The viral tissue titre of immune herring has since been reported as being below that of moribund herring or is undetectable (Kocan *et al.*, 2001a). This implies that antibody titre against VHSV is not positively correlated to the level of protection or infection and, therefore, herring immune to VHSV will not exhibit the classic mammalian secondary antibody response of an increase in titre with re-exposure. In fact, tissue viral titre has been shown to decrease to below detection limits ($<10^{2.6}$ pfu/g) within 30 d post-exposure in juvenile and adult herring that experienced an epizootic of VHSV shortly after capture (Kocan *et al.*, 2001a). A lack of neutralizing antibody in the serum of suspected immune herring following VHSV challenge, as occurred in the present study, has also been seen in PS herring (Kocan *et al.*, 2001a). Absence of a detectable secondary, booster, effect when exposed to a pathogen for the second, or more, time(s) has also been reported in rainbow trout resistant to challenge with infectious hematopoeitic necrosis virus (IHNV), another fish rhabdovirus (Corbeil *et al.*, 1999). Given that antibodies cannot be detected in the serum of immune VHSV epizootic survivors, it would appear that a very low titre might be sufficient to provide protection. This is also seen in rainbow trout, where very low serum antibody titres (1:10 or 1:20 dilution of serum at which agglutination with the viral antigen stops) are protective against IHNV (LaPatra *et al.*, 1994). The lack of antibodies against VHSV in immune herring is substantiated by the findings of Davis *et al.*, (1999) which show that IgM, the primary fish immunoglobulin, is only significantly associated with *I. hoferi*. It suggests

that cell-mediated immunity plays a greater role in immunity against VHSV than humoral immunity does.

Sub-samples of both juvenile and adult herring tested negative for VHSV during the first week of captivity supporting an absence of VHSV shortly after capture. However, mortality rates of 27.1% (juveniles) and 18.2% (adults) were seen during the holding periods, prior to acclimatization of herring to experimental tanks. Since completing this study, information has been published that could explain the apparent disparity between the negative screening results and the belief that VHSV was present in the pre-experimental period, resulting in acquired immunity in some of the survivors. Firstly, the natural prevalence of VHSV in juvenile wild caught herring has been estimated at <1%, meaning that the chance of detecting virus from small numbers (<100) of freshly captured herring was small (Kocan *et al.*, 2001a). Secondly, there was potential for sufficient loss of viral titre in the dead fish submitted such that the titre was below detectable levels. The relationship of the time from death to freezing on VHSV titre remains unknown but it has been suggested that this could affect titre detectability (Kocan *et al.*, 2001a). In addition, it has been demonstrated that each freeze-thaw cycle of tissue/serum can result in up to a 90% loss of VHSV titre (Hershberger *et al.*, 1999, Kocan *et al.*, 2001b).

Dry ice was used during transportation of samples from the field site to the testing laboratory. The journey lasted a minimum of 3 to 4 hours and partial thawing may have occurred before the samples were restored at –80°C at the PBS laboratory prior to analysis (Kieser, pers. comm., 2003). Therefore, given that thawing does reduce VHSV titres (Kocan *et al.*, 2001b), it was possible that VHSV was present in these samples but went undetected due to the loss of titre below detectable levels. Clinical signs consistent with VHSV were present in dead fish (moderate to severe focal skin reddening) (Meier, *et al.*, 1994; Meyers *et al.*, 1994; Marty *et al.*, 1998) during the pre-experimental periods when mortality ranged from 18.5 to 27.1%. Therefore, VHS epizootics may have occurred during the pre-experimental periods. There were no gross lesions evident when challenges and WSFO exposures were initiated but there was potential for sub-clinical infection to be present.

Tissue viral titre has been detected in herring in spawn-on-kelp pounds within the first week of herring entering the pounds, suggesting that any type of confinement will precipitate an outbreak of VHSV (Hershberger *et al.*, 1999). It has been shown that if even 1/200 captured herring is infected (0.5%), the amount of VHSV that is shed into the transport water ($>10^6$ pfu/h) is sufficient to be infective and will kill SPF herring (Kocan *et al.*, 2001a). The course of the epizootic is also such that it may occur during the typical pre-experimental acclimation period, beginning within a week of capture and lasting one month. Consequently, if such an epizootic occurred in this study, then some of the fish may have been immune to the virus, and some sub-clinically infected before experimental challenge. Immunity to VHSV following an epizootic has been documented up to 60 d after mortality has ended (Kocan *et al.*, 2001a) but the absolute duration of immunity following exposure remains unknown. Immunity against re-infection has been seen in herring as young as 4 months of age following massive epizootics in captivity (Kocan *et al.*, 2001a). The juvenile herring in this experiment were approximately 10 months of age. The use of younger herring was desired as they may have been more susceptible to re-infection or naïve. Unfortunately, it was not possible to use < 4 month old fish due to the high captive mortality rates encountered (68.5 to 87.0%) during the 5 months prior to establishing this challenge. It was also not possible to examine juveniles for serum antibody titre due to the small blood volumes obtained. Consequently, the presence of possible sub-clinical infection could only be inferred from the presence of VHSV in 9.5% of the dead fish and the presence of clinical signs in some sampled fish.

The failure to induce significant clinical disease was not thought to be due to the challenge methodology as it was based on a previously validated challenge model using a virulent VHSV stock solution (Kocan *et al.*, 1997) that was cytotoxic to cell culture medium. The same methods were previously used to expose SPF herring to VHSV with 60 to 100% mortality although the SPF fish were naïve to VHSV and therefore had no possibility of acquired immunity (Kocan *et al.*, 1997). It is possible that environmental factors had an impact on the ability to induce clinical VHSV *via* the water-borne route. Water temperatures at the time of the adult challenge in this study (8.5 to 12.4°C) were periodically slightly above the upper limit of the temperature range known to induce VHSV in rainbow trout (1 to 12°C) (Meier *et al.*, 1994) and most natural outbreaks occur when water temperature is 10°C (Wolf, 1988). Therefore, water temperature had the potential to interfere with the adult challenge. However, it was not known if the optimum

water temperature range for inducing VHSV was the same in herring as that reported for rainbow trout at the time of conducting the challenge. In the juvenile challenge, water temperature ranged from 8.1 to 12.0°C.

In juvenile herring, the increase in phagocytosis activity in the WSFO/VHSV-challenged fish at day 14(7) occurred in conjunction with a significant difference in plasma glucose compared with control fish. The elevated phagocytosis activity of macrophages in these fish suggested that the combined exposure to WSFO and the VHSV challenge were interacting to up-regulate this activity. This implied an active, competent immune system at 7 d post-VHSV challenge and was not suggestive of immunosuppression. The increase in phagocytosis did not persist, as there was no significant difference in phagocytosis activity at 14 d post-challenge. It appears that any up-regulation of phagocytosis activity was short-lived and that any synergistic effect occurring due to the concurrent challenges was not sustained. In addition, at 14 d post-challenge, a significant proportion of the WSFO/VHSV-challenged herring had external lesions and viral titre was detected in the tissue of some of the dead fish. This may support a loss of ability to fight the effects of this combination of xenobiotic and pathogen in the face of clinical VHSV infection.

Elevation of LSI (1.34 to 1.35) in juveniles either exposed to only WSFO or challenged only with VHSV respectively, above control levels, was indicative of a relative liver hypertrophy (increased liver size). Similar increases in liver condition index, have been seen in several other fish studies in response to polluted water systems and PAHs (Sloof *et al.*, 1983; Fabacher and Baumann, 1985; Payne *et al.*, 1988) and following exposure to pollution/specific toxicants (pulp mill effluent and oiled sediments, Oikari and Nakari, 1982; Fletcher *et al.*, 1982). Increased LSI (1.38 ± 0.9) has also been associated with external lesions and liver discolouration in winter flounder chronically exposed to PCBs and PAHs in polluted water (Khan, 2003).

In the adult challenge, the differences in response to WSFO and VHSV at the Tier 1 and 2 levels did not persist beyond one sampling point. Despite the lack of clinical disease following VHSV-challenge, it was possible that the statistically significant changes with VHSV challenge were indicative of a non-specific secondary stress response (alterations in plasma biochemistry) in sub-clinically infected fish that had no visible clinical signs of

disease. The reduction in CF seen early into the adult post-VHSV challenge period occurred in the VHSV-challenged and WSFO/VHSV-challenged groups only. As there was no reduction in the WSFO only group at this point, it was possible this might be attributable to the viral exposure. However, because CF did decrease in all groups, including controls, on day 49(28) and day 63(42), the earlier decrease in both groups challenged with VHSV was more likely due to a combination of VHSV and a captivity effect rather than VHSV alone. Conversely, this decrease could be a function of sampling order over time as CF could not be monitored within the same fish over time.

The decrease in plasma lysozyme in all adult exposure groups of herring compared with control fish at day 49(28) suggested that WSFO and VHSV were acting to a similar degree to lower lysozyme and that they were not having an additive effect. Yet, it was unlikely that this constituted evidence of immunosuppression, as the degree of change was small and was not evident at the later sample points. The significant decrease in Lct in VHSV-only challenged adults relative to controls, at the recovery sample time, suggested that at least some fish were likely sub-clinically infected because of the challenge. On day 77(56), 59% of the VHSV-only challenged herring had no detectable Lct, which was supportive of leucopenia within this group, which has been associated with VHSV infection (Noga, 1996).

The elevation in median Hct in the VHSV-challenged (to 51.6%) and in WSFO/VHSV-challenged fish (to 49.2%) compared with control fish at the recovery sample exceeded the limits of the IQR only (31.3 to 46.9%) and was not consistent with VHSV infection (in which anemia is commonly observed). Release of splenic stores of red blood cells and fluid movements between tissue compartments (resulting in dehydration) are mechanisms that can greatly elevate Hct (i.e., by up to 50%) in response to stress (Gallaugher and Farrell, 1998). Therefore, the elevation in Hct could be due to either of these stress mechanisms as there were no gross skin lesions, which, if present, could also have resulted in dehydration (Law, 2001).

The overall response of juveniles and adults among exposure groups in these two VHSV challenge experiments was quite different with no variable being affected within both age classes. In juveniles during the 14 d post-VHSV challenge, significant differences in LSI, glucose, and phagocytosis were observed in either the VHSV-challenged, or

WSFO/VHSV-challenged juveniles compared with control fish. Although there were some statistically significant differences in Tier 1 and 2 variables in adults following exposure to WSFO and VHSV, there was no consistency to these changes and no difference in the cumulative mortality rate between groups. Only CF varied significantly in the VHSV-challenged and WSFO/VHSV-challenged adults during the initial 14 d post-challenge. Changes in adult VHSV-challenged and WSFO/VHSV-challenged fish, relative to control fish, were seen in Lct, Hct, and plasma lysozyme during the recovery period (day 28 to 56 post-VHSV challenge). Again, these age class differences in response to disease challenge further supported age separation in experimental design and analysis.

In this study, exposure to WSFO, in concentrations relevant to the PWS exposure, did not affect the apparent established immunity to VHSV in terms of morbidity or mortality. Yet, the differences seen in Tier 1 and 2 variables suggested the VHSV challenge may have served as a non-specific stressor in sub-clinically infected fish (no clinical signs). In particular, the significant increase in phagocytosis in the WSFO/VHSV-challenged juvenile fish may support an immunostimulatory response to the presence of sub-clinical VHSV infection. Sub-clinical viral infection as a response to exposure to weathered crude oil has previously been reported in adult Pacific herring (Carls *et al.*, 1998) but has not previously been studied in juveniles. The leucopenia evident in WSFO/VHSV-challenged adults in this study could also reflect sub-clinical infection but the absence of a concurrent anemia further supported the lack of overt, clinical disease.

Assessment of disease susceptibility in these challenges was compromised by the probable acquired immunity against re-infection in some herring by the time of the challenges. Nonetheless, these experiments showed that once immunity against VHSV was established, that exposure to low concentrations of WSFO did not significantly affect mortality of juvenile or adult herring to VHSV *via* water-borne challenge. The response of adult herring in the current study was contrary to the findings of Carls *et al.* (1998) in which mortality of adult herring due to VHSV, and VHSV prevalence, was correlated with exposure to weathered crude oil. Carls *et al.* (1998) suggested a causal association between oil exposure, expression of VHSV and mortality. It was proposed that oil exposure might activate VHSV in sub-clinically infected and asymptomatic carrier fish. It is possible that differences in the toxicity of the PBCO WSFO mixture that I used *vs.* the

weathered crude oil mixture could account for the contradictory response of adult herring between the Carls *et al.* (1998) study and the current study.

3.5. CONCLUSIONS

The factors: fish age and size; sample schedule; WSFO concentration; variation in holding time; sample size; and the selection of variables evaluated all appear to affect whether or not a significant difference in the immunotoxicological response was detected. Therefore, caution should be used when applying these results to herring from other populations, or of a different age class, as it is evident that demographic differences can be anticipated. These experiments validate, for herring, the need to consider immunotoxicological results in the context of the specific factors associated with the fish, the toxin and the conditions under which the experiment was conducted.

An identical suite of variables for each population was not evaluated. Therefore, it is not known whether variables that differed with WSFO exposure, but were not evaluated in all experiments, varied consistently across populations and, or, age classes. For instance, ion concentrations were not tested in the juvenile herring due to the small size of juveniles and a lack of plasma availability, but they were statistically significant in the adult WSFO-exposed BS herring. In future studies, electrolytes could be evaluated in juvenile and QI herring to determine if they change with WSFO exposure and to comprehensively include or exclude them as indicators of immunotoxicological change.

In conclusion it can be stated that:

1) There were few consistent changes in variables with respect to exposure status, magnitude or timing. However, statistically significant associations with WSFO exposure were evident in both age classes of herring that were biologically plausible.
2) The lack of a consistent response was a critical limitation in making causal conclusions about the effect of WSFO on the immune response of herring, because most statistical associations were not replicated between experiments.

3) The statistical differences across experiments were suggestive of episodic stress responses (alterations in plasma biochemistry, plasma lysozyme and phagocytosis) in association with WSFO exposure. Additionally, the high background plasma

glucose concentration in the adult herring infers chronic stress in captive adult herring.

4) Of all the variables measured, only plasma lysozyme, lactate and phagocytosis changed significantly in association with WSFO exposure alone, or in combination with VHSV challenge, in both juveniles and adults. However, the direction and magnitude of change were not consistent for plasma lysozyme and the decrease in lactate in adults may be attributable to a size difference as plasma lactate was negatively correlated to length, which differed in this case among control and WSFO-exposed fish. In addition, phagocytosis increased with time in both control and WSFO-exposed juvenile herring and in both control populations of adult herring.

5) The significant changes that occurred over time in control fish, which exceeded the T_0 95th PE reference ranges, identified variables that could be considered less stable. In juveniles, these were Hct and Lct whereas in adults these were plasma glucose, lactate, lysozyme and phagocytosis. Several variables showed an additional statistically significant interaction between holding time and experimental sampling time in both age classes (Hct, Lct, plasma glucose and lactate, and phagocytosis) supporting a captive time effect common to both juvenile and adult herring. There is a need for additional studies to discover the basis of these background changes with holding time in general and with experimental time.

6) The future use of VHSV as a challenge pathogen to assess Tier 3 of the immunotoxicological evaluation system should be done with caution due to the endemic nature of VHSV and subsequent immunity in wild herring populations. The likelihood that capture and confinement will precipitate an epizootic shortly after capture could be incorporated into the experimental design of future studies to utilize the captivity-induced VHSV exposure.

7) Knowledge of the effect of prior exposure to VHSV plus subsequent WSFO exposure and re-exposure to VHSV on other immunotoxicological variables was gained. Still, the exact relationship between prior VHSV exposure, immunity, re-infection *via* challenge, serum antibody and tissue viral titres, sub-clinical infection, and the impact of WSFO exposure on these aspects of herring immunity remains to be determined.

8) Experiments did not reveal a suite of indicators that predict immunological change to oil exposure.

9) In juvenile herring, WSFO exposure increased mortality against the background rate (control fish) but there was little additional mortality after termination of oil exposure or in association with pathogen challenge.

Chapter 4 - Concluding Discussion

The cautionary approach towards interpreting immunotoxicological results in wild Pacific herring exposed to crude oil: - what is the biological significance and relevance to *EV*OS?

"uncertainty is at the core of the scientific process"

(Cairns, 1992)

This research endeavoured to evaluate the responses of wild Pacific herring to sublethal concentrations of WSFO using a three-tiered suite of immunotoxicological variables commonly used in fish immunotoxicology (Anderson, 1990; Anderson and Zeeman, 1995; Zelikoff, 1993). This discussion chapter summarizes the key issues encountered throughout this study as well as provides direction for future, related research. The chapter is divided into separate sections that address the following subjects: variation and normality in wild fish, statistical *vs.* biological significance, the applicability of the immunotoxicological approach, the implications of WSFO exposure on immune function, on individual fish, on populations and on the relevance to the PWS declines, disease and mortality in wild fish and future recommendations. The questions that this research strived to answer are restated at the end of the discussion for each section along with comments on whether or not they were answered by the results of this research.

4.1. Variation and normality in wild fish

Understanding the inherent variation in basic biological measures in fish is critical to comprehending the importance of changes in these variables with toxicant exposure (Elston *et al.*, 1997). Pacific herring are a pelagic species that can currently only be obtained from the wild. While SPF herring have been successfully reared at the MFS laboratory, it was not feasible to either transport any of these fish to BMSC, or to use them at MFS for my experiments or to rear them at BMSC. As an experimental species, wild-caught herring lack the uniformity that is commonly seen in captive-bred species such as rainbow trout. The wide range of variability, evident in most of the measured variables, was evidence of this lack of homogeneity. For example the 95th PE range in Hct, for adult herring was shown to be as wide as 5.3 to 59.1 % (Chapter 2) compared to 32 to 45% in rainbow trout (Barham *et al.*, 1980) and the correlation analysis showed it was not simply related to overt disease states and was unlikely methodological. In Chapter 2, inherent variability was evaluated through characterization of the distribution, range and magnitude of variation in the variables measured by examining the control fish.

Factors affecting the variability of these parameters were identified and included age, size, gender, the presence of gross pathological lesions, holding time and experimental sampling time. The influence of these factors on the measured variables was examined. The resulting 95th PE reference ranges were generated with the intent to aid in assessing the relevance of statistically significant changes in treatments *vs.* controls and

the impact of time on variables. Under my experimental conditions, I could not find a stable and narrow expected range for most variables and it appeared that age, size, gender and prevalence of lesions (juveniles only) were associated with the range and median. These factors warrant further study to elicit the underlying causes and define the consequences of the degree of variation.

The adjective normal has several definitions that depend on the circumstance in which it is used. Dictionary definitions of normal include:

"1. agreeing with the regular and established type
2. in the context of infectious disease, not immunized or infected
3. statistically speaking, the values of a variable follow a bell-shaped distribution."
(Blood and Studdert, 1988)

In biological terms, normal is always context specific rather than an absolute value meaning that the reference ranges I established in this study are applicable to PNW herring of similar age and size when held under similar captive conditions. It was not possible therefore to extend these absolute values and expect them to be normal for herring in the wild. It was possible though to determine how WSFO exposure affected measures of central tendency and the spread of the IQR by comparing control and exposed fish under the same conditions. This study attempted to identify what constitutes the "regular type" for herring in order to be able to classify responses to time or treatment as "normal" or not.

The use of the 95th PE method to set reference range boundaries resulted in wide ranges for most variables. However, the non-normal distribution of most variables precluded the use of the Gaussian distribution (mean $\pm$ 2 SD) which would have narrowed the range for many variables. The use of this range would have been biased when Gaussian assumptions such as normality were not met. Therefore, the non-parametric 95th PE was used as it is applicable regardless of the distribution of the sample population (Miller *et al.*, 1983) and provided a more comprehensive picture of the degree of intraspecific variation evident in wild herring. Others have reported the IQR (25th and 75th percentiles) to define reference ranges (Harms *et al.*, 2002) but this does exclude 50% of the sample population and thus excludes much of the natural variation in the case of herring. Nevertheless, both the 95th PE and the IQR were determined and reported for completeness and future reference against other species in which only the IQR is reported. Even so, the net effect of using the 95th PE *vs.* the IQR

to assess time and treatment effects was minor in terms of the number of additional statistical changes that fell between the 2.5th and 25th percentile or the 75th and 97.5th percentile.

The intraspecific variation in herring was in part attributable to captivity effects given the degree of variability in control fish with varying holding periods and over the course of experiments. The populations used in the current study may have been under sufficient stress from captivity to exceed the threshold of stability for the variables examined. In which case, alterations in indicators of immunological status following WSFO exposure would need to have been of sufficient magnitude to over-ride individual variability in order to potentially have an impact on function and overall well-being. To definitively test if captivity had a significant impact on the reference ranges it would be necessary to sample herring for the hematological, biochemical and immunological variables immediately after capture. This would eliminate the influence of primary and secondary stress effects due to transport, prolonged handling and long-term captivity and may produce different 95th PE reference ranges.

Conversely, capture stress may also contribute to variability and it may be that reference ranges would still be large for all variables as was the case in the PWS herring biochemical parameters which were sampled onboard the collection vessel (Marty *et al.*, 1998). To further investigate whether wide reference ranges are "normal" in wild fish, other wild, marine fish species could be sampled, both after varying periods of captivity and immediately following capture, to provide a comparison. The greater variability in captive herring compared with other captive fish populations may be largely due to the negative effects of captivity on these wild populations. Therefore, until the basis for this variability is discovered and new holiding methods are developed, their continued use as an experimental animal has to take this variation into account.

"What constitutes the representive Pacific herring in terms of the central tendency and variation of hematological, biochemical, and immunological status?"

This question was partially answered by examination of the effect of demographic factors, disease status indicators, and time on the degree of variance of selected hematological, biochemical, and immunological variables. Reference range profiles were determined for both juvenile and adult herring that can serve as guidance to those using herring of a similar age, size and health status within the PNW. However, due to

the range of factors shown to influence the variables examined, no single definition of a "normal" Pacific herring can be determined. Instead it can be stated that the 95th PE range to be used, which should encompass 95% of captive herring, is dependent on fish age, size, lesion status, gender, and holding and experimental sampling time to varying degrees. As such, reference range profiles were generated separately for T_0 (baseline fish) and T_P (pooled controls) and used as appropriate depending on whether or not a given variable differed significantly with T_E. The captivity effect in control and treated fish was evaluated against the T_0 range, subdivided by T_H as necessary. Treatment effect was assessed against the T_P for variables that were stable over T_E. For variables that did change with T_E the 95th PE range of the time-matched specific control fish was used to control for the effects of captivity time alone as the T_0 range would not have adequately represented the "normal" range.

Future work could focus on quantifying the difference in captivity effect with time between control and exposed fish. For example, if the control value for a variable at a given time interval is within T_0 95th PE and the exposed value at the same time interval is not it implies, in the absence of a statistically significant difference to support a treatment effect, that there is a difference in captivity effect as the magnitude of change relative to baseline is different.

4.2. Statistical *vs.* biological significance

Much of the existing knowledge of the effect of toxicants or pathogens on fish hematology, biochemistry, and immunological variables has been limited to documenting a statistically significant physiologic effect but does not integrate the biological significance of changes in response variables (Adams, 1990; Heath, 1995). Biological significance means that a change in a performance variable has the potential to affect growth, fecundity, or survival (Heath, 1990; Beyers *et al.*, 1999a), and extends to the population (Moore, 2002).

This study was unable to find a consistency in the changes in the measurement variables that would contribute to a decrease in survival. To be meaningful, a change at the cellular level needs to translate to an effect upon a function, such as immunological performance and ability to resist pathogens. For example, to assess if an elevation in plasma glucose is detrimental, the threshold of change in glucose above baseline levels, above which point there is a high probability of association with a change in a specific

function of biological importance for fish, needs to be known. These thresholds are not yet known for herring, and few have been established for other fish. Therefore, the criteria for determining the relevance of a specific statistically significant change among groups of fish with toxicant/pathogen exposure was based on change beyond the 95th PE reference range, or the presence of supporting concurrent changes in at least one other variable, or change occurring consistently in more than one experiment, or was documented as 'relevant' in the fish immunotoxicological literature with other fish species showing a similar environmental stressor/toxicant exposure effect. Table 7.1 summarizes the statistically significant changes and highlights those that were also considered to be biologically relevant based on the above criteria.

Nevertheless, there was no apparent WSFO concentration-response pattern within the range of concentrations used and there was no consistent response within and between experiments. The magnitude, lack of persistence, and lack of consistent sequence in the timing of the changes seen in the Tier 1 and 2 variables following WSFO exposure were also insufficient to support a causal effect due to WSFO. Therefore, it can not be stated that WSFO caused the observed differences between treatment groups. However, WSFO exposure may have been a factor in the greater cumulative mortality (Tier 3) of WSFO-exposed and WSFO/VHSV-challenged juvenile herring in the time series experiment. Still, the experiment needs to be repeated with additional pathological evaluation of all dead fish to rule out other pathogens before concluding that WSFO caused mortality.

"Does sub-lethal exposure to WSFO lead to meaningful changes in immune function in Pacific herring?"

Sporadic statistically significant changes were detected in both juvenile and adult herring that suggested a possible episodic secondary stress response (glucose and lactate in juveniles; Hct and chloride in adults) and macrophage function was altered in juvenile fish exposed to WSFO/VHSV concurrently. These changes were generally of small magnitude though, and none of the variables that changed significantly in WSFO-only exposed fish exceeded the 95th PE reference range. This implied that despite differences between WSFO-exposed and control fish being statistically significant, and the differences being biologically plausible for effecting a functional impact, that the degree of variation seen in most instances was to be expected for captive herring. However, the high background median plasma glucose observed in adults is suggestive

of a chronic captivity stress which may have precluded dectection of changes in glucose in association with WSFO exposure. Further work to elucidate the acute and chronic stress reponse of herring is required before generating a definitive conclusion regarding their captive stress response and the subsequent effect on immune function.

Juveniles exposed to WSFO plus *V. anguillarum* showed elevation of Lct in the 80 g WSFO group and elevation of antibody titre in the 40 g and 80 g WSFO/*V.anguillarum* fish that exceeded the 95th PE. Therefore, this shows that the statistical boundaries set to restrict a reference range govern which variables will exceed or fall below the range and be deemed "abnormal". The fact that relatively few variables changed from the spread observed in control fish, even when 50% of the variation in controls was excluded suggests that (a) there was still a large range within the 25th to 75th percentile range or (b) that the magnitude of changes associated with WSFO exposure were minimal for most statistical differences.

There were statistical changes associated with WSFO exposure that were outside the IQRs. These included the increase in total plasma protein and lysozyme in juvenile herring, the decrease in A/G and increase in thrombocytes, and the decrease in plasma chloride and lactate after the recovery period in adult herring. The additional changes that exceed the reference range boundaries with the use of the IQR *vs.* the 95th PE range illustrates the impact of excluding 50% of the variation from the population *vs.* excluding only 5%.

4.3. Immune function implications

In juvenile herring, it could only be concluded that WSFO was a contributing factor in the elevated mortality rate in WSFO-exposed fish. Dead fish were only tested for VHSV tissue titre to evaluate their VHSV infection status but the infection status of other pathogens was not known. In addition, fish exposed to both WSFO and challenged concurrently with VHSV had elevated phagocytotic activity that was suggestive of transient immunostimulation.

Table 4.1 Summary of statistically significant changes with respect to the direction of change, timing of change and age of herring. Arrows signify the direction of change; age is indicated as J= juvenile (0+year), A= mature adult. Where two entries exist for "day," the first refers to time since initiation of WSFO exposure and the number in parentheses refers to time since pathogen challenge. Highlighted cells indicate that there was more than a single statistical change at a given time point/the change(s) occurred over >1 experiment for the population and highlighted plus number boxed indicates the change in the median value was greater than the 95th PE reference range. *=Proportional mortality. #=Size difference existed. TP = total protein Δ = change

Variable	WSFO			VHSV			WSFO/VHSV			*V.anguillarum*		
	↓/↑	Day	J/A	↓/↑	Day	J/A	↓/↑	Day	J/A	↓/↑	Day	J/A
CF	↓	7	J	↓	10	A	↓	14	A			
LSI	↓	7	J	↑	21	J						
SSI	↓ ↑	7 21	A A									
GSI	↓ ↑	21 63	A A									
Hematocrit (%)	↑ ↓ ↑	4 21 77	A A A	↑	56	A	↑	77 (56)	A			
	Modality ΔJ											
Leucocrit (%)	Modality ΔJ			↑ ↑ ↓	10 42 56	A A A				↑	70	J
										Modality Δ J		
White blood cells	↑	6	A									
	Thrombocytes											
Glucose	↓ ↑	14 21	J J	↓	7	J	↓	14 (7)	J			
Lactate	↑ ↓ ↓#	4 63 70#	J J A#									
Total protein	↑	21	J									
Albumin	↓	21	A									
A/G	↓	21	A									
Chloride	↑ ↓	21 63	A A									
Phosphorus	↓	21	A									
Calcium	↓	21	A									
Lysozyme	↑ ↓ ↓ ↑#	4 28 49 70#	J A A #	↓	49 (35)	A	↓	49 (35)	A			
Vibrio titre										↓	70	J
Respiratory burst	↑	70	A									
Phagocytosis	↑ ↓ ↓	21 28 70	A A A				↑	14 (7)	J			
Mortality	↑	14 - 42	J	↑	7*	J	↑	35 (28)- 63 (56)	J			

BS juvenile herring exposed to WSFO were susceptible to *V. anguillarum.* In addition, a simultaneous significant difference in both antibody titre and Lct following WSFO exposure and *V. anguillarum* challenge occurred. These alterations had the potential to be immunologically important as both variables are indicators of ability to fight pathogens and can be altered when fish are infected (Angelidis *et al.*, 1987; Jansson and Ljungberg, 1998; Alcorn and Pascho, 2002). The low incidence of clinical VHSV in juveniles exposed to WSFO and challenged with this virus supported prior exposure to the pathogen as all naïve herring are susceptible to VHSV (Kocan, pers. comm., 2004). Similarly, the general lack of response to challenge with *I. hoferi* also suggested prior exposure and immunity to this pathogen.

In both the *V. anguillarum* and VHSV challenge experiments, it was suspected there had been prior natural infection with each pathogen respectively due to the response of the control fish. If this was true then it appeared that the immunity of juveniles that survived the prior pathogen exposure was better against the virus, VHSV. It also implied that immunomodulatory changes (differences at Tier 1 and 2) due to WSFO were insufficient to affect cell-mediated immunity (CMI), the key component of defense against viral pathogens, but were impacting humoral-mediated immunity (HMI) which is more involved with the defense against bacterial pathogens. Evaluation of the specific immune response of rainbow trout vaccinated against *V. anguillarum* supports the protective role of humoral-mediated immunity against bacteria (Palm *et al.*, 1998). The differences in immunological response to challenge pathogens suggests that in juvenile herring, acquired CMI against viral pathogens is stronger and more effective than HMI against bacterial pathogens in fish of the same age. VHSV can result in high mortality when a population experiences stressors capable of decreasing immune surveillance (Carls *et al.*, 1998). Therefore, early, strong, immunity against VHSV in juveniles surviving an epizootic may be necessary for the population to endure.

WSFO exposure had no impact on mortality in BS or QI adult herring in any of the trials, alone or in combination with VHSV challenge. Therefore, the statistical changes in plasma biochemistry, lysozyme and phagocytosis that may have indicated a transitory, secondary stress response at Tier 1 and 2, which occurred in association with WSFO exposure, were of insufficient degree to have an impact on survival and did not increase disease susceptibility in adult herring. However, the possibility that WSFO exposure

increased the relative risk for future mortality cannot be ruled out as sampling did not extend beyond 77 days post-exposure/challenge and post-experimental, long-term mortality was not assessed.

4.4. The applicability of the 3-tiered approach in this study

The use of this approach to assess toxicant effects on the immune system of herring revealed some advantages and limitations. The theoretical advantages include examination of toxicant exposure at varying levels of biological organization (cellular, organismal). In addition, the assays assess the functional effect of a toxicant *in vivo* thus providing a more comprehensive evaluation than *in vitro* assays that expose only cells or tissue to a toxicant (Wester *et al.*, 1994; Anderson and Zeeman, 1995). Limitations of the approach were due to constraints surrounding experimental design and sampling but others were inherent to the study species. Sample sizes were relatively small and inconsistent which placed limitations on the type of data analysis that was possible, lowered statistical power, and prevented a complete characterization of biological variation. Small blood volume limited the suite of indicators that could be applied in several experiments, thus preventing a full comparison of effects between ages and populations for each variable. It was also possible that some of the variability in the variables measured could be attributable to methodological measurement error, and subjectivity surrounding macrophage cell counts and differential WBC counts. However, the greatest limitations were likely the unknown infection and exposure history associated with wild herring and the identified captivity effects.

Inherently, wild fish populations are a "mixed-bag" in terms of their immunological competence, their previous exposure history, and their response to captivity. To be a useful tool, the pathogen challenge test (Tier 3) needs to be applied to control and challenged fish with the same immune stressors and immunological status. The challenge pathogen also needs to be reliably capable of inducing clinical disease, as a significant difference in mortality is the ultimate test of whether or not exposure to a xenobiotic such as crude oil has an effect at the organismal level. If the groups being challenged have a different immunological starting point then it is difficult to attribute any differences in disease, antibody titre, or mortality to the effects of a toxicant alone. Therefore, given the endemic nature of VHSV, the possibility of immunity prior to experimental challenge and the lack of clinical disease seen in the challenges in this study, it seems reasonable to question the use of VHSV in future pathogen challenge

tests designed to evaluate the Tier 3 immunological response of wild Pacific herring under captive conditions. Furthermore, the use of the pathogen challenge test in wild fish, such as herring, must recognize that differential immunological states might exist between treatment groups prior to the initiation of experiments that could affect conclusions. The solution here might be to evaluate immune status *via* serum antibody titre, in a non-terminal manner, prior to initiating the exposure and challenge and to re-test serum antibody titre and also tissue viral titre again when terminal sampling occurs. This may allow differentiate between carriers and actively infected fish but optimizing a protocol to reliably anesthetize and recover herring would be essential.

In this study, no single variable was affected consistently by exposure to WSFO. As well, there was no association effect between the tiers of the immunotoxicological suite of indicators in relation to WSFO exposure. With the suite of variables used, thrombocytes was the only one (in WSFO-exposed adults) that exhibited a change of sufficient scale to supersede the effects of inherent variation within the population. In addition, small sample sizes and variation from measurement precision added to overall variation, thus obscuring any small-scale differences due to WSFO exposure. Consequently, with the experimental design and herring used, large effects due to WSFO exposure would have been necessary to distinguish between real and random effects. Accordingly, it cannot definitively be concluded that WSFO had no effect but that the high level of variation (from all sources) may have obscured detection of subtle effects, and made it impossible to identify variables that were consistently predictive of exposure to WSFO in herring. Nonetheless, if subtle physiological effects do not have any survival effect (i.e., in rainbow trout plasma lactate must be > 12.2 mmol/L to decrease repeat swimming, Jain and Farrell, 2003) then ultimately the biological significance of any masked, slight effects would be minimal in terms of mortality.

It would appear that the use of the Tier 1 and 2 variables to assess response following a pathogen challenge (Tier 3) are best used to establish differences in the health of surviving fish and associations with the Tier 3 challenge agent. The probable prior exposure to pathogens, known or unknown, in wild herring populations used for experimental challenges made it difficult to attribute any significant differences observed in immunotoxicological measures solely to the challenge pathogen. This was evidenced by the failure to induce clinical VHS in pre-spawning adults that were 78% *I. hoferi*

positive and that appeared to be resistant to VHSV and in both WSFO/VHSV challenges. It was also evident in the 0+year herring challenged with *I. hoferi* that were survivors of an epizootic of VHS shortly after capture.

The development of disease subsequent to an infection from challenge with a pathogen depends on factors of the host, the pathogen, and the environment. In these experiments, the failure to induce clinical disease appeared to be primarily due to host immunity factors. The pathogens used (VHSV and *I. hoferi*) had previously been shown to be virulent in SPF herring at the doses used (Kocan *et al.*, 1997, Kocan *et al.*, 1999), although this fact was established after the current experiments had started. The water temperatures during the pathogen challenges were within the optimum ranges, therefore, it was unlikely that the failure to induce clinical disease can be due to improper water temperature. However, the inability to induce clinical disease following a challenge is in itself a measure of the immunocompetence of the population being challenged. Exposure of these same fish to a different pathogen could have had a different outcome.

"Does the immunotoxicological approach lend itself to the assessment of the impact of xenobiotic exposure on the immune status of Pacific herring?"

Due to the degree of variation inherent to herring in the variables evaluated, the small sample sizes, and the limitations of the study design it was not possible to comprehensively determine if this immunotoxicological approach was appropriate or not. Tier 3 challenge using *V. anguillarum* following WSFO exposure did result in suppression of antibody titre (Tier 2) compared with juvenile fish only challenged with the pathogen but not in a WSFO-concentration dependent manner. This suggests that the immunotoxicological approach to assessing xenobiotic effect can assess immune response. However, in the VHSV challenges, probable prior immunity to VHSV impeded an accurate assessment of this methodology. The extent of the biological variation seen in herring in this study made it hard to distinguish between real and random effects. Age, size, gender, the presence of skin lesions in juveniles, differences in holding time and differences in experimental sampling time were all factors identified as having an impact on the variability of the selected measurement variables. Therefore, future research must consider and/or control for these effects in design and analysis.

4.5. Individual fish health implications

It has been shown that oil can result in a general stress response, which may be adaptive or detrimental (Heath, 1990; Nolan *et al.*, 2003). The current experiments showed that exposure to sublethal concentrations of WSFO did not have an adverse effect on the immune system of adult herring. Juveniles exhibited changes in some Tier 1 variables indicative of secondary stress effects, decreased immune response, and increased cumulative mortality in association with WSFO exposure. Variability in immune function of fish following toxin exposure has been associated with age in other species. For example, juvenile Japanese medaka were more susceptible to adverse immune function effects following exposure to PCB 126 (Duffy *et al.*, 2002). Therefore, in juvenile herring, WSFO exposure may be detrimental depending on the duration of the exposure, immunity at the time of exposure and the specific pathogens encountered.

Several of the significant changes in variables supportive of a transitory secondary stress response with time (Hct, Lct, glucose, lactate, plasma lysozyme, and phagocytosis) exceeded the respective 95th PE reference ranges concurrently in control and WSFO exposed fish suggesting an underlying captivity effect that may be inevitable in captive herring. Similar captivity stress has been seen in other fish species (Torres *et al.*, 1986). Of the variables measured, none remained consistently altered through all subsequent sample points to support association of a chronic effect due to WSFO exposure in the sample populations. However, several variables that peak at different times need to be measured concurrently (plasma cortisol, lactate and glucose) to comprehensively assay the classical primary and secondary stress response. Furthermore, measurement of ACTH and α-MSH may allow bettercharacterization of the background level of acute and chronic stress responses in captive herring. This study suggests that captivity had a greater impact on herring health than exposure to WSFO given the response of both controls and WSFO-exposed fish with time, the differences seen due to variation in holding time in controls and the change in both controls and exposed fish due to time that exceeded the 95th PE range.

With regards to the herring used in the present study, the lack of knowledge of past exposure and experience history implied lack of knowledge of their stress tolerance capacity. It was also possible that physiological compensatory mechanisms masked the true degree of the stress response (Adams, 1990). This can be expected to be

especially true for wild populations where predation is a considerable cause of mortality (Hourston and Haegele, 1980). In herring, predation mortality may increase in association with epizootics (Quinn *et al.*, 2001) due to the culling of diseased fish. This implies that epizootics result in decreased fitness which should be detectable physiologically. It is possible that the "fitness" variables measured in the current study were not correct for herring.

4.6. Population level implications

For the WSFO exposure to have implications at the population level, the changes seen in disease resistance at the individual level need to be linked to measures of population performance and success such as mortality rate. The changes seen in juvenile herring suggest that exposure to sub-lethal WSFO could decrease the antibody response against *V. anguillarum* if the population encountered the pathogen shortly after oil exposure. This may affect the population by lowering the number of surviving herring of that year class if the reduction in antibody titre renders the fish incapable of recovering from infection. However, the threshold for effective antibody levels is not yet defined for herring to the best of this authors' knowledge. From the results of this study alone, the same cannot be stated for VHSV due to the uncertainty surrounding the mortality of herring exposed to WSFO plus VHSV. Exposure of adult herring to WSFO, either alone or in conjunction with VHSV, had no significant impact on mortality. This was to be expected given that immunity to VHSV from probable prior exposure existed. Therefore, this level of sublethal WSFO exposure did not increase susceptibility to VHSV in captive herring. Thus, based on the experimental results, an impact at the population level would not be anticipated. However, the pre-experimental mortality, 18.5% in adults, and 10.7 to 27.1% in juveniles likely resulted in more resistant sub-groups remaining for the WSFO exposure and pathogen challenges. Therefore, it is possible that similar exposures in the wild could have a greater impact, as the less resistant fish might still be present.

The wide range of variation in the variables measured for herring may be an indicator of stress at the captive population level. In brook trout, the effects of a sublethal exposure to toxaphene on growth and development were examined using a population dynamics model to analyze for population-level stressor effects (Power, 1997). It was shown that once a threshold of stability was crossed, with respect to mechanisms that offset mortality due to stressors, that variability in the abundance of adults increased as a

function of stress at the population level (Power, 1997). In juvenile herring, the lack of sustained elevation of glucose in WSFO-exposed, VHSV-challenged and WSFO/VHSV-challenged fish may support an inability to maintain a secondary stress response or conversely may indicate they were better acclimated to the effects of captivity than control fish due to the effects of WSFO-exposure and/or VHSV challenge. In this respect, general stress can be viewed as an adaptive response at the individual fish level rather than an adverse event (Weyts *et al.*, 1999; Nolan *et al.*, 2003).

Stress at the level of the population, such as exposure to an oil spill, can occur from either changes in the environment's carrying capacity or changes in the innate capacity of an average population member to survive and reproduce in the absence of intraspecific competition (Shuter, 1990). Habitat occupation, individual well-being, and the balance between recruitment and mortality rates are the primary population characteristics that determine if a stressor like WSFO will have an impact. The presence or absence of pathogens (such as VHSV and *I. hoferi*) and immunity status are factors that contribute to individual well-being. Compensatory mechanisms in wild populations are generally concentrated at the start or end (after sexual maturity) of the life cycle, and the sensitivity of a population to any given stressor depends on when in the life cycle the compensatory forces occur (Shuter, 1990). The results of this research suggest herring may be most at risk at the start of the life cycle. In general, juvenile herring showed changes associated with stress in other species (greater changes in plasma glucose and lactate), whether from captivity, WSFO exposure, or pathogen challenge more often than adults. It is possible then that juveniles exposed to WSFO or pathogens may have greater potential for population level effects than similar exposure of adults if the changes associated with stress exceed thresholds that are detrimental to survival .

"Can the results from captive PNW herring be extrapolated to explain the population declines in PWS herring?"

The concentrations of TPAH used in this research were comparable to those reported from PWS in the weeks following the *EV*OS (Table 3.1). While the duration of exposure in most experiments approximated the likely exposure period for adult herring in PWS, exposure duration was shorter than the likely exposure of juveniles in PWS. However, exposures of durations any longer than were employed would have been truly problematic given the issues of change with time within control fish, and the level of experimental mortality in most instances.

With the age of fish used (primarily 0+year and adults), and the results obtained, it was not possible to determine with any certainty if the PWS declines and increased prevalence of disease could be attributed to the *EV*OS. WSFO exposure may be a contributing factor, but it needs to be integrated with much broader evaluations of population level responses of herring to environmental disturbances. Stressors, such as oil, do not occur in isolation of other abiotic and biotic environmental components. Factors such as capture, nutritional status, other disease, and spawning likely contribute to the periodic epizootics of VHSV that occur in wild herring. From the pathogen challenge experiences with VHSV both at BMSC and MFS, it would seem unlikely that VHSV would lead to chronic immunosuppression given that fish surviving the captivity-induced epizootics appear to have strong immunity against re-infection. It is feasible, however, that VHSV, alone or in conjunction with other environmental factors and pathogens, could have played a significant role in the PWS declines given the high mortality associated with VHSV epizootics. In addition, the existence of the closed pound spawn-on-kelp fishery in PWS has the potential to amplify the prevalence of VHSV and to further contribute to episodic VHSV epizootics (Hershberger, *et al.*, 1999).

4.7. Disease and mortality in wild fish

Several factors complicate assessing disease in wild fish. One of the difficulties encountered is the lack of exposure history to xenobiotics and to pathogens (Secombes *et al.*, 1991). Indeed, a lack of detailed knowledge of the immunological status of wild herring added a layer of uncertainty to this study. The PS herring used in this study were all survivors of documented laboratory epizootics of VHS (Kocan *et al.*, 1999, 2001a). Therefore, this confounded the results of the pilot juvenile *I. hoferi* challenge and prevented induction of clinical or subclinical VHS in adult fish as they had already acquired immunity. The presence of acquired immunity to VHSV likely also occurs in wild herring populations.

It was also suspected that confinement-induced VHS accounted for some of the higher preexperimental mortality rates (34.2 to 81.6%) seen in the herring held at BMSC despite the initial negative screening results. If this were the case, then the survivors were either immune carriers or sub-clinically infected fish at the time of the experimental challenges. Effectively, this may have lead to selection of a more resistant sub-sample of captive herring that were subsequently used for the experimental exposures (WSFO and pathogens). This absence of knowledge of prior exposure for the various herring

populations used in this research made it difficult to know if fish were naïve, subclinically infected or immune to challenge pathogens and whether previous exposure to xenobiotics, such as petroleum hydrocarbons, had led to acclimation.

Disease can be defined as a continuum from acute mortality to benign or inconsequential syndromes that share a departure from the normal structure and function of the host, the severity of which is determined by factors affecting the host, pathogen, and environment (Hedrick, 1998). Disease has also been defined as any impairment that interferes with, or modifies, the performance of normal functions, including responses to environmental factors such as toxicants and climate, nutrition, infectious agents, inherent or congenital defects, or any combination of these factors (Wobeser, 1981). Hedrick's (1998) modification for wild fish of the web of causation for diseases put forth by Wobeser (1994) is a comprehensive integration of the multitude of factors affecting the host, pathogen, and environment. These factors span a wide range and include host population density, habitat deterioration, water temperature, dissolved gases, toxins, stress, pathogens, impaired immunity, reproductive and nutritional status, altered behaviour, inter/intraspecific competition, hatchery/wild fish interaction and human intervention (Hedrick, 1998). Therefore, the problem of establishing causation between disease and toxicant exposure in wild fish populations is compounded by a multitude of environmental and ecosystem level factors in addition to host and pathogen factors.

Many fish are carriers of pathogens and only become symptomatic, and therefore more easily detected, when the fish is under stress (Anderson, 1990). In addition, populations of fish consist of subsets that differ in their susceptibility to any given pathogen (Reno, 1998). Determining which host, pathogen and environmental factors are important, how best to measure them and how to interpret the results remains a challenge (Espelid *et al.*, 1996). Although difficulty does exist in determining causative links between disease, pollution, immunosuppression, and environmental stress in wild fish populations (Sindermann, 1993, Vethaak and Jol, 1996), it may be possible to use fish that have exposure/infection during a known epizootic to test the impact of pathogen and environmental stressors.

Ideally, the immunological response (serum/tissue antibody titre) to pathogens endemic to the fish species being studied should be documented prior to conducting a pathogen challenge to establish if there are active infections or immunity. This would provide more certainty regarding the influence that natural pathogen exposure may have on any differences in the mortality and variables measured following experimental pathogen challenge. To obtain the initial immunological response of herring to a pathogen it is ideal to use a naïve population. The gold standard is to use SPF individuals, which are laboratory-raised from eggs under barrier conditions to prevent exposure to pathogens. While, this artificial situation does not represent the true situation for wild herring, it allows primary evaluation of how a pathogen affects herring in the absence of other confounding immunological factors.

4.8. What is the relevance of exposure of captive wild Pacific herring to sublethal concentrations of crude oil to the *EVOS* ?

The WSFO exposure concentrations used in this study resulted in comparable water TPAH concentrations as reported in PWS following the *EVOS* (Table 3.1). In fact the water TPAH concentrations were higher than those reported for the *EVOS* at the peak of the experimental exposure and could be considered to have represented a worst case scenario for exposure following a spill. As previously stated, the duration of exposures mimiced the exposure in PWS for adults but not as well for juveniles which were potentially exposed for several months. Consequently despite using the maximum possible WSFO exposure concentration for almost a month, at TPAH concentrations relevant to the field *EVOS* exposure concentrations, no major effects upon the variables measured occurred. The mortality that occurred both during the pre-experimental and experimental periods in this study may have represented a culling of the population, such as might have happened with the *EVOS* and with reported epizootics world-wide. From this perspective, although my reference ranges and results are specific to PNW herring held under certain captive conditions in absolute terms, the relative results obtained from the PNW herring may be an indicator of the probable impact of sublethal exposures in PWS following the *EVOS*.

4.9. Recommendations for future fish studies

In future fish immunotoxicological studies, additional measures of overall organismal function such as swimming performance, schooling and feeding behaviour, predator avoidance, reproduction, and growth could be included as adjuncts to cumulative mortality following pathogen challenge depending on the specific goals of each study.

Incorporation of these physiological performance measures into the existing immunotoxicological tiered framework may identify correlations between altered physiology and changes in Tier 1 and 2 variables with toxicant exposure that have the potential to affect survival. This could then provide a clearer picture of the biological significance of exposure to toxicants like sublethal concentrations of WSFO. In addition, it may be possible to link concentration response results with bioenergetics modeling to determine the cost of exposure on the physiology, immunology and the energy budget (Beyers *et al.*, 1999b). However, in regard to captive herring, it is also possible that the intra-specific range of variation in additional measures of organismal function would be as wide as the range in the Tier 1 and 2 immunotoxicological variables evaluated in the present study. Nonetheless, if adverse immunological changes at the cellular and biochemical level of organization can be consistently predicted by changes in behavioural characteristics it would permit evaluation of toxicant exposure in a less invasive manner (Cooper, 1997). Beyers *et al.*, (1999b) advocate a bioenergetics-based stressor-response model (SRM) to evaluate multiple stressors. This provides a method to apply laboratory-generated relationships to field situations where natural conditions change with time. This method integrates the effects of natural variability and chemical exposure.

Tier 3 evaluation using pathogen challenges proved problematic due to the probable occurrence of epizootics prior to experimental challenge which resulted in challenges generally failing to produce clinical disease. This may always remain a problem given that VHSV epizootics often occur in wild herring within a week of captivity (Kocan *et al.*, 2001a). However, this "natural" challenge could be incorporated into the design of future studies that examine the impact of potential immunotoxicants so that the confinement-induced epizootic is taken advantage of. Measurement of the transport water titre, tank viral titre, tissue viral titre from dead fish and freshly killed fish, and serum antibody titre at more frequent intervals and measures of CMI would further elucidate these aspects of VHSV-immunity in herring, while also allowing an examination of the effect of WSFO exposure during an epizootic. The rapid development of VHSV within captive herring populations also speaks to not acclimating herring prior to toxicant exposure if VHSV is the pathogen of interest. Otherwise, by the time acclimation (one month or more) is over they will either have died from VHSV, be immune, or be in the process of developing immunity, which then complicates evaluating disease

susceptibility. In retrospect, it seems more prudent to take advantage of the presence of VHSV, as mentioned above to assess the combination of exposure to this pathogen and a toxicant.

To assess disease susceptibility following toxicant exposure it would appear that *V. anguillarum* is a better challenge pathogen than VHSV in captive herring. Antibody titre was a useful measure for assessing response to *V. anguillarum* but not for VHSV. Since completion of the current study, it has been shown that serum antibody titre is generally not found in immune VHSV carrier herring (Kocan *et al.*, 2001a). Viral tissue titre was not significantly associated with WSFO exposure and its absence likely indicated immunity to VHSV not the naïve state. As a tool to assess disease susceptibility, it appears that *V. anguillarum* is more likely to induce clinical disease in captivity, and therefore allows a means of testing proportional mortality. In contrast, the use of VHSV as a pathogen challenge agent is complicated due to the potential for captivity-induced VHS to have occurred prior to the challenge.

It was difficult to be confident that treatment effects were not confounded by differences in the pre-existing immune status between treatment groups. The use of SPF fish eliminates these possible confounders but is not realistic to the true situation for wild herring populations in which confounding factors are unknowable. This potential for variation in prior exposure to pathogens and toxicants in herring used in laboratory studies will always exist. Consequently, to better equalize these factors a much larger sample size for each treatment group is needed to permit evaluating WSFO effects under experimental conditions so that the chances of differential immune status influencing exposure results are minimized.

In final conclusion:

1) The application of a well-established immunotoxicological methodology for fish was not well suited to captive herring, which were found to have a large degree of intraspecific variation in the variables measured. This was an important conclusion of this study.

2) The degree of variability in most measurement variables, the changes in variables over time in response to captivity alone, and the level of mortality encountered prior to initiating experiments suggested that Pacific herring are not an ideal experimental fish for laboratory immunotoxicological studies. However, limitations on comprehensively defining variation did exist as a function of experimental design and small sample size which decreased statistical power. Without any *a priori* knowledge of the extent of variability in herring, duplicate tanks with 6 to 10 fish per sample were generally used. For future studies, an increase in sample size and replication should be employed based on determining the power required to detect treatment differences, with $\alpha=0.05$, in the face of wide intra-specific variation to lessen the risk of a type II error (falsely accepting the null hypothesis). If these changes were incorporated into a future study and the level of change in control fish over time were still evident, then it would be advisable to determine if the same effects occurred when herring are held in larger holding tanks as tank size may be an important factor. Knowledge of the influence of biological variation on reference ranges for the selected variables was gained and could be built upon.

3) There was a definite separation between age classes of herring supporting not combining fish of varying age for experiments. Also it would be preferable if size range in treatment groups is minimized. In future studies, it may be possible to improve the reference ranges by using greater numbers of herring that have been pre-selected for size and that are within very narrow size categories and that have been accurately aged (otolith and annuli determination) to control for the influence of age and size. This should produce a greater number of more refined reference ranges. Normally the acceptable variation in size within a treatment group is < 2 times the range but this may be too large for herring. Unfortunately though the additional handling to pre-screen for size could constitute another factor to control for. Therefore size screening would be best done at the time of initial distribution to tanks to miminize the degree of additional handling.

4) Exposure to WSFO alone and in combination with VHSV and *V. anguillarum* was associated with statistically significant changes in some Tier 1 and 2 variables for both juvenile and adult herring and increased mortality in juveniles in association with WSFO exposure. However, these changes were often not consistent in terms of

direction or magnitude of change but were biologically plausible for a potential physiological consequence and supported in the literature. Of all the variables measured, only a few can be recommended as being specifically informative of pathology and were not attributable to WSFO exposure. These variables are plasma lysozyme and Hct in the evaluation of juvenile herring with skin lesions; Lct and antibody titre after *V. anguillarum* challenge in juvenile herring. Several variables could be useful in future studies to evaluate the effect of captivity on herring and included Hct and Lct in juvenile herring, and plasma glucose, lactate, lysozyme and phagocytosis in adult herring. With regard to WSFO exposures, mortality may be the best method of evaluating exposure effects because it avoids measuring survivors and the resulting biases and effects this may have on the central tendency and distribution of variables.

5) The key question became whether or not the statistical changes in immunotoxicological variables were biologically relevant and meaningful for herring. As no precedent benchmarks existed to judge the relevance of these changes, the criteria established in this study to assess them were somewhat arbitrary. As such, these criteria may require future modification once more is known about the physiological consequences of reductions/elevations in the suite of variables, once thresholds of stability for each variable are known, and reference ranges are refined with larger numbers of fish. In particular, determining why such variability and bimodality exists for some variables and their relationship to mortality warrants further study.

6) Future herring studies could be done on a larger scale exposure using net pens to avoid fish bumping into the sides of tanks and to facilitate using schooling as a behavioural measure. This may permit exposures of longer duration, e.g. 3 months, which would more closely resemble the *EVOS* exposure for juvenile herring. However, sampling herring from net pens would be problematic and would likely result in additional logistical issues to ensure random samples were obtained.

7) There is a need to use a consistent holding time and experimental sampling times for comparisons between experiments and/or studies to eliminate the effect of time on measurement variables. In addition, obtaining sufficient baseline data, immediately

following capture and immediately prior to all future experiments is recommended to better characterize the effects of time. Additionally, comparison of control fish held in the larger holding tank concurrently with control fish in the smaller experimental tanks could be performed to determine if the changes in control fish over time is a function of tank size which could then be controlled for in subsequent experiments.

APPENDIX 1: ASSAY PROTOCOLS

1) Hematology Assay Protocols
2) Biochemistry Assay Protocols
3) Lysozyme Assay Protocol
4) Macrophage Cell Isolation Protocol
5) Phagocytosis Assay Protocols
6) Respiratory Burst Assay Protocol
7) Preparation of Bacterial Cultures for Disease Challenge
8) Serial dilution plates
9) Microbial Identification Tests
10) Antibody Agglutination Protocol (Plasma titre determination)
11) Cell Viability Counts and Cell Concentration Determination
12) Extraction of PAHs from Water Samples
13) Viral Assay
14) VHSV Challenge Exposure
15) Oil protocol
16) Culture of *I. hoferi* spores
17) Cell Media and Solutions

1) HEMATOLOGY ASSAYS

Introduction

In fish, Hct can be obtained using the centrifuge method to produce a packed cell volume, the percentage of the volume of whole, unclotted blood occupied by the red blood cells. It has been shown that a systematic and significant error is associated with the ordinal sampling of Hct capillary tubes (Korstrom *et al.*, 1996). To reduce this error, a maximum of 3 tubes of blood was collected per fish. The volume of the white cell layer that forms between the packed red cells and the plasma layer estimates Lct.

Materials and Supplies

Microcentrifuge
Microcapillary tubes (heparinized, 100,uL Critoseal (VWRbrand™)
Digital calipers (VWRbrand™)
Dissecting microscope

Protocol

1 A hematocrit tube (small, thin, glass, heparinized) was filled with blood from the fish and the end sealed with Critoseal™.

2 The sample was placed into a micro-centrifuge with the sealed end facing outwards, and the number of the slot recorded. The samples had to be balanced; therefore, a blank tube was filled with water to act as a counterbalance if necessary.

3 The lid was secured before starting the centrifuge to avoid sample loss.

4 The sample spun for a 3-minute cycle.

5 Once spun, measurements were taken using digital calipers and a dissecting light microscope. The limit of detection was to 2 decimal places.

6 Three measurements were taken from the tube, the entire distance from the end of the plasma column (clear/yellow) to the far end of the red column; the red column (the red blood cells); the small band of whitish cells between the plasma and red blood cells.

7 From these measurements, the Hct and Lct were determined as follows:

$$\text{Hct} = (\text{length of red cell column/total length}) \times 100$$

$$\text{Lct} = (\text{length of white cell column/total length}) \times 100$$

2) BIOCHEMISTRY ASSAYS

Materials and supplies

Multiplate Spectrophotometer (Bio-Rad® Microplate Reader "Benchmark")
(read to 3 decimal places for all assays)

Sigma Diagnostics kits

Glucose: (HK): Procedure No. NR 16-20 Chloride: Procedure No. 461
Lactate: Procedure No. 735-10 Albumin: (BCG): Procedure No. 631
Protein: Microtitre Plate - Standard Procedure (Bio-Rad)

Falcon brand 96-well plates Sterile pipet tips
10µL, 100µL, 200 µl Pipettors

Assay Protocols

a) Glucose

1 A 1:100 sample to reagent volume ratio was used and was incubated for a minimum of 5 min, to a maximum of 1 h. Optical density was measured @ 340 nm.
2 The following calculation was used to determine plasma glucose concentration:

Concentration (mg/dL) of sample =Δ A x TV x MW x 100/6.22 x LP x SV x 1000
Δ A = absorbance of sample at 340 nm minus absorbance of blank at 340 nm
TV = total volume (mL) MW = molecular weight of glucose (180.16)
LP = light path in cm SV = sample volume (mL)
6.22 = millimolar absorptivity of NAD at 340nm 1000 = converts µg to mg
(For SI units (mmol/L) multiply final concentration in mg/dL by 0.0555)

b) Lactate

1. A 1:100 sample to reagent volume ratio was used and was incubated for a minimum of 5 min to a maximum of 10 min. Optical density was measured @ 540 nm.
2 The following calculation was used to determine plasma lactate concentration:

Concentration (mg/dL) = absorbance sample/absorbance standard x 40*
* Concentration of lactate in standard = 40 mg/dL (For SI units multiply by 0.111)

c) Chloride

1 A 1:100 sample to reagent volume ratio was used and was incubated for a minimum of 5 min, to a maximum of 1 h. Optical density was measured @ 490 nm.
2 Use the following calculation to determine plasma chloride concentration:

Concentration (mEq/L) =(Absorbance sample - absorbance blank/absorbance standard - absorbance blank) x concentration of standard

d) Albumin

1 A 1:100 sample to reagent volume ratio was used and was incubated for 1 min or less. Optical density was measured @ 655 nm.
2 The following calculation was used to determine plasma albumin concentration: Concentration (g/dL) = (Absorbance sample - absorbance blank/ absorbance standard - absorbance blank) x concentration of standard

e) Protein

1 A 1: 20 sample to reagent volume ratio was used and was incubated for a minimum of 5 min, to a maximum of 1 h. Optical density was measured @ 595 nm.

3) LYSOZYME ASSAY (Ellis, 1990)

Introduction

This assay was used to determine the lysozyme concentration in herring plasma. It is based upon the lysis of the lysozyme-sensitive, Gram-positive bacterium *Micrococcus lysodeikticus.* The concentration of lysozyme in the samples was determined from a standard curve calculated from the clearance zones of the Hen Egg Whie Lysozyme (HEWL) standards.

Materials and supplies

Micrococcus lysodeikticus (Sigma, M-3770)
0.50% agarose (Sigma, A-6013)
0.06M Phosphate buffered saline (Sigma, P-4417)
3-mm cork borer or biopsy punch
Hen Egg White Lysozyme (HEWL) (Sigma, L-6876)
0.02 M Na Cl
Petri dishes
Calipers (VWRbrand™)
1.5 mL Eppendorf tubes

Protocol

Lysoagar Plates
0.60 mg/ml *Micrococcus lysodeikticus* (Sigma M3770)
0.02 M Na Cl (1.169g/L)
0.50% Agarose (Sigma A6013)
- In phosphate buffer (0.06 M, pH 6.0) (Sigma, P-4417)

1 The mixture was heated and stirred until all agarose dissolved.
2 25 mL were dispensed per Petri plate (15 cm diameter plate).
3 The lysoagar was left to solidify (overnight minimum). It can be stored in a refrigerator for 2 to 4 weeks.
4 Uniform wells were punched into the agar (10 to 16 wells/plate) with a 3mm diameter sterilized biopsy punch.

Lysozyme Standards

A series of standards were made from HEWL. The initial stock solution contained100 mg of HEWL/5 mL of phosphate buffered saline (0.06 M, pH 6.0). A range of standards of concentrations 100-20,000 µg/mL were made by using the following chart to make 1 mL of each standard in a 1.5-mL Eppendorf tube. Standards were run every time an assay was performed to control for any variation in incubation temperature and duration.

Standard No.	Concentration (µg/mL)	Volume of stock solution (mL)	Volume of PBS buffer (mL)
1	20,000	1.000	0
2	15,000	0.750	0.250
3	11,200	0.565	0.435
4	7,500	0.375	0.625
5	3,750	0.187	0.813
6	2,250	0.1125	0.8875
7	1,500	0.0750	0.925
8	750	0.0375	0.9625
9	150	0.0075	0.9925

Sample Preparation

Plasma remained on ice after removal from the freezer. It was removed from the microcapillary tube and placed on Parafilm paper and was then pipetted into the wells.

Lysoplate Assay

Ten µL aliquots of each sample were dispensed into duplicate or triplicate wells (depending on the amount of plasma available), and at least one standard per plate was included. Some samples were diluted with sterile saline when the volume of plasma was low. Time of completion of each plate was recorded. A couple of "standard-only" plates were also run simultaneously during each assay. The plates were left at room temperature for 20 h. Lysis was measured as a function of the diameter of the clearance zones in the gel surrounding the wells using digital calipers (VWRbrand™) to 2 decimal places of accuracy and recorded in the order in which the plates were completed. A dissecting microscope was used to aid in determining measurements.

Lysozyme Concentration Calculation

This was calculated using a logarithmic regression analysis as lysozyme concentration increases logarithmically with clearance zone. The following equation was used.

$Y=A + B (\log X)$ where Y=diameter of clearance zone
X=lysozyme concentration (µg/ml)
B=the slope, "X coefficient" (determined from standard curve)
A=the Y intercept (determined from standard curve)

4) MACROPHAGE CELL ISOLATION (Secombes, 1990)

Introduction

Macrophage cells were isolated using a continuous Percoll density gradient. Percoll is a colloidal silica medium, supplied as a 23% (w/w) colloid with a density of 1.13 ± 0.005 g/mL. It was diluted using 1.5M NaCl (Cheetham *et al.*, 1998). The density of 1.075 g/mL was used for the isolation of Pacific herring macrophages as this optimized the number harvested (Garduño and Kay, 1994).

Materials and supplies

Tissue sieves
L-15 medium (Sigma, L-4386)
12-well sterile cell culture plates
Fetal bovine serum (FBS) (Sigma, F-2442)
Penicillin/Streptomycin (Sigma, P-0781)
Microbeakers (disposable)
Spoonula
Sterile 3-cc syringe plungers
Sterile glass Pasteur pipettes
15-mL plastic centrifuge tubes
4% Trypan blue solution (Sigma, T-8154)
70% Ethanol
Laminar flow-hood
Centrifuge, refrigerated
Hemocytometer
Percoll (1.075 g/ml) (Sigma, P-49937)
1.5M NaCl
Distilled, de-ionized water
Laminar flow-hood
Diff-Quik stain (Gibco)
6 mL-plastic centrifuge tubes

Protocol

1 Small tissue sieves made from one inch PVC tubing and 80 µm nylon mesh were sterilized by soaking in 70% ethanol, rinsing in distilled, de-ionized, sterile water and placement under an UV light in a laminar flow hood for four h.

2 The head kidney was placed immediately into a sterile micro-beaker containing 2 mL of cold L-15 medium, supplemented with penicillin/streptomycin and 0.5% FBS.

3 The sterile tissue sieves were placed into the wells of a 12-well sterile cell culture plate and 2 mL of L-15 medium, supplemented with penicillin/streptomycin and 0.5% FBS, was added to each well and maintained on ice.

4 The head kidney was transferred to the tissue sieve and forced through the nylon mesh with a sterile 3-cc-syringe plunger (pre-sterilized by autoclave). A small amount of L-15 medium was used to flush remaining tissue through the sieve into the culture plate well. A sterile glass Pasteur pipette was used to transfer the cell suspension solution from each well into a sterile 15-mL tube.

5 Cell isolation solution (L-15 with 2% FBS) was added to bring each cell suspension to 5 mL. A glass Pasteur pipette was used to underlay the cell suspension with 3mL of Percoll (density 1.075 g/dL) to create a discontinuous density gradient. Care was taken to gently underlay the Percoll to create two distinct layers. The Percoll/cell suspension mixture was centrifuged for 30 min, at 5°C, at 10,000 rpm.

6 Using another sterile glass Pasteur pipette, the band of cells at the Percoll-media interface was transferred into a sterile 6-mL plastic tube. This layer contained macrophages and some neutrophils. It was generally whitish in colour but can appear blackened depending on the concentration of melanin-containing macrophages present. This macrophage cell suspension was placed on ice until performing the macrophage function assays. A cell viability and concentration count were performed prior to performing these assays to ensure sufficient cells (Protocol No. 11).

5) PHAGOCYTOSIS ASSAY PROTOCOLS

Introduction

Head kidney macrophage cells were incubated with Congo red stained yeast cells in a 1:40 ratio. The number of yeast cells phagocytosed per macrophage was primarily used, while the phagocytic index was used in the initial adult experiment. The former method measured the optical density of the resulting yeast/macrophage solution to 2 decimal places and involved comparison to a standard curve whereas the latter method involved the use of a light microscope to manually score the yeast within the macrophage cells.

Materials and supplies

Congo red (Sigma, C-6767)
Yeast cells (*Saccharomyces cerevisceae*, Sigma, YSC-1)
Percoll, density = 1.075, (Sigma, P-4937)
Spectrophotometer (Bio-Rad® plate reader)
Centrifuge
490-510 nm filter
HBSS (Sigma, H9269)
PBS (Sigma, P-4417)
Trypsin-EDTA solution (Gibco 1.5 g/L trypsin, 0.4 g/L EDTA in PBS)

Protocol

Preparation of Congo red yeast solution

Three mL of Congo red solution, 0.87% in PBS was added to 1.5 g of yeast cells (dry wt), *Saccharomyces cerevisceae*, (Sigma, YSC-1) and was incubated at room temperature for 15 min. Then 7 mL of distilled water were added and thoroughly mixed before autoclaving for 15 min to kill and fix the yeast. The yeast cells were rinsed several times using HBSS to remove excess stain until the supernatant was clear. The resulting solution was stored at 4°C and was stable for at least several months. Prior to use, the cells were resuspended at 4 x 10^7 cells/mL in HBSS or culture medium.

Phagocytosis assay (Seeley *et al.*, 1990; Kaminiski *et al* 1985)

1 The macrophage cell suspension was diluted to 2 x 10^6 cells/mL and stained yeast cells were resuspended as above.
2 1 mL of cell suspension was mixed with 2 mL of the yeast cell suspension (4.0 x 10^7 yeast cells/ml) providing a yeast cell-macrophage ratio of 40:1. The mixture was incubated at room temperature for 90 min in duplicate.
3 Following incubation, 5 mL of ice-cold HBSS was added to each tube, and each sample was under laid with 3 mL of Percoll, 1.075 g/mL.
4 The tubes were centrifuged at 850 g for 3 min to separate the macrophages from the free yeast cells. The macrophage cells were at the Percoll-media interface and were harvested with sterile glass pipettes and washed with HBSS.
5 At this point, the cell suspension could be used directly to score percent phagocytic cells (the number of cells ingested by each macrophage counted using a light microscope). For this method, 200 macrophages were counted (in duplicate) and the percentage of cells containing yeast particles was determined to give a phagocytic index.
6 Alternatively, the pellet of macrophage cells was resuspended in 120 µL of trypsin-EDTA solution (1.5 g/L trypsin and 0.4 g/L EDTA in PBS) to solublize the macrophages and incubated at room temperature over night.
7 The absorbance of 50 µL samples, in duplicate for each sample, was read at 510 nm (to 3 decimal places) against a blank containing the trypsin-EDTA solution using a 96-well plate and plate reader. Results were compared with a standard curve prepared by using a series of standard solutions from serial dilutions of the Congo red yeast stock solution.

6) RESPIRATORY BURST ASSAY

Introduction

This assay evaluated the respiratory burst activity of head kidney macrophages following incubation with nitroblue tetrazolium (NBT). The respiratory burst can be defined as the production of reactive oxygen species with strong bacteriocidal activity following stimulation of phagocytes (Secombes *et al.*, 1988). It was tried using two different methods: a) head kidney macrophage cell suspension in a welled-slide assay and b) whole blood with zymosan A challenge in a slide assay.

Materials and supplies

0.2% NBT solution (Sigma, N-6876)
Glass cover slips
Moist chambers
PBS (Sigma, P-4417)
Sterile pipet tips
Hemocytometer
Photomicroscope
0.4% trypan blue solution (Sigma, T-8154)
Tissue sieves
Zymosan A (Sigma, Z-4250) 100µL pipettor

Protocol

a) Welled plate assay

1 Aseptically dissected head kidney was placed in HBSS or L-15, homogenized through tissue sieves. The resulting cell suspension was incubated in welled plates for 90 min.
2 Cell viability counts were conducted on the cell suspensions (Protocol No. 11).
3 NBT solution was added to the plates and incubated again in moist chambers for 90 min.
4 The welled plates were read using a photomicroscope to count the ratio of activated (blue in appearance) *vs.* non-activated cells (green-yellow appearance).

b) Whole blood slide assay (Stasiak, 1996; Stasiak and Baumann, 1996)

1 One drop of fresh fish blood was placed onto a clean glass cover slip and incubated in a moist chamber for 30 min.
2 The blood was gently washed off with PBS until there was no blood visible. 50µL of a 0.2% NBT solution with zymosan A (activator) was added to the cover slip, which was inverted onto a clean glass slide.
3 The slide was incubated in the moist chamber for 30 min.
4 Following incubation, the slide was evaluated using a photomicroscope and 4 fields at 280x magnification were photographed.
5 Scoring of the fields was done by calculating the percentage activated *vs.* percentage non-activated macrophages per field. Activated macrophages were blue while non-activated ones had a green-yellow glow.

7) PREPARATION OF BACTERIAL CULTURES FOR DISEASE CHALLENGE

Introduction

V. anguillarum (*Listonella*) is a commonly encountered, pathogenic, marine bacterium and was used as a challenge agent. A pure bacteria stock culture, which had been stored at -80°C in sterile glycerol, was obtained from SFU. The challenge dose of *V. anguillarum* was grown in an overnight culture that was monitored by determining the optical density at 650 nm and comparing this to a known growth curve (Koenig, 1996, pers. comm.). The culture was used once the optical density (OD) was >2.5 as this ensured the ideal phase of the growth curve. It was placed on ice once the desired concentration was reached.

Materials and supplies

4 L Erlenmeyer flask
500 mLTrypsin soy broth (TSB) +1.5% NaCl
Shaker incubator
Overflow glass jar
Air source and air pump
Antifoam agent (3 to 5 drops)
1 capsule (1.5 mL Eppendorf) from previous overnight culture of *V.anguillarum* (*Va*).

Protocol for overnight growth of culture

1 500 mL of TSB+1.5% NaCl was placed into the 4 L flask.
2 One capsule of *Va* (stored at -80°C) and 3 to 5 drops of the antifoam agent were added aseptically to the flask and an air pump was attached to aerate the culture.
3 The flask was placed into the shaker incubator at room temperature for 24 hours
4 After 24 h, the OD_{650nm} was checked until it reached ~2.50. The culture was placed on ice until ready to use.

The design also included a glass jar for any overflow of culture.

8) SERIAL DILUTION PLATES

Introduction

A sub-sample of the bacterial culture was used to make serial dilution plates once the final OD reading was made and the stock placed on ice to verify the concentration of the bacterial culture solution used for the bath challenge. This confirmed that the concentration of the bacterial suspension, based on optical density, was accurate as determined by calculating the cfu/ml from dilution plates. The dilutions were plated on trypsin soy agar (TSA) +1.5% NaCl plates as most *Vibrio* species have absolute requirements for Na^+ for growth and need a minimum of 0.5% (w/v) NaCl media (Hjeltnes and Roberts, 1993).

Materials and supplies

Glass test tubes
TSA plates (make about one week prior to use).
Sterile pipet tips
Sterile PBS (Sigma, P-4417)
100µL pipettor

Protocol

1 A row of test tubes, labeled 1 to 10, was used and 5 mL of the stock bacterial culture was placed into the first test tube and mixed gently. This was dilution = 10^0. To make the next dilution, 0.5 mLfrom test tube 1 was placed into tube 2 and 4.5 mL PBS was added to make 10^{-1}. This process was continued until the final tube had been mixed to produce the dilution 10^{-5}.
2 A 25 µL drop from each dilution tube was streak plated on TSA plates. This allowed determination of the cfu/25 µL and back calculation was done to determine the concentration concentration of bacteria at each dilution.

9) MICROBIAL IDENTIFICATION TESTS

Introduction

V. anguillarum is a facultative anaerobe, is oxidase and catalase positive, and ferments carbohydrates anaerobically. It is also sensitive to the *Vibriostatic* agent, 0/129 (Fryer and Rohovec, 1993). When cultured it usually produces colonies that are round, raised, convex, and cream-coloured. To determine if dead fish challenged with *V. anguillarum* were infected with the bacteria, the head kidney from all juvenile herring that died post-challenge was streaked on TSA +1% NaCl plates. The presence or absence of *V. anguillarum* was determined by using standard diagnostic microbiological procedures (Frerichs, 1993; Noga, 1996).

Materials and supplies

TSA +1% NaCl agar plates	Glucose test media	Oxidase test strips
Streak loop	Hanging drop slide	Glass slides
Aseptic head kidney tissue	Gram stain kit (Difco)	

Antibody coated Latex beads (Microtek® International Ltd., Saanichton, BC) Novobiocin (0/129) disks (Unipath®, Nepean, Ontario)

Protocol

Setting up the streak plate

1 The inoculating loop was flamed prior to use and between each streak. The cooled loop was streaked through the head kidney. The first streak was made, back and forth at one edge/side of plate.
2 The loop was dragged through the end of the first streak to pick up a small amount of bacteria. This enabled each successive streak to have fewer bacteria and therefore permit isolation of individual colonies.
3 The second streak was made at 90 degrees to the first. This was repeated until 4 streaks had been made, and a tail was made with the final (fourth) streak.
4 The plate was turned upside down to reduce condensation, and incubated at room temperature. The plate was checked for growth over the next 48 h.

Confirmation of V. anguillarum

1 Colony morphology, Gram-stain, hanging drop motility test, oxidase production test, and glucose fermentation test were used to screen for *V. anguillarum.*

2 All samples that passed these preliminary tests (Gram-stain negative, motile, oxidase-producing, glucose positive) were then tested for sensitivity to the antibiotic Novobiocin (0/129) at 150 µg/mL dose (Unipath®, Nepean, Ontario) and for agglutination with specific antibody coated latex beads (Microtek® International Ltd.).

The minimum inhibitory concentration for most *Vibrio spp.* is 1-5 µg/mL. Therefore, any organisms resistant to this antibiotic disc were very unlikely to be a *Vibrio* spp.

Checking for agglutination

1 Using a grease pencil, 2 circles were drawn, the size of a quarter, on a glass microscope slide. A sterile loop of bacterial culture was taken from the plate and suspended to a light cloudy suspension in approximately 0.5 mL PBS.
2 Using a pipettor,one drop of the bacterial culture suspension (50 µL) was added to each circle. Five µL of the specific antibody coated latex bead solution was added to one circle and 5 µL of PBS was added to the other circle as a negative control.
3 The slides were rocked gently for 20 to 120 seconds and observed for agglutination. A positive response was classified as clumping of the antigen-antibody mix (visible to naked eye) as specific complexes were formed. This test is a rapid method of detecting fish pathogens (Toranzo *et al,* 1987).

10) ANTIBODY AGGLUTINATION PROTOCOL

Introduction

At the end of the 6-week observation period, following *V. anguillarum* challenge, the survivors from each tank were euthanized and their plasma tested for the presence and quantification of *V. anguillarum* specific antibodies. This test is based on the assumption that if plasma contains antibodies it will agglutinate when exposed to a suspension of *V. anguillarum.* A drop of serum was added to a drop of cultured *Vibrio anguillarum* suspension. If the serum contained antibodies then an agglutination (clumping) reaction was seen, similar to the positive control. A series of 2-fold dilutions of serum in phosphate buffered saline (PBS) in 96-well microtitre plates (Falcon™) was used to determine the titre. The titre is defined as the last dilution in which obvious agglutination is seen. It is expressed as the reciprocal of the corresponding serum dilution. To control for cross-reactivity, the serum was also tested for antibodies to both *V. salmonicida* and *V. ordalli* (Roberson, 1990).

Materials and supplies

96-well plates
Sterile pipet tips
Sterile saline
10 µL and 10µL pipettor
Fish serum
Titre recording sheets
Parafilm strips
Latex bead antibodies for *V. anguillarum* (Microtek® International Ltd.)

Protocol

1 25 µL of saline was placed in each well of a 96 well plate, except for the first column.

2 The serum was diluted 1:1 in saline.

3 10 µL of diluted serum was added to the 1st and 2nd wells, and 2 fold serial dilutions were made through to the 11th well. Twenty-five µL was removed from the last well.

4 Latex bead antibodies were used as the postive control (Microtek®).

5 25 µL of the *V. anguillarum* bacterial suspension was added to each well. The suspension was made by suspending colonies in saline or by diluting stock solution with saline.

6 The plate was covered with Parafilm and mixed by rotating the plate on a flat surface and was then incubated at room temperature overnight.

7 The titre was determined by recording the last well in which there was visible agglutination.

NB

Undiluted serum produces clearer/more easily readable agglutination but 5 µL of plasma was all that was available from many fish. Therefore, the samples were diluted down to 5 µL saline and 5 µL plasma. In other species a dilution of 1:10 has been used, however this dilution did not work with herring.

11) CELL VIABILITY COUNTS AND CELL CONCENTRATION

Introduction

The dye, trypan blue, is commonly used in cell assays to determine cell viability. Viable cells do not take up the dye and therefore remain clear whereas non-viable cells take up the blue colour of the dye. The following two dilutions were commonly used when assessing cells, depending upon the gross cell concentration. More concentrated solutions were further diluted prior to conducting the viability counts.

Materials and supplies

50 µL 0.4% trypan blue (Sigma, T-8154)
30 µL HBSS/L-15 (Sigma, H-9269/L-4386)
20 µL macrophage cell suspension,
(Dilution factor =5)

80 µL 0.4% trypan blue (Sigma, T-8154)
10 µL HBSS/L-15 (Sigma, H-9269/L-4386)
10 µL macrophage cell suspension
(Dilution factor=10)

10 µL and 100 µL pipettors
Sterile pipet tips
Timer

Hemocytometer
Light microscope

Protocol

1 After combining the macrophage cell suspension, trypan blue and diluent (HBSS or L-1 5), the resulting solution was left to stand at least 5 min but no longer than 15 min before evaluation.

2 The cells were counted and evaluated using a hemocytometer, with 2 standardized measurement chambers, each of 0.5 cm^3. A 10 µL aliquot of the dye-media-cell solution was added to each chamber. Five of the squares on the centre grid were counted from each chamber (4 corners, and the middle). An average was taken over 5 squares from each chamber. As well as counting the cells, the cells were scored as alive, dead or red cells. Ideally there should be very few red cells and greater than 95% alive cells.

Cell viability calculation

Count 5 squares: average of 5 squares x 50,000 x df (dilution factor)

$A \times B \times C \times 10^4$ where A= volume of cells
B= dilution factor in trypan blue
C= mean number of unstained cells (i.e. unstained count divided by number of areas counted)
10^4 = conversion of 0.1 mm^3 to mL

Cell concentration calculation - cells/mL

Cells per ml = the average count per square x df x 10^4 (counting 10 squares)

12) EXTRACTION OF PAHs FROM WATER SAMPLES

Materials and supplies

Surrogate spiking solution (from Auke Bay Lab, NOAA, AK)
250 µL Hamilton syringe
1 L Teflon separatory funnel
Glass cone tipped centrifuge tubes
2 mL-glass vials, with screw top Teflon liners
Teflon squirt bottle (for rinsing)
Dichloromethane (Sigma, 15,479-2)
4 L solvent bottles (empty) x 2 - labeled "A" and "B"
Pipet bulbs (1-2 mL)
Boiling chips
250 mLamber bottles
Water bath
Hexane (Sigma, 27,050-4)
Disposable glass pipets
100 mL glass cylinder

Protocol

1 A 4 L solvent jug labeled "A" was rinsed with 3 successive 100-mL aliquots of the water to be sampled and was then filled to 3.8 L with the water sample.
2 250 µL of surrogate spiking solution was added to jug "A" with a cleaned 250 µL Hamilton syringe. This syringe was cleaned by filling it with 3 successive aliquots of dichloromethane, discarding the dichloromethane after each filling. 100 mL of dichloromethane was also added to jug "A."
3 Jug "A" was shaken by inverting the jug followed by return to the upright position within a period of about 2 seconds for each inversion/return cycle for 60 cycles. The jug then rested upright on a stable flat surface undisturbed for at least 2 min to allow the dichloromethane phase to separate out as a layer on the bottom of the jug.
4 As much of the water phase as was easily practical was poured into jug "B" using a cleaned funnel, without allowing any of the dichloromethane layer to be transferred into jug "B." The remaining contents of jug "A" were poured into the cleaned 1 L separatory Teflon funnel. The funnel was left undisturbed until the upper aqueous layer separated completely from the lower dichloromethane layer.
5 The separated dichloromethane layer was drained from the separatory funnel through the stopcock into a clean amber-boiling flask. The remaining aqueous layer was drained from the separatory funnel into jug "B." The boiling flask was labeled with a sample identification number (SIN) listed on the chain-of-custody sheet supplied by the Auke Bay lab. Steps 4 to 6 were repeated with jug "B."
6 As much of the water phase as was easily practical was discarded from jug "B." The remaining dichloromethane and water (ca 100 mL or so of water) was poured into the separatory funnel used in step 5. The separatory funnel was again left undisturbed until the upper aqueous layer separated completely from the lower dichloromethane layer. The separated dichloromethane layer was drained into the boiling flask used in step 7, so that the 2 dichloromethane extracts were combined.
7 Two-3 boiling chips were placed into each boiling flask just before the volume reduction of the extract. The flask was placed on the steam bath at a medium setting to obtain a gentle boil and boiling continued until the volume of extract was about 1.5 cm from the bottom of the bottle and was removed from the heat.
8 The extract from the boiling flask was poured into a conical tip centrifuge tube labeled with the appropriate SIN. The boiling flask was rinsed with 2 several mL portions of dichloromethane pouring the rinsate into the centrifuge tube each time.
9 Fresh boiling chips were put into the centrifuge tube and this was boiled on a steam bath to 2 mL without letting the extract boil to dryness and 1 mL of hexane was added and the extract was boiled again to 2 mL. Then, another 1 mL of hexane was added and boiled to about 1.5 mL. The final extract was transferred with a glass pipet to a 2-mL screw top vial labeled with the appropriate SIN. All vials were tightly capped and transported to Auke Bay Laboratory for analysis.

13) VIRAL NEUTRALIZATION ASSAY

Introduction

This assay involves incubation of fish tissue, serum or water samples with *Epithelioma papulosum cyprini* (EPC) cells to determine whether the virus, VHSV, was present. Monolayers of the cells were first grown in 12-well tissue culture plates under sterile conditions. The suspension of fish tissue/serum or water samples was added in a series of dilutions to the welled plates. After a 7-day incubation period, the plates were stained with a crystal violet preparation and scored for the presence and number of plaque forming units (pfu), which were indicative of the presence of the virus (Kocan *et al.*, 1997). The virus effectively created small "holes" (plaques) in the monolayer of EPC cells if it was present. All water samples were filtered with a 0.22 µm filter prior to performing the assay. The negative control for the water samples consisted of Leibovitz's-15 with FBS and seawater but no virus. VHSV stock solution was used as a positive control. This method was also used to confirm the titre and virulence of each VHSV stock solution. The detection limit for the test is $10^{2.6}$ pfu/g of tissue.

Materials and supplies

12-well tissue culture plates	Stomacher (to homogenize fish)
Crystal violet solution (Sigma, C-3886)	L-15 medium (Sigma, L-4386)
Epithelioma papulosum cyprini cells	MEM-5T

VHSV stock solution (from MFS, Nancy Elder)

Protocol

1 EPC cells were plated at 500,000 cells per well.
2 Cells were left overnight at 20°C to allow them to grow to form a complete monolayer (1,000,000 cells/well of a 24-well plate is ideal).
3 The virus stock solution was diluted using MEM-5T. 100 µL of viral stock was placed into the first pair of wells and 100 µL into a microcentrifuge tube containing 900 µL of MEM-5T. The tube was vortexed.
4 100 µL was transferred into each of the second pair of wells and 100 µL to another microcentrifuge tube with 900 µL of medium. This process was repeated to dilute the viral stock to achieve dilutions from $1/10^{1}$ to $1/10^{12}$.
5 Fish tissue was homogenized using a stomacher machine and the resulting weight of tissue suspension was diluted 5 times with L-15 medium.
6 100 µL aliquots of dilutions of the tissue suspension/serum/water samples ($1/10^{1}$ to $1/10^{12}$) were prepared as per steps 4 and 5 for the virus stock solution.
7 All virus stock, tissue suspension, serum and water sample dilutions were incubated with EPC cells for 7 d at 15°C.
8 After incubation, the wells were stained by adding 1 mL of crystal violet solution to each well, incubating for 30 min, then rinsing gently with water.
9 Plaque forming units (pfu) per well were counted with a backlit illumination tray.
10 Pfu counts were used to calculate the titre of the viral stock prior to use and to determine the pfu/ml of tissue, serum, and water samples based on the dilution used.

14) VHSV CHALLENGE EXPOSURE

Materials and supplies

VHSV stock solution (from MFS, Nancy Elder)
Sterile syringes
FBS medium (in MEM (20%))
5-mL sterile plastic test tubes

Protocol

Pathogen challenge (Kocan *et al., 1997)*

The pathogen challenge protocol was conducted according to Kocan *et al.*, (1997). Fish were fed *ad libitum* daily during the post-challenge period. The VHSV stock solution was verified as viable, and the titre confirmed, by incubation with EPC cells prior to use as a pathogen challenge.

1 The water within each tank was lowered and the VHSV stock solution added to each exposure tank (controls included).

2 Good aeration was maintained throughout the 1 h static bath challenge and the water flow was restored to the pre-challenge level after the 1 h period.

3 All tanks were observed twice daily for mortalities for an 8-week observation period post-challenge.

Water samples

1 Double strength FBS in L-15 with glutamine (20%) was prepared and used to dilute the 1 mL water samples 1:1 for a 10% solution.

2 An initial water sample was taken at 5 min post-exposure.

3 One mL water samples were collected hourly for the next 3 h post exposure.

4 At 24 h post-exposure, another 1 mL water sample was collected.

5 Step 4 was repeated at the same time for the next 5 d.

6 After the 5 d, a 1-mL sample was taken hourly for 3 consecutive h, turning off the water intermittently each time prior to collecting the sample.

7 All samples were frozen at -80°C and later evaluated as per Protocol No. 13.

15) OIL PROTOCOL

Introduction

The design of the PVC column was modified from Moles *et al* (1985). Water does not pick up the oil droplets as it passes through the column and this results in production of a water-soluble fraction of crude oil.

Prior work with Siporax® biofilter rings that were oil-soaked for 4 to 5 h resulted in an uptake of approximately 0.9 g oil per biofilter ring. 120 g of beads was considered equivalent to 2000 sq ft of oil (Kocan, pers. comm., 1996).

Materials and supplies

Prudhoe Bay Crude Oil (PBCO)
Scale
Mesh-bottom plastic container
2 foot PVC custom-designed column
Cable ties and string
Fumehood
Nalgene beaker
Marbles
Siporax® biofilter rings (Aquatic Eco-Systems®, Inc, Part No. BF820)

Protocol

A pilot oil-soaking was performed to determine whether time of soaking changed the amount of uptake per Siporax® biofilter ring. There was no difference between 1, 4 or 6 h soak times with approximately 0.73 g absorbed per filter ring. Therefore, in the interest of time, all experiments involved a 1 h soaking of filter rings, plus drainage overnight, prior to use. A blindly selected set of filter rings (n=10) was weighed before and after oil soaking during each experiment to determine the average oil uptake (Table A1.2).

Flow rates were equalized for each tank during each experiment. A flow rate of 2 L/min was used for the 265 L tanks and 5 L/min for the 450 L tanks.

Table A1.2. Uptake of oil by Siporax® biofilter rings

Experiment	Average dry weight (g)	Average wet weight (g)	Oil uptake/bead (g)
BS juveniles	1.42	2.15	0.73
BS juveniles	1.43	2.15	0.71
QI adults	1.46	2.17	0.71
QI adults	1.45	2.15	0.71
BS adults	1.38	2.18	0.81
BS adults	1.77	2.48	0.71
Average	1.49 ± 0.06	2.21 ± 0.05	0.73 ± 0.02

16) CULTURE OF *I. hoferi* SPORES

Introduction

I. hoferi spores can be cultured from tissue with germinating spores from recently dead fish and spores can be maintained for up to 70 - 110 d.

Materials and supplies

1% glucose
10% FBS L-15 (f/s), 2% P/S, 10% FBS (Sigma, F2442)
Incubator set at ~15°C
6-mL sterile plastic culture tubes
I. hoferi reference culture (from MFS, Dick Kocan)
200 mmol glutamine/100 mL
100 µg gentamicin/mL (10 mg/100mL)

Protocol (Kocan *et al.*, 1999)

1 Whole tissue from lesions on the skin, heart and other viscera were removed and incubated with L-15 (f/s) media supplemented with 10% FBS, 2% P/S, 1% glucose, glutamine (200 mmoL/100 mL) and gentamicin (100 µg/mL) at 15°C.

2 Tissue was aseptically transferred to fresh media every 7 d.

3 Cultures were maintained for at least 2 weeks prior to determining if the sample was positive or negative for *Ichthyophonus* spores.

4 Each culture was scored as negative or positive by comparing to a reference culture for the presence of characteristic germinating bodies and spores.

17) CELL MEDIA AND SOLUTIONS

Macrophage overnight media
L-15 (f/s), 2% FBS

Macrophage isolation media
L-15 (f/s), 2% FBS, 2% P/S, 10 IU heparin/mL

Macrophage maintenance media
L-15 (f/s), 5% FBS, 2% P/S

Macrophage cell attachment media
L-15 (f/s), 1% FBS

Tissue culture initiation solution (100 mL)
Fungazone 2 mL
Gentamicin 200 µL
Dispense into 10-mL tubes and refrigerate

Percoll gradient solution - Pacific herring (for a density of 1.075 g/dL)

For 100 mL:

53 mL	Percoll
37 mL	Distilled-deionized water
10 mL	1.5M NaCl

Prepare using aseptic technique in a laminar flow hood and refrigerate the unused portion as soon as possible.

Heparin solution (Sigma)

For 10 mL of a 10,000 units/mL stock solution use 0.575 g in 10 mL physiological saline

10,000 units/mL x 1 mg/174 USP units x 10 mL = 574.713 mg

To dilute this to 100 units/mL, 1 mL of the stock solution was added to 100 mL of sterile physiological saline or distilled water.

TSA + 1.5%
NaCl 3 g TSB
1.5 g NaCl
1.5 g agar agar
100 mL water

This quantity made approximately 10 plates. The mixture was autoclaved and poured into sterile petri dishes. (For 500 mL use 7.5 g NaCl and 15 mg TSB)

NB T solution
10 mg NBT/5 mL in 0.85%

APPENDIX 2

1) Water PAH concentration data: juvenile and adult exposures.

2) Mortality data.

3) Inter-quartile range tables: juvenile and adult herring.

4) Immunotoxicological studies table.

5) Gender divided references for Hct, Lct, SSI and cortisol in adult herring.

6) Evaluation for between tank variability in juvenile and adult herring.

Table A2.1 The absolute quantity of individual PAH compounds in ng/L from water samples at various times after exposure of juvenile herring to the WSF of crude oil using 120 g PBCO as the initial exposure concentration. The sum PAHs is the sum of all compounds in a sample excluding the substituted analytes (2,6-dimethylnaphthalene, 2,3,5-trimethylnaphthalene, and 1-methylphenanthrene). Analysis performed at the Auke Bay Laboratory, Alaska (Chapter 3).

Time elapsed (days)	Immediate	1 d	2 d	4 d	7 d	14 d	21 d
Analyte concentrations (ng/L)							
Naphthalene	14126.00	2199.85	1128.55	761.49	201.96	14.89	11.51
2-methylnaphthalene	11362.40	1702.97	975.25	847.43	289.30	13.09	10.03
1-methyl naphthalene	9400.61	1397.73	802.30	674.43	241.73	5.79	6.07
2,6-dimethylnaphthalene	3201.63	435.37	242.36	307.91	138.86	6.52	4.11
C-2 naphthalenes	13306.80	1785.73	1000.84	1245.00	554.94	19.00	15.41
2,3,5-trimethylnaphthatene	1462.78	129.88	54.39	140.40	50.07	2.23	1.59
C-3 naphthalenes	8507.79	744.18	359.62	818.78	420.75	22.92	13.77
C-4 naphthalenes	3193.41	203.43	75.73	308.98	168.25	24.13	10.27
Biphenyl.	1246.29	220.24	132.13	118.30	57.63	3.97	3.35
Acenaphthylene	0.00	6.36	3.61	1.14	0.25	0.79	0.58
Acenaphthene	75.56	11.74	6.81	16.18	10.81	1.53	1.92
Fluorene	598.23	82.61	46.84	58.72	29.26	4.51	2.47
C-1 fluorenes	955.14	96.84	42.65	110.14	65.15	31.75	21.99
C-2 fluorenes	1227.79	98.58	43.87	128.63	82.95	19.92	8.79
C-3 fluorenes	1061.78	65.05	18.90	103.46	58.77	15.77	2.17
Dibenzothiophene	944.62	89.79	46.51	75.45	38.73	5.49	2.88
C- dibenzothiophenes	1580.31	.98.63	37.09	125.43	70.74	9.40	5.22
C-2 dibenzothiophenes	2302.26	120.01	34.06	174.57	105.53	16.85	3.63
C-3 dibenzothiophenes	1590.77	79.50	19.18	122.75	80.15	34.70	10.08
Phenanthrene	1144.47	103.32	52.08	96.56	45.66	9.78	4.61
1-methylphenanthrene	643.01	42.24	15.26	54.63	29.83	2.33	1.95
C-1 phenanthrenes/anthracenes	2831.80	178.12	66.84	230.11	125.79	12.86	11.51
C-2 phenanthrenes/anthracenes	3094.58	173.17	46.63	258.17	153.95	24.97	7.02
C-3 phenanthrenes/anthracenes	2193.90	116.83	27.78	179.50	110.82	62.85	9.53
C-4 phenanthrenes/anthracenes	586.24	31.39	7.49	42.88	25.90	17.60	1.21
Anthracene	24.22	2.61	0.68	7.00	7.53	6.20	11.37
Fluoranthene	19.24	2.48	1.21	3.22	2.06	1.85	1.44
Pyrene	51.68	6.38	2.71	6.17	5.86	3.42	0.49
C-1 fluoranthenes/pyrenes	227.13	12.27	3.02	21.62	11.36	6.83	1.17
Benz-a-anthracene	19.67	0.95	0.17	1.61	1.09	0.44	0.10
Chrysene	160.72	9.27	2.36	13.23	8.61	4.25	0.70
C-1 chrysenes	261.75	12.58	3.08	21.29	14.66	7.16	0.83
C-2 chrysenes	322.71	16.05	3.11	23.76	20.89	6.84	1.53
C-3 chrysenes	125.02	5.76	0.00	10.21	4.55	0.00	0.00
C-4 chrysenes	16.72	0.86	0.00	1.39	0.77	0.00	0.00
Benzo-b-fluoranthene	94.01	6.87	1.76	7.79	5.89	4.87	2.00
Benzo-k-fluoranthene	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Benzo-e-pyrene	103.51	6.75	1.70	7.94	7.21	2.87	0.80
Benzo-a-pyrene	0.00	0.00	0.21	0.54	0.00	0.20	0.67
Perylene	4.31	0.00	0.00	0.00	0.39	0.00	0.26
Indeno-123-cd-pyrene	0.00	0.00	0.00	0.00	0.18	0.00	0.00
Dibenzo-a,h-anthracene	12.95	0.63	0.00	1.00	0.88	0.00	0.00
Benzo-g,h,i-perylene	44.05	4.08	1.69	4.31	3.80	2.32	1.64
Sum PAHs (ng/L)	**82818.45**	**9693.61**	**4996.47**	**6629.18**	**3034.78**	**419.83**	**187.04**

Table A2.2 The absolute quantity of individual PAH compounds in ng/L from water samples at various times after exposure of adult herring to the WSF of crude oil using 120 g PBCO as the initial exposure concentration. The sum PAHs is the sum of all compounds in a sample excluding the substituted analytes (2,6-dimethylnaphthalene, 2,3,5-trimethylnaphthalene, and 1-methylphenanthrene). Analysis performed at the Auke Bay Laboratory, Alaska (Chapter 3). *Separate experiment (see section 3.2.1.2. for detailed description and discussion of high value).

Time elapsed (days)	Immediate	1 d	2 d	4 d	7 d	21 d	28 d*
Analyte concentrations (ng/L)							
Naphthalene	1936.84	3347.46	748.89	263.85	155.27	9.44	664.11
2-methylnaphthalene	1127.36	5157.03	574.30	278.69	194.78	8.00	1601.13
1-methyl naphthalene	901.01	3972.65	452.85	223.76	158.38	5.11	1262.16
2,6-dimethylnaphthalene	222.78	2582.12	151.23	47.67	51.14	4.12	972.29
C-2 naphthalenes	917.55	9486.66	648.82	193.48	209.86	16.06	3709.55
2,3,5-trimethylnaphthatene	46.61	940.93	30.55	7.46	14.10	2.62	387.18
C-3 naphthalenes	291.45	7687.77	261.57	80.34	82.38	18.54	2267.98
C-4 naphthalenes	38.16	3052.63	64.33	17.89	16.89	15.89	570.69
Biphenyl.	136.47	590.46	83.19	35.45	25.80	3.39	224.84
Acenaphthylene	3.12	4.68	0.54	0.28	0.46	0.15	0.00
Acenaphthene	7.26	42.16	5.88	10.05	1.59	1.06	17.01
Fluorene	46.67	357.72	27.96	14.47	10.58	3.54	141.17
C-1 fluorenes	92.43	743.09	39.16	29.87	15.42	9.98	247.61
C-2 fluorenes	42.39	1120.82	33.37	14.86	14.64	11.71	256.05
C-3 fluorenes	17.64	952.48	24.06	12.52	1.42	6.42	116.54
Dibenzothiophene	44.72	834.41	37.87	11.26	11.57	2.83	214.45
C- dibenzothiophenes	38.49	1698.11	38.45	12.52	8.82	4.89	250.67
C-2 dibenzothiophenes	35.34	2456.40	42.94	10.45	6.71	9.17	217.12
C-3 dibenzothiophenes	37.50	1658.01	27.70	14.91	7.51	7.10	107.28
Phenanthrene	52.84	1078.91	48.00	15.95	14.11	4.91	384.20
1-methylphenanthrene	18.57	728.59	17.14	5.16	5.25	2.29	115.12
C-1 phenanthrenes/anthracenes	82.36	3217.39	80.15	30.80	18.76	9.00	516.91
C-2 phenanthrenes/anthracenes	53.19	3615.89	65.76	17.81	14.09	15.83	332.93
C-3 phenanthrenes/anthracenes	30.61	2461.10	49.61	16.65	2.63	11.74	165.96
C-4 phenanthrenes/anthracenes	3.33	633.57	11.48	4.56	2.19	2.78	49.54
Anthracene	24.87	20.21	4.48	7.53	4.10	1.40	8.92
Fluoranthene	2.56	20.61	1.78	2.05	1.15	1.37	31.66
Pyrene	4.79	30.19	3.25	3.06	2.47	2.73	47.08
C-1 fluoranthenes/pyrenes	5.20	259.75	5.49	2.58	1.65	2.33	19.85
Benz-a-anthracene	0.34	23.23	0.38	0.34	0.05	0.23	1.50
Chrysene	3.06	175.78	3.15	1.12	0.71	1.69	14.47
C-1 chrysenes	3.05	302.77	4.61	0.70	0.70	2.08	17.35
C-2 chrysenes	12.48	316.74	5.68	0.42	0.57	1.34	15.59
C-3 chrysenes	0.94	118.12	1.96	0.00	0.00	0.00	4.93
C-4 chrysenes	0.00	17.88	0.17	0.61	0.00	0.00	3.09
Benzo-b-fluoranthene	4.60	25.47	4.26	0.94	0.50	0.00	21.19
Benzo-k-fluoranthene	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Benzo-e-pyrene	1.88	33.50	0.58	0.18	0.24	0.70	19.55
Benzo-a-pyrene	0.42	0.00	0.00	0.47	0.28	0.12	6.52
Perylene	0.00	1.61	0.00	0.00	0.07	0.09	1.50
Indeno-123-cd-pyrene	0.00	0.00	0.00	0.00	0.00	0.00	23.15
Dibenzo-a,h-anthracene	0.00	5.62	0.43	0.00	0.00	0.00	0.00
Benzo-g,h,i-perylene	2.16	13.72	0.74	0.37	0.35	0.60	90.44
Sum PAHs	**6003.07**	**55534.60**	**3403.87**	**1330.79**	**986.70**	**192.22**	**13644.70**

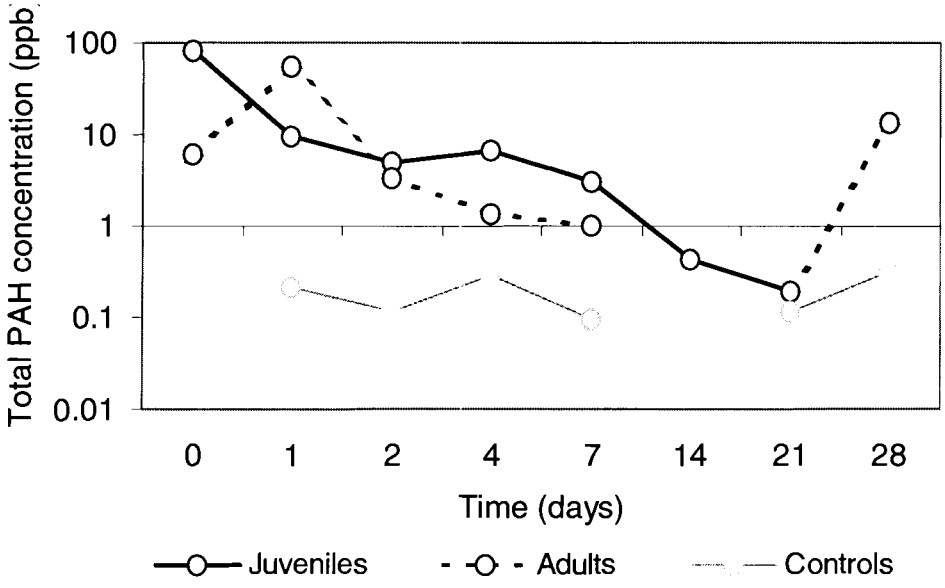


Figure A2.1 Total PAH concentration (ppb) in water samples collected at varying times after initiation of oil exposure of juvenile and adult herring.

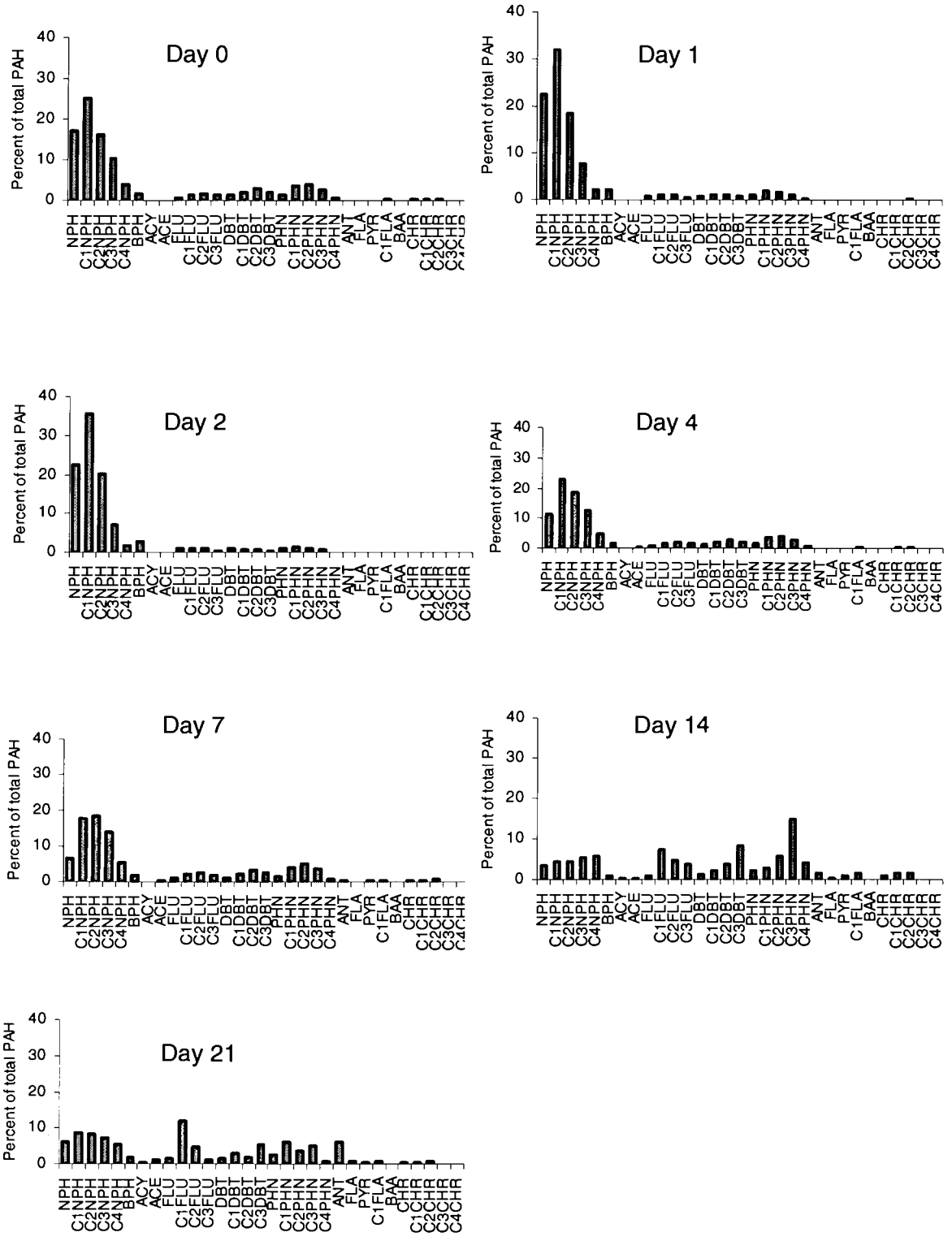


Figure A2.2. The relative proportions of individual PAH compounds as a percentage of the total PAH recovered from water samples at various times after starting exposure of juvenile herring to the WSF of crude oil.

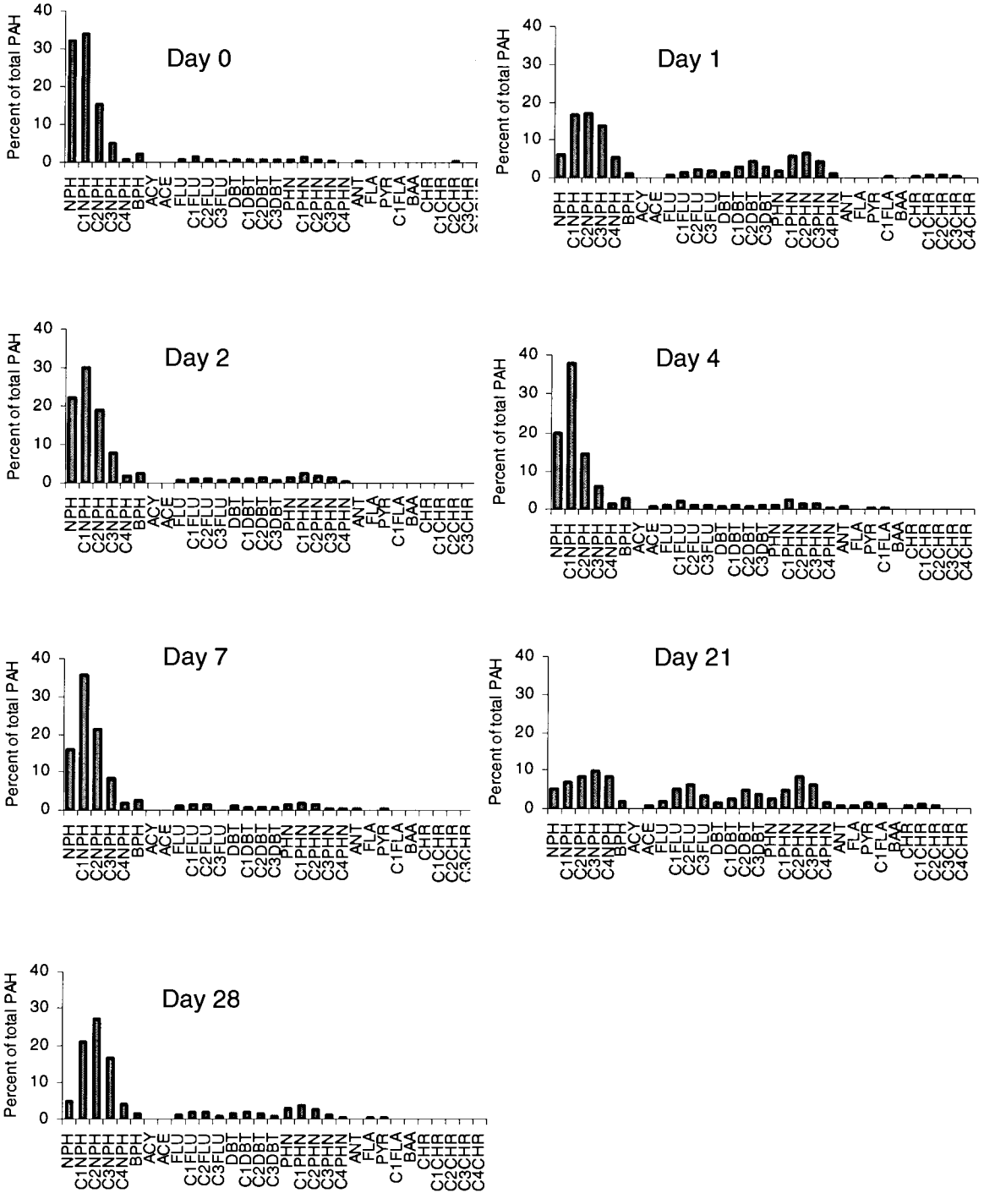


Figure A2.3. The relative proportions of individual PAH compounds as a percentage of the total PAH recovered from water samples at various times after starting exposure of adult herring to the WSF of crude oil.

Table A2.3 Number of mortalities, percentage mortality during period, and per day, for holding, acclimation and experimental periods and overall in various Pacific herring populations. BS=Barkley Sound, QI=Quadra Island, PBS=Pacific Biological Station, UK=unknown, holding period=day of capture to day moved to experimental tank, acclimation period=day after moved to experimental tank to day prior to starting experiments, pre-experimental period=holding period plus acclimation period.

Year	1996	1996	1996	1997	1997	1997	1998	1998	1998	1998	1999		
Age	Adults	0+yr	0+yr	Adults	Adults	Adults	Adults	0+yr	0+yr	0+yr	1+yr		
Population	BS	BS	BS	BS	QI	QI	BS	BS	BS	BS	QI		
												TOTALS	AVERAGE
Date obtained	02/25/96	08/23/96	08/23/96	02/27/97	07/09/97	10/17/97	03/04/98	07/20/98	08/25/98	09/23/98	02/23/99		
Moved to expt. tanks	06/09/96	08/28/96	08/30/96	04/28/97	07/31/97	11/03/97	06/04/98	08/04/98	09/25/98	11/22/98	n/a		
Date expt started	06/29/96	08/28/96	09/02/96	07/04/97	09/25/97	11/05/97	07/07/98	n/a	n/a	11/22/98	05/18/99		
Date expt ended	07/16/96	11/12/96	09/16/96	07/04/97	12/04/97	01/25/98	09/15/98	08/24/98	11/22/98	02/26/99	05/18/99		
Total for experiment (N)	260	1119	171	1500	544	688	944	2300	2000	700	160	10386	944.2
Total acclimated (N)	260	999	150	1360	158	523	200	1000	1260	480	0	6390	580.9
Total sampled (N)	110	190	80	30	62	415	132	0	0	216	80	1315	119.5
Subset sent to PBS				30		40	30	30		30	30	190	31.7
Holding period (days)	105	6	8	86	23	18	92	16	32	61	84	531	48.3
Holding morts (N)	UK	120	21	110	386	125	714	1270	740	190	15	3691	369.1
Holding mortality (%)	UK	10.72%	12.28%	7.33%	70.96%	18.17%	75.64%	55.22%	37.00%	27.14%	9.38%	35.54%	32.4%
Mortality per day	UK	20.00	2.63	1.28	16.78	6.94	7.76	79.38	23.13	3.11	0.18	6.95	16.1
Standardized mortality rate	UK	**0.019**	**0.016**	**0.002**	**0.048**	**0.011**	**0.014**	**0.048**	**0.014**	**0.005**	**0.001**	**0.001**	**0.02**
Acclimation period (days)	19	0	2	67	55	1	32	20	58			254	28.2
Acclimation morts (N)	89	0	3	708	58	2	53	730	630			2273	252.6
Acclimation mortality (%)	34.23%	n/a	2.00%	52.06%	36.71%	0.38%	26.50%	73.00%	50.00%	n/a	n/a	35.57%	34.4%
Mortality per day	4.68	n/a	1.50	10.57	1.05	2.00	1.66	36.50	10.86	n/a	n/a	8.95	8.6
Standardized mortality rate	**0.028**	**n/a**	**0.010**	**0.011**	**0.008**	**0.004**	**0.010**	**0.057**	**0.011**	**n/a**	**n/a**	**0.002**	**0.02**
Pre-experimental period (days)	124	6	10	153	78	19	124	36	90	61	84	785	71.4
Pre-experimental morts (N)	89	120	24	818	444	127	767	2000	1370	190	15	5964	542.2
Pre-experimental mortality(%)	34.23%	10.72%	14.04%	54.53%	81.62%	18.46%	81.25%	86.96%	68.50%	27.14%	9.38%	57.42%	44.3%
Mortality per day	0.72	20.00	2.40	5.35	5.69	6.68	6.19	55.56	15.22	3.11	0.18	7.60	11.0
Standardized mortality rate	**0.004**	**0.021**	**0.020**	**0.005**	**0.020**	**0.017**	**0.013**	**0.043**	**0.012**	**0.006**	**0.002**	**0.001**	**0.0**

Table A2.4. The median, inter-quartile range (IQR), and coefficient of variation (CV) for a profile using all control juvenile herring *vs.* one using only the time zero controls. PE=percentile estimation, CV=coefficient of variation, CF=condition factor, LSI=liver-somatic index, Hct=hematocrit, Lct=leucocrit, mo.=month, TP=total protein, mø=macrophage, BS=Barkley Sound, PS=Puget Sound, T_H=holding time, d= days. For use with juvenile herring ranging from 1.6 – 7.8 g and 6.4 – 9.8 cm.

Variable	T_H (d) and age	All controls				Time zero only controls			
		Median	N	IQR	CV	Median	N	IQR	CV
CF	BS, 6 d	0.63	39	0.59 – 0.76	15.5	0.63	10	0.59 – 0.72	12.7
	BS, 61 d	0.82	84	0.70 – 1.04	14.8	---	---	---	---
	PS, 77 d	0.79	72	0.75 – 0.86	9.8	0.81	30	0.77 – 0.85	9.1
LSI	<11 mo.	1.21	72	0.94 – 1.54	34.2	---	---	---	---
	11 + mo.	0.79	11	0.50 – 0.88	29.9	---	---	---	---
Hct (%)	<11 mo. 6 d	25.4	39	15.0 – 31.3	44.5	30.9	10	25.7 – 33.3	27.2
	<11 mo. 61 d	18.0	70	9.1 – 31.0	62.7	---	---	---	---
	<11 mo. 77 d	31.6	46	18.3 – 43.4	48.3	38.7	30	26.6 – 49.7	36.9
	11 + mo.	42.0	50	29.6 – 45.9	38.2	---	---	---	---
Lct (%)	<11 mo, 6 d	0.71	39	0 – 3.76	112.6	0.82	10	0.70 – 1.13	54.9
	<11 mo, 6 1 d	0.95	70	0 – 1.82	105.6				
	<11 mo. 77d	1.17	46	0.47 – 1.62	126.7	1.41	30	1.17 – 1.91	101.8
	11 + mo. 61 d	1.52	12	0 – 3.39	113.1				
	11 + mo. 77 d	0.47	38	0 – 0.98	91.2	---	---	---	---
Lympho-cytes (%)	BS, 6 d	72.2	14	51.9 – 79.4	28.9	---	---	---	---
	PS, 77 d	50.5	46	28.0 – 64.0	42.2	42.5	30	28.8 – 58.3	40.3
Monocytes (%)		0	60	0.00 – 1.00	210.0	0.0	30	0.0 – 1.0	209.7
Neutrophils (%)		6.0	60	2.8 – 11.8	119.8	6.0	30	3.0 – 10.3	124.0
Thrombo-cytes (%)	BS, 6 d	15.7	14	9.0 – 19.8	76.8	---	---	---	---
	PS, 77d	38.5	46	21.5 – 58.5	55.8	42.5	30	28.3 – 58.5	50.2
Eosinophils (%)		0	39	0.00 – 0.00	511.8	0.0	30	0.0 – 0.0	547.7
Yeast/mø		6.00	40	4.41 – 8.07	50.4	---	---	---	---
Vibrio titre		0	9	0.0 – 90.0	267.2	---	---	---	---
Lyso-zyme (µg/mL)	BS, 61 d	2.12	28	2.07 – 2.21	---	---	---	---	----
	PS, 77 d	265.3	37	85.4 – 620.3	207.7	306.8	29	113.5 – 659.3	197.9
Glucose (mmol/L)	<11 mo.	3.59	60	2.53 – 5.59	72.6	---	---	---	---
	11+ mo.	2.32	12	1.54 – 3.27	34.5	---	---	---	---
Lactate (mmol/L)	<11 mo.	3.31	51	2.21 – 5.14	51.6	---	---	---	---
	11+ mo.	10.88	10	8.26 –12.59	26.2	---	---	---	---
Albumin (g/dL)		0.85	11	0.70 – 0.96	18.4	----	---	---	---
TP (g/dL)	<11 mo.	1.41	44	0.78 – 1.97	85.2	---	---	---	---
	11+ mo.	2.00	9	1.26 – 4.57	84.7	---	---	---	---
Globulin (g/dL)		0.72	9	0.32 – 2.44	89.7	----	---	---	---
Albumin/globulin		1.13	9	0.36 – 2.13	114.7	----	---	---	---

Table A2.5. Supplemental to Table A2.4 for juvenile herring, using only control fish with no lesions for variables significantly impacted by the presence of lesions. No time zero juvenile fish were examined by post mortem so their gross lesion status was unknown.

Controls with no gross lesions				
	Median	N	IQR	CV
Hct (%) < 11 mo.	19.5	65	9.2 – 31.2	62.2
Lysozyme (µg/mL)	2.12	27	2.07 – 2.21	5.9

Table A2.6. Comparison of the median, inter-quartile range (IQR), and coefficient of variation (CV) for a profile using all control adult herring *vs.* one using only the time zero controls. LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, Hct =hematocrit, Lct=leucocrit, ALP=alkaline phosphatase, T_H=holding time, d=days, PS=Puget Sound. Applicable for adult herring ranging from 16.5 – 117.4 g and from 12.9 –24.0 cm.

Variable	T_H (d)	All controls pooled				Time zero only controls			
		Median	N	IQR	CV	Median	N	IQR	CV
CF	19	0.89	129	0.79 – 0.89	10.8	---	---	---	---
	78	0.79	31	0.74 – 0.85	10.5	---	---	---	---
	90	0.91	75	0.83 – 1.01	14.3	0.95	30	0.82 – 1.27	12.1
	124	0.69	108	0.62 – 0.77	20.2	0.78	31	0.47 – 1.39	5.1
	153	0.99	30	0.93 – 1.05	11.5	0.99	30	0.93 – 1.05	1.5
LSI	19	0.87	49	0.70 – 1.05	28.6	---	---	---	---
	78	0.78	31	0.65 – 0.86	20.1	---	---	---	---
	124	1.00	99	0.82 – 1.26	33.7	0.77	22	0.62 – 0.90	31.2
SSI	All – 124	---	---	---	---	0.12	22	0.08 – 1.05	58.9
	19	0.20	49	0.11 – 0.29	50.7	---	---	---	---
	78	0.09	31	0.08 – 0.11	45.0	---	---	---	---
	124	0.10	72	0.07 – 0.17	64.1	---	---	---	---
GSI	19	0.52	49	0.33 – 0.95	131.4	---	---	---	---
	78	0.32	31	0.15 – 0.48	104.3	---	---	---	---
	124	0.79	60	0.62 – 1.00	258.3	0.88	10	0.75 – 1.00	238.7
Hct (%)	All	---	---	---	---	39.0	89	31. 3 – 46.9	31.1
	19	35.3	125	23.4 – 42.9	42.1	---	---	---	---
	78	41.1	31	37.6 – 47.9	16.7	---	---	---	---
	90	40.5	75	35.1 – 46.3	18.9	---	---	---	---
	124	39.2	108	29.6 – 47.3	34.3	---	---	---	---
	153	39.0	28	29.9 – 57.8	27.2	---	---	---	---
Lct (%)	All	1.00	367	0.57 – 1.64	82.3	---	---	---	---
	PS 90	---	---	---	---	0.68	30	0.30 – 0.90	60.7
	124	---	---	---	---	0.56	31	0.0 – 2.48	120.0
	153	---	---	---	---	2.03	28	1.35 – 2.93	49.1
Lympho-cytes (%)	90	31.1	60	22.2 – 38.3	39.4	31.9	30	25.2 – 38.4	4.2
	124	54.6	27	37.8 – 67.8	33.4	---	---	---	---
Neutro-phils (%)	90	22.6	60	13.5 – 32.3	61.7	24.0	30	14.4 – 32.9	64.1
	124	29.0	27	15.0 – 50.0	61.4	---	---	---	---
Thrombo-cytes (%)	90	45.1	60	38.1 – 57.2	37.8	42.5	30	33.9 – 52.4	43.9
	124	16.3	27	8.7 – 21.4	41.6	---	---	---	---
Glucose (mmol/L)	78	11.9	31	9.9 – 15.3	25.6	---	---	---	---
	124	7.8	86	5.5 – 11.0	46.0	10.5	10	8.7 – 12.9	24.9
Lactate (mmol/L)	78	4.6	31	3.9 – 6.4	35.5	---	---	---	---
	124	2.1	67	1.5 – 7.2	116.7	1.78	10	1.4 – 2.0	31.0
Albumin (g/dL)		0.77	62	0.21 – 1.09	157.5	0.41	8	0.33 – 0.56	59.3

Variable	T_H (d)	All controls pooled				Time zero only controls			
		Median	N	IQR	CV	Median	N	IQR	CV
Total protein (g/dL)		4.64	66	3.97 – 5.09	47.0	3.64	8	1.69 – 4.04	46.8
Globulin (g/dL)		3.72	61	2.86 – 4.14	47.7	2.90	8	1.29 – 3.78	53.8
Albumin/globulin		0.20	61	0.10 – 0.40	379.2	0.16	8	0.11 – 0.43	86.7
Cortisol (ng/mL)		12.6	59	8.02 – 16.7	47.6	11.3	10	5.3 – 15.2	47.7
Chloride (mEq/L)		119	48	100 – 131	18.8	125	8	106 – 139	14.5
Phosphorus (mg/dL)		2.72	50	1.56 – 3.09	38.1	3.16	10	2.84 – 3.49	18.0
Calcium (mg/dL)		18.4	50	15.8 – 22.0	24.1	15.9	10	15.07 –17.60	8.9
ALP (U/L)		20.7	9	13.9 – 24.2	38.9	---	---	---	---
Lysozyme (µg/mL)	19	1.01	127	0.13 – 0.17	69.4	---	---	---	---
	78	0.04	26	0.04 – 0.06	29.4	---	---	---	---
	90	1.26	68	0.91 – 9.40	68	10.07	24	9.21 –10.96	22.2
	124	2.59	86	2.30 – 3.35	92.3	2.21	10	2.16 – 2.53	9.8
Macrophage (mø) count		1.52×10^7	37	1.18×10^7 – 2.16×10^7	68.7	1.48×10^7	10	1.03×10^7 – 2.48×10^7	58.0
Yeast/mø	19 d	2.32	49	0.66 – 4.07	34.8	---	---	---	---
	78 d	3.46	31	2.54 – 4.79	54.4	---	---	---	---
	90 d	2.91	23	0.53 – 5.00	77.4	5.03	7	4.36 –6.49	19.1
	124 d	2.78	60	1.99 – 3.95	31.8	---	---	---	---
	153 d	---	---	---	---	1.27	10	0.92 – 1.52	32.2
% Phagocytosis		81.2	9	80.1 – 84.2	3.96	---	---	---	---
Respiratory burst		0.20	31	0.17 –0.33	102.7	---	---	---	---

Table A2.7. Examples of immunotoxicological studies detailing the stressor, the exposure where applicable, the variables measured and the general findings. Hct=hematocrit, Lct=leucocrit, B(a)P=benzo-a-pyrene.

Species	Stressor	Variables	Findings	Reference
Cyprinus carpio L. (Fancy carp)	Crowding	Total protein, lysozyme, bactericidal complement, cortisol, glucose, chloride.	Increased cortisol; decreased lysozyme, total protein, and disease resistance.	Yin, *et al.*, (1995)
Dicentrarchus labrax (European sea bass)	Cadmium & B(a)P (Water-borne and injection)	Phagocytosis, respiratory burst.	Decreased phagocytosis, respiratory burst variable.	Lemaire-Gony *et al.*, (1995)
Salmo trutta (Brown trout)	Seawater transfer	Phagocytosis, lysozyme, cytotoxicity.	Increase in phagocytosis and lysozyme.	Marc *et al.*, (1995)
Oncorhynchus mykiss (Rainbow trout)	Diesel oil-based drilling mud extracts (injection)	Serum immunoglobulin, hemolytic complement, lysozyme, mitogenesis.	Decreased lysozyme, increased mitogenesis.	Tahir and Secombes (1995)
Oncorhynchus mykiss (Rainbow trout)	Chlorinated resin acids (flow-through exposure)	Biochemistry, swimming, disease resistance.	Increased Lct, decreased cortisol and impaired disease resistance.	Kennedy *et al.*, (1996)
Oncorhynchus mykiss (Rainbow trout)	PAH-contaminated water	Phagocytosis, respiratory burst, B cell numbers.	Decreased phagocytosis and respiratory burst.	Karrow *et al.*, (2003)
Salmo trutta *Oncorhynchus mykiss* (Brown/rainbow trout)	Chronic stress	Disease resistance	Increased mortality in presence of chronically elevated cortisol.	Pickering and Pottinger (1989)
Ictalurus punctatus (Channel catfish)	Tributylin (injection)	Humoral immunity and oxidative burst.	Neutrophilia, specific antibody secreting cell most sensitive	Rice *et al.*, (1995)
Oncorhynchus spp.	Chlorinated and aromatic hydrocarbons	Disease resistance	Increased probability of disease-related impacts.	Arkoosh *et al.*, (1998)
Oreochromis niloticus (Nile tilapia)	*Chlorpyrifos*	Chemotaxis, phagocytosis chemiluminescence.	Lower pronephros cell counts and decreased phagocytosis.	Holladay *et al.*, (1996)
Limanda limanda (L.) (Dab)	Oil-contaminated sediments	Hct, lymphocyte numbers, lysozyme, respiratory burst.	Variable Hct and lymphocyte numbers, decreased lysozyme, and respiratory burst.	Tahir *et al.*, (1993)

Table A2.7. Continued.

Species	Stressor	Variables	Findings	Reference
Limanda limanda *(L.)* (Dab)	Sewage sludge (water-borne)	Hematology, serum protein, melano-macrophage centres.	Decreased serum protein, lysozyme equivocal, increased MMC's in high exposure.	Secombes *et al.*, (1991)
Oncorhynchus mykiss (Rainbow trout)	Dissolved copper from copper tailings run-off	Hct, Lct, CF, WBCs, respiratory burst.	Decreased Hct, Lct, and lymphocytes.	Dethloff *et al.*, (2001)
Oncorhynchus tshawytscha (Chinook salmon)	o,p-Dichlorodiphenyl-dichloroethylene (10 ppm)/Dimethyl sulfoxide	Plasma lysozyme, IgM, mortality.	Decreased expression of IgM by splenic leucocytes.	Milston *et al.*, (2003)
Crangon crangon (Common shrimp)	Harbour dredge spoils (Water-borne)	Hemolymph volume, total hemocyte count, phenoloxidase activity.	Increased hemolymph, decreased hemocyte count and phenoloxidase activity.	Smith *et al.*, (1995)
Mytilus edulis (Blue mussel)	PAHs (water-borne)	Phagocytosis, neutral red uptake.	Phagocytosis inhibited, uptake affected.	Grundy *et al.*, (1996)
Ictalurus punctatus (Channel catfish)	Tributylin and PCBs	Antibody response to *V.anguillarum*, respiratory burst activity.	Decreased antibody response and respiratory burst activity.	Regala *et al.*, (2001)
Oncorhynchus mykiss (Rainbow trout)	Acute stress from handling, confinement and thermal shock	Coagulation time and leukocyte phagocytosis of yeast.	Decreased clotting time, increased thrombocytes, and increased phagocytosis.	Ruis and Bayne, (1997)
Tautogolabrus adspersus (Cunner)	Water-accommodated fraction of crude oil	Organ indices, aryl hydrogen hydroxylase activity (AHH).	Induction of AHH, 5.8 fold in males, 7.5 fold in females. Inhibition of gonad size increase.	Walton *et al.*, (1983)
Heteropneustes fossilis (Freshwater catfish)	Bleached kraft paper mill effluent	Respiratory burst and lipid peroxidation.	Increased respiratory burst activity and increased peroxidative tissue damage.	Fatima *et al.*, (2000)
Dicentrarchus labrax (Seabass)	Phenolic compounds, intraperitoneal injection	Hemoglobin, Hct, glucose, BUN, ALP.	Hypoglycemia, low blood urea nitrogen (BUN), decreased ALP.	Roche and Bogé (2000)
Cyprinus carpio (Common carp)	Cortisol implantation and *Aeromonas hydrophila* challenge	Passive hemolytic plaque and indirect haemagglut-ination assays, glucose.	Suppression of antibody-mediated responses, blood glucose increased with time.	Lumanlan-Mayo (2000)
Rutilus rutilus L. (Roach)	Bleached kraft pulp and paper mill effluent	Hct, Lct, cortisol, WBCs, glucose, chloride, total protein, and calcium.	Increased cortisol, glucose, and chloride. Decreased Hct, Lct, protein, calcium, lymphocytes.	Jeney *et al.*, (1996)

Table A2.8. The median, 95th PE, and CV for a profile using all control adult herring *vs.* one using only the time zero controls for variables affected by gender. PE=percentile estimation, CV=coefficient of variation, SSI=spleen-somatic index, Hct=hematocrit, Lct=leucocrit, ALP=alkaline phosphatase, T_P = pooled controls, T_0 = time zero only controls. BS= Barkley Sound, QI=Quadra Island, PS=Puget Sound, M=male, F=Female, U=unknown gender. Applicable for adult herring ranging from 16.5 – 117.4 g and from 12.9 – 24.0 cm.

Variable	T_H (d)	M/F	T_P controls				T_0 controls			
			Median	N	95th PE	CV	Median	N	95th PE	CV
SSI	124	All	---	---	---	---	0.12	22	0.02 – 0.30	58.9
	19	F	0.21	30	0.05 – 0.42	45.0	---	---	---	---
	78	F	0.09	14	0.07 – 0.26	46.8	---	---	---	---
	124	F	0.13	33	0.02 – 0.37	59.4	---	---	---	---
	All	M	0.10	71	0.03 – 0.36	68.8	---	---	---	---
Hct (%)	All	All	---	---	---	---	39.0	89	6.6 – 57.7	31.1
	All	F	37.4	118	11.4 – 57.8	33.4	---	---	---	---
	19	M	30.0	18	7.1 – 58.8	44.5	---	---	---	---
	78	M	40.8	13	27.8 – 52.4	18.5	---	---	---	---
	90	M	43.8	13	31.2 – 55.2	17.3	---	---	---	---
	124	M	43.7	47	20.7 – 65.4	24.3	---	---	---	---
	153	M	41.8	19	25.5 – 57.8	20.0				
	19	U	37.0	77	4.5 – 62.8	43.4	---	---	---	---
	78	U	41.4	4	35.5 – 43.8	9.3	---	---	---	---
	90	U	40.2	49	24.3 – 52.3	18.0				
	124	U	23.5	10	9.6 – 46.4	50.3				
Lct (%)	124	BS, QI	---	---	---	---	0.56	31	0.0 – 2.48	120.0
	153	BS, QI					2.03	28	1.35 – 2.93	49.1
	All PS		0.72	75	0.0 – 1.48	60.2	0.68	30	0.03 – 1.39	60.7
	All	F BS, QI	0.96	118	0.0 – 3.64	80.2	---	---	---	---
	19	M	1.11	18	0.57 – 4.05	65.6	---	---	---	---
	78	M	1.02	13	0.55 – 2.03	40.0	---	---	---	---
	124	M	1.16	47	0.00 – 4.61	80.7	---	---	---	---
	153	M	2.17	19	0.92 – 5.17	47.1	---	---	---	---
	All	U	1.07	91	0.00 – 4.30	89.6	---	---	---	---
Cortisol (ng/mL)		F	14.8	28	5.4 – 29.3	41.8	11.7	8	5.4– 18.9	38.9
		M	11.2	31	3.3 – 31.1	52.1	5.0	2	4.9 – 5.1	4.0

Table A2.9. The median, inter-quartile range (IQR), and CV for a profile using all control adult herring *vs.* one using only the time zero controls for variables affected by gender. PE=percentile estimation, CV=coefficient of variation, SSI=spleen-somatic index, Hct =Hct, Lct=leucocrit, ALP=alkaline phosphatase. T_P = pooled controls, T_0 = time zero only controls. BS= Barkley Sound, QI=Quadra Island, PS=Puget Sound, M=male, F=Female, U=unknown gender. Applicable for adult herring ranging from 16.5 – 117.4 g and from 12.9 –24.0 cm.

Variable	T_H (d)	M/F	T_P controls				T_0 controls			
			Median	N	95th PE	CV	Median	N	95th PE	CV
SSI	124	All	---	---	---	---	0.12	22	0.08 – 1.05	58.9
	19	F	0.21	30	0.14 – 0.29	45.0	---	---	---	---
	78	F	0.09	14	0.08 – 0.13	46.8	---	---	---	---
	124	F	0.13	33	0.07 – 0.19	59.4	---	---	---	---
	No effect		0.10	71	0.07 – 0.19	68.8	---	---	---	---
Hct (%)	No effect	All	---	---	---	---	39.0	89		31.1
	No effect	F	37.4	118	26.4 – 44.2	33.4	---	---	---	---
	19	M	30.0	18	19.0 – 41.9	44.5	---	---	---	---
	78	M	40.8	13	34.6 – 47.9	18.5	---	---	---	---
	90	M	43.8	13	36.6 – 49.2	17.3	---	---	---	---
	124	M	43.7	47	34.7 – 50.2	24.3	---	---	---	---
	153	M	41.8	19	37.1 – 47.6	20.0	---	---	---	---
	19	U	37.0	77	26.5 – 44.8	43.4	---	---	---	---
	78	U	41.4	4	36.6 – 43.8	9.3	---	---	---	---
	90	U	40.2	49	34.9 – 42.3	18.0	---	---	---	---
	124	U	23.5	10	13.6 – 32.4	50.3	---	---	---	---
Lct (%)	124 BS, QI		---	---	---	---	0.56	31	0.00 – 2.48	120.0
	153 BS, QI						2.03	28	1.35 – 2.93	49.1
	All PS		0.72	75	0.41 – 0.95	60.2	0.68	30	0.03 – 1.39	60.7
	All	F BS, QI	0.96	118	0.56 – 1.59	80.2	---	---	---	---
	19	M	1.11	18	0.74 – 2.13	65.6	---	---	---	---
	78	M	1.02	13	0.73 – 1.34	40.0	---	---	---	---
	124	M	1.16	47	0.56 – 1.85	80.7	---	---	---	---
	153	M	2.17	19	1.35 – 2.97	47.1	---	---	---	---
	Gender unknown		1.07	91	0.00 – 1.88	89.6	---	---	---	---
Cortisol (ng/mL)	No effect	F	14.8	28	11.2 – 18.4	41.8	11.7	8	7.2 – 15.3	38.9
	No effect	M	11.2	31	7.6 – 14.6	52.1	5.0	2	4.9 – 5.1	4.0

Table A2.10. Evaluation for between tank variability for juvenile, control herring used in determining T_0 and T_P reference range profiles using tank medians and the Kruskal-Wallis test at the 95% confidence level. Sample size (N) refers to the number of tanks evaluated for each variable. Hct=hematocrit, Lct=leucocrit, CF=condition factor, LSI=liver-somatic index, A/G=albumin/globulin ratio, mø= macrophage.

Variable	N	p-value
Body mass (g)	15	0.4497
Fork length (cm)	15	0.4497
CF	15	0.4497
LSI	4	0.3916
Hct (%)	16	0.4514
Lct (%)	15	0.4514
Lymphocytes (%)	7	0.4232
Monocytes (%)	7	0.4232
Neutrophils (%)	7	0.4232
Thrombocytes (%)	7	0.4232
Glucose (mmol/L)	4	0.3916
Lactate (mmol/L)	4	0.3916
Albumin (g/dL)	3	0.3679
Total protein (g/dL)	4	0.3916
Globulin (g/dL)	3	0.3679
A/G	3	0.3679
Yeast phagocytosed/mø	4	0.3916
Lysozyme (µg/mL)	8	0.4289

Table A2.11. Evaluation for between tank variability for adult, control herring used in determining T_0 and T_P reference range profiles using tank medians and the Kruskal-Wallis test at the 95% confidence level. Sample size (N) refers to the number of tanks evaluated for each variable. Hct=hematocrit, Lct=leucocrit, CF=condition factor, LSI=liver-somatic index, SSI=spleen-somatic index, GSI=gonad-somatic index, A/G=albumin/globulin ratio, ALP=alkaline phosphatase, mø= macrophage.

Variable	N	p-value
Body mass (g)	14	0.4478
Fork length (cm)	14	0.4478
CF	14	0.4478
LSI	11	0.4405
SSI	8	0.4335
GSI	7	0.4289
Hct (%)	14	0.4478
Lct (%)	14	0.4478
Lymphocytes (%)	6	0.4159
Monocytes (%)	3	1.0000
Neutrophils (%)	6	0.4159
Thrombocytes (%)	6	0.4159
Glucose (mmol/L)	7	0.4232
Lactate (mmol/L)	6	0.4232
Albumin (g/dL)	3	0.3916
Total protein (g/dL)	3	0.3916
Globulin (g/dL)	3	0.3916
A/G	3	0.3916
Cortisol (ng/mL)	2	0.3173
Chloride (mEq/L)	2	0.3173
Phosphorus (mg/dL)	2	0.3173
Calcium (mg/dL)	2	0.3173
ALP (U/L)	2	0.3679
Macrophage cell count	4	0.3916
Respiratory burst activity	3	0.3679
Yeast phagocytosed/mø	11	0.4405
Lysozyme (µg/mL)	13	0.4457

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