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EFFECTS OF EARLY REARING HISTORY ON SELECTED ENDOCRINE AND
IMMUNE FUNCTIONS IN JUVENILE PACIFIC SALMONIDS

by

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ABSTRACT

The effects of different early rearing conditions on plasma cortisol concentration, immune function, hematological profile, and disease resistance were examined in populations of hatchery-reared and wild chinook salmon (*Oncorhynchus tshawytscha*) and hatchery-reared, wild, and colonized coho salmon (*O. kisutch*). These features were examined during smoltification and after an acute stress. The effect of initiating feeding with a wild-type diet as compared to a commercially prepared diet was also examined in chinook salmon fry as one aspect of rearing history that is different between fish reared in the hatchery and those in the wild.

During smoltification and following periods of acute stress, wild chinook salmon and wild and colonized coho salmon had significantly higher concentrations of plasma cortisol. Hatchery-reared juveniles showed less sensitivity to stress and lower concentrations of plasma cortisol during the smoltification period and after an acute stress. Antibody producing cell (APC) number and disease resistance to *Vibrio anguillarum* were not significantly different between the hatchery and wild chinook salmon. These features were also similar among the hatchery, wild and colonized coho salmon smolts, despite significantly higher levels of circulating cortisol in the wild and colonized smolts. White blood cell to red blood cell (wbc/rbc) ratios were slightly higher in wild fish than in their hatchery-reared counterparts in chinook and coho salmon juveniles.

Significant elevations in plasma cortisol concentration after an acute stress was still evident retained in the wild and colonized coho salmon juveniles even after holding them for 6 months in an artificial rearing environment. Disease resistance in the wild fish significantly decreased over that time. Following the 6-month rearing period, the initial numbers of APC in the wild fish were significantly higher than those in their hatchery counterparts. This difference precludes the ability to conclude that a cause-effect relationship exists between a high cortisol response and decreased specific immune function.

The use of a live diet to initiate feeding in chinook salmon fry compares favorably to feeding a commercial diet in that the activity of lysozyme and wbc/rbc ratios were higher in this group. Specific immune function was correlated with body weight while non-specific immune defense was not.

There appear to be physiological differences between hatchery-reared, wild and colonized coho and chinook salmon. Rearing history may be a determinant in the survival of hatchery-reared salmonids released into the natural environment.

TABLE OF CONTENTS

ABSTRACT.....	ii
TABLE OF CONTENTS.....	iv
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
ACKNOWLEDGEMENTS.....	x
GENERAL INTRODUCTION.....	1
 CHAPTER 1 Cortisol and immune function in chinook and salmon smolts.....	 4
Introduction.....	5
Materials and Methods.....	11
Results.....	19
Discussion.....	34
 CHAPTER 2 Cortisol and specific immune responses to stress in juvenile chinook and coho salmon.....	 39
Introduction.....	40
Materials and Methods.....	42
Results.....	48
Discussion.....	60
 CHAPTER 3 Diet and immune function at first feeding in chinook salmon fry	 64
Introduction.....	65
Materials and Methods.....	70
Results.....	74
Discussion.....	80
GENERAL CONCLUSIONS AND RECOMMENDATIONS.....	84

REFERENCES.....	86
APPENDICES.....	94

LIST OF TABLES

Table 1.1. Size of wild and colonized coho smolts and their hatchery counterparts prior to release.....	13
Table 1.2. Size of wild chinook salmon and of their hatchery counterparts prior to release.....	13
Table 1.3. Size of wild and colonized coho salmon and their hatchery counterparts prior to release.....	14
Table 2.1. Size of coho salmon juveniles after one month of rearing in a common environment.....	45
Table 2.2. Size of chinook salmon juveniles after one month of rearing in a common environment.....	45
Table 2.3. Size of coho salmon after 6 months rearing in a common environment.....	45

LIST OF FIGURES

Figure 1.1. Mean plasma cortisol concentrations in May, 1990 of migrating wild chinook smolts and of their hatchery counterparts prior to release.....	21
Figure 1.2. Mean plasma cortisol concentrations in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release.....	22
Figure 1.3. Mean plasma cortisol concentrations over the month of May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release.....	23
Figure 1.4. Mean hematocrit in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release.....	24
Figure 1.5. Mean hematocrit over the month of May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release.....	25
Figure 1.6. Mean hematocrit in May, 1990 of migrating wild and chinook smolts and of their hatchery counterparts prior to release.....	26
Figure 1.7. Mean lymphocyte, total leukocyte and thrombocyte ratios in May, 1990 of migrating wild chinook smolts and their hatchery counterparts prior to release.....	27
Figure 1.8. Mean lymphocyte, total leukocyte and thrombocyte ratios in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release.....	28
Figure 1.9. Mean lymphocyte, total leukocyte and thrombocyte ratios in May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release.....	29
Figure 1.10. Mean number of antibody producing cells leukocytes in May, 1991 of migrating wild smolts and their hatchery counterparts prior to release.....	30
Figure 1.11. Mean number of receptor sites for cortisol in May, 1991 on leukocyte whole cell preparations of 7-10 anterior kidneys of migrating wild coho smolts and of their hatchery counterparts prior to release.....	31
Figure 1.12. Total cumulative mortality (%) and time to death (d) for chinook salmon juveniles in response to a challenge by bath immersion with <i>Vibrio anguillarum</i>	32

Figure 1.13. Total cumulative mortality (%) and time to death (d) for coho salmon juveniles in response to a challenge by bath immersion with <i>Vibrio anguillarum</i>	33
Figure 2.1. Means \pm s.e. of plasma cortisol concentration of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress.....	49
Figure 2.2. Means \pm s.e. of hematocrit values of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress.....	50
Figure 2.3. Means \pm s.e. of the plasma cortisol concentration (ng/ml) of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress.....	51
Figure 2.4. Means \pm s.e. of lymphocyte (top) and leukocyte (bottom) number per 10^3 red blood cells of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress.....	52
Figure 2.5. Means \pm s.e. of hematocrit values of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress.....	53
Figure 2.6. Means \pm s.e. of the plasma cortisol concentration of hatchery, wild and colonized chinook salmon juveniles in response to a 30-60 sec handling stress after 6 months in a common rearing environment.....	54
Figure 2.7. Means \pm s.e. of hematocrit values of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment.....	56
Figure 2.8. Means \pm s.e. of leukocyte number per 10^3 red blood cells of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment.....	57
Figure 2.9. Means \pm s.e. of antibody producing cell (APC) number per 10^6 leukocytes in the anterior kidney of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment.....	58
Figure 2.10. Cumulative mortality (%) and mean time to death (d) of hatchery, wild and colonized coho salmon juveniles in response to a bath immersion challenge with <i>V. anguillarum</i> after 6 months rearing in a common environment....	59

Figure 3.1. Means + 1 s.e. of the number of antibody producing cells (\overline{APC})/million viable leukocytes in the head kidney of chinook salmon fry fed either live or frozen krill or a commercially prepared diet75

Figure 3.2 Means + 1 s.e. of lysozyme activity (U) and concentration ($\mu\text{g}/\text{ml}$) in the plasma of chinook salmon fry fed either live or frozen krill, or a commercially prepared diet76

Figure 3.3 Hematological profile of chinook salmon fry fed either live or frozen krill or a commercially prepared diet (OMP).....77

Figure 3.4. a; Means + 1 s.e. of hematocrit and b; condition factor ($\overline{C.F.}$) of chinook salmon fry fed either live or frozen krill(*E. pacifica*), or a commercially prepared diet78

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General Introduction

Intensive aquaculture is used as a tool in fisheries management. The release of large numbers of intensively cultured juveniles to supplement wild fish stocks is a common practice in North America. Over the last decade, survival rates (smolt to adult) of coho (*Oncorhynchus kisutch*) and chinook (*O. tshawytscha*) hatchery-reared salmon smolts have declined (McIntyre, 1985). Survival of wild coho salmon is reported to be 1.5 to 2 times higher than in their hatchery counterparts (Felton et al., 1990). A similar comparison involving chinook salmon on the Lewis River, Washington, (a tributary of the Columbia River) reported that wild fish had average survival rates 2.6 times higher those of hatchery fish (Felton et al., 1990). These higher survival rates imply that differences between hatchery-reared and wild fish may significantly affect the survival of hatchery fish in the wild.

Differences between wild and hatchery fish have been observed with respect to their morphology (Hjort and Schreck, 1982), behavior (Smith, 1987), and physiology (see Iwama et al., 1990, Shrimpton et al., 1990, Woodward and Strange, 1987). There may be a physiological advantage to rearing fish in the wild environment. Reisenbichler (1988) found that stocks of salmon transferred into environments other than those in which they were locally adapted performed poorly in comparison to indigenous populations and that wild fish from different river systems were more similar to each other than a wild stock and its hatchery

counterpart. A fish that is reared in the wild may be physiologically adapted to survive those conditions, and, thus, preferentially suited to this environment over others, including that of the hatchery.

A series of experiments were conducted to test the hypothesis that rearing conditions in hatcheries could affect the survival of hatchery salmon smolts at the time of seawater entry. The effects of rearing history on cortisol release, immune function and on disease resistance were examined in hatchery-reared and wild chinook salmon and in hatchery-reared, wild and colonized coho salmon.

The first set of experiments, described in Chapter 1, was a series of field experiments designed to describe the physiological state of salmonid smolts reared under different conditions just before entry into seawater. Endogenous cortisol concentration, immune function, and disease resistance were examined. The second set of experiments, described in Chapter 2, examined the effect of stress-induced increase in plasma cortisol concentration on immune function and disease resistance in fish with different rearing histories. The consistency of these responses following the transfer into and subsequent rearing of wild, colonized, and hatchery fish in common environmental conditions was also examined. The third set of experiments, described in Chapter 3, was designed to examine food type (live or prepared) as one aspect of intensive culture different from natural rearing that may influence survivorship. The possible effects of feeding a live diet as opposed to a

commercially prepared diet to chinook salmon fry were studied.

By undertaking these experiments, the hypothesis that physiological differences between salmon reared in the natural environment and their hatchery-reared counterparts may be influence the survival of hatchery releases in the wild was tested. Several objectives were intended; these were: 1) to determine the effect of early rearing history on endogenous cortisol concentration, immune function and disease resistance in salmonid juveniles at the time of seawater entry; 2) to examine the cortisol stress response and its effect on specific immune function and disease resistance in these fish; and 3) to examine the effect of different diet types on specific and non-specific immune defense in salmon fry.

Chapter 1

Effects of early rearing history on the cortisol response and specific immune function in chinook and coho salmon smolts.

Introduction

Rearing history is believed to play a major part in determining ocean survival of stocked hatchery salmon. Fagerlund et al. (1983) found that return rates of adult coho salmon were inversely proportional to their hatchery rearing density. Furthermore, Hosmer (1979) found that Atlantic salmon (*Salmo salar*) reared in water with a reduced flow rate had significantly reduced rates of returning adults. The fish reared in Salmonid Enhancement Program (SEP) hatcheries are assumed to be genetically similar to their wild counterparts because of the policy of SEP hatcheries to randomly select brood stock from returning adults. Any physiological differences observed are thought to be due to the divergence of physical conditions in the artificial rearing environment as compared to the natural environment. For example, differences in conditions such as the temperature of the rearing water, dissolved oxygen concentrations, or rearing densities, or the presence of suspended solids may be important (see Appendix A for examples of conditions in SEP hatcheries).

Pickering (1989) suggested that conditions, such as those listed above, and those resulting from human perturbations rarely occur in a natural environment, and can cause a condition of chronic stress within a hatchery population. In the wild, variable conditions are the rule. Warren (1985) suggested that smolt quality must be defined as conformance to requirements. In that regard, the character of fish desired then depends on the

objective of the artificial propagation program. If one wishes to produce smolts that have high rates of survival in the natural environment, they should resemble those wild fish that presumably are more successful at doing so.

In physiological comparisons and performance tests, the effect of prior rearing on certain parameters will be affected by the present environment in which the fish are held (Schreck, 1981). Thus, the study of the physiology of wild fish will be affected by holding them in an artificial environment. In the following field experiments comparing naturally-reared and hatchery-reared juvenile salmon, the different environmental rearing conditions may be considered as the treatments. Performance tests, such as a disease challenge, may be used to perturb the equilibrium state in order to discern possible differences in physiological states.

In the spring of 1990, physiological differences in corticosteroid concentrations and immune function were examined in smolting populations of chinook and coho salmon reared in different rearing environments. Physiological differences were once again examined in 1991 in the same coho salmon population. The three rearing histories examined were wild rearing, colonized rearing, and hatchery rearing. Wild smolts were fish that developed from eggs spawned naturally in the river. Hatchery smolts were fish artificially propagated from eggs of returning adults in the river and maintained for the entire rearing period in a hatchery before smolt migration. Colonized (or outplanted) fish were of hatchery origin but were transported and released in

the upper watershed of the river as fry. At the time of smolt migration, colonized smolts will have spent the larger proportion of their early rearing history in the natural environment. Colonization occurred in an area of the upper watershed where the outplanted fish were physically separated from the wild fish to minimize competition between these groups. The management practice of colonization was undertaken within the coho salmon population, affording the opportunity to examine fish with three different rearing histories in 1990. In 1991 however, comparisons were made only between wild and hatchery coho smolts as very few colonized fish were obtained during the month of sampling. No colonization was undertaken by hatchery management in the chinook salmon population under study.

Physiological differences between wild and hatchery salmon may be the cause of differing relative success rates of hatchery-reared and naturally-reared salmon to survive in the wild. One aspect that affects survival rates of fish is disease. The ability to generate an immune response directly affects the health of the fish, and hence is closely related to their ability to resist disease. Fish have been shown to be more susceptible to disease during periods of adverse environmental conditions and at the time of smoltification (Wedemeyer et al., 1984). Certain hormonal changes, such as those that occur during the physiological process of smoltification, have been shown to affect the immune system, and cause changes to haematopoietic tissues and cells (Ellis, 1981).

During the period leading up to seawater entry, there is

an increase in the concentration of endogenous corticosteroids in the plasma of juvenile salmonids (Barton et al. 1985; Young, 1989), affecting physiological changes such as increased Na^+ and Cl^- secretion, water permeability and ATPase activation on the gill (for review see Folmar and Dickoff, 1980). The primary corticosteroid of teleost fish is cortisol (Barton and Iwama, 1991; Patiño et al., 1987). During the period of smoltification, interrenal cells producing cortisol in the anterior kidney undergo hypertrophy and increase the secretion of this hormone (Young, 1986). Increased secretion of cortisol is also associated with the response to stress (Patino et al, 1987). This elevation in plasma cortisol has been shown to result in various types of immunosuppressive effects in teleosts (McLeay, 1975; Ellsaesser and Clem, 1986; Tripp et al., 1987, Maule et al., 1987).

It is generally considered that endocrine receptors are involved in the integration of the endocrine and immune systems (Coffey and Djeu, 1986). The effects of corticosteroids on the cells of the immune system and the target tissues involved in the physiological changes of smoltification in salmon may be mediated similarly by specific corticosteroid receptors of these cells (Maule, 1989). During smoltification, changes in the affinity and number of these receptors on leukocytes in the anterior kidney have been observed (Maule, 1989). The net effect of exposure to cortisol was an increase in the number of these receptors on leukocytes and may be responsible for the induction of immunosuppression at this physiological state in development

(Maule, 1989). Differences in changes affected by cortisol may depend not only on the magnitude of the measurable circulating cortisol response, but on the sensitivity of the target tissues to cortisol (Pickering et al, 1989).

There is evidence that the regulation of cortisol secretion is mediated by negative feedback of cortisol on the hypothalamic-pituitary-interrenal axis (Fryer and Peter, 1977). Continued cortisol secretion from the interrenal cells will inhibit further interrenal response (Donaldson et al., 1981). Exposure to chronic stress has been shown to cause a decrease in immune function despite acclimation of the fish to the stressor. Fish that were chronically stressed had levels of circulating cortisol that were lower than those of the unstressed fish, while suppression of immunological defense systems continued (Pickering and Pottinger, 1989).

The first objective in this chapter was to establish the magnitude of plasma cortisol changes as it relates to smoltification in naturally-reared and hatchery-reared juvenile salmonids. The second objective was to assess immune function and disease resistance at the time of smoltification, and to determine the relationship between endogenous cortisol concentration and immune function at that time.

The hypothesis tested was that enhanced interrenal activity over the period of early rearing in the hatchery environment effects changes to interrenal tissue and their function, resulting in a down regulation of these systems. This process may reduce the capacity of hatchery salmon in a variety of ways

to respond to challenges they must overcome in order to survive, and, thus, be a potentially major determinant of survival at the time of seawater entry.

Materials and Methods

Fish

On May 16, 1990, juvenile wild chinook salmon smolts were netted from the Big Qualicum River during their nocturnal migration as they passed through open gates in a fish counting fence. Colonized fish were distinguished from wild fish on the basis of size. The following day, their hatchery counterparts were randomly sampled from the concrete raceways of the Big Qualicum River fish hatchery, Qualicum Beach, British Columbia (Department of Fisheries and Oceans, Canada).

On May 26, 1990 and on May 10, 19, and 25, 1991, juvenile wild and colonized, coho salmon smolts and their hatchery counterparts were sampled as described above, from the Quinsam River and Quinsam River fish hatchery, near Campbell River, British Columbia.

In 1990, approximately 1000 wild and hatchery chinook salmon and 1000 wild, colonized and hatchery coho salmon were brought to the Aquaculture Unit of the Department of Animal Science at the University of British Columbia. Wild, colonized and hatchery fish were held separately in 1000-L oval tanks continuously supplied with dechlorinated Vancouver tap water (7-8°C, pH 6.1, total hardness 4.2 mg/L, [O₂] 10 ppm). Fish were fed a maintenance diet of EWOS grower pellets (EWOS Canada, Surrey, B.C.) at 1 to 2 % body weight daily. Wild fish were initially fed on freeze dried krill (Argent Laboratories,

Richmond, B.C.) and gradually introduced to pellets over a period of two weeks. In July, 1990 these fish were subjected to disease challenge experiments.

Experimental procedures and sampling

Fish were rapidly netted from their respective environments (naturally-reared fish from the river, hatchery-reared fish from their rearing channels) and transferred to a bucket containing a lethal dose (200 mg/l) of buffered tricaine methanesulfonate (MS222). This dose has been shown to inhibit a further rise in plasma cortisol concentration in salmon after anesthetization (Barton et al., 1986). After the fish were anesthetized, the caudal peduncle was severed and the blood was collected in heparinized capillary tubes. The plasma was separated by centrifugation within 15 min of collection and stored at -20°C for determination of plasma cortisol concentration. Fork length (Ln) and weight (W) were measured and used to calculate condition factor(K) using the following formula $K = [W(g)/Ln^3(cm) \times 100]$ (Pickering and Duston, 1983). The basic body morphology of fish used is presented in Tables 1, 2 and 3.

Table 1.1. 1990. Mean \pm S.E. of weight, length and condition factor of migrating wild and colonized coho salmon smolts and of their hatchery counterparts prior to release, n=32.

Group	Weight (g)	Length (cm)	condition factor
Hatchery	24.1 \pm 1.0 ^a	13.7 \pm 0.2 ^a	0.93 \pm 0.01 ^a
Wild	8.7 \pm 0.5 ^b	9.4 \pm 0.2 ^b	0.95 \pm 0.02 ^a
Colonized	17.5 \pm 0.8 ^c	12.5 \pm 0.2 ^c	0.91 \pm 0.02 ^a

note: Values with superscripted letters in common are not significantly different between groups for each variable ($p \geq 0.05$).

Table 1.2. 1990. Mean \pm S.E. of weight, length and condition factor of migrating wild chinook salmon smolts and of their hatchery counterparts prior to release, n=32.

Group	Weight (g)	Length (cm)	condition factor
Hatchery	6.1 \pm 0.2 ^a	8.3 \pm 0.1 ^a	1.08 \pm 0.01 ^a
Wild	3.2 \pm 0.1 ^b	6.8 \pm 0.1 ^b	1.01 \pm 0.06 ^a

note: Values with superscripted letters in common are not significantly different between groups for each variable ($p \geq 0.05$).

Table 1.3. 1991. Mean \pm S.E. of weight, length and condition factor of migrating wild and colonized coho salmon smolts and of their hatchery counterparts prior to release $n=24$, except for the colonized group ($n=6$).

Group	Weight (g)	Length (cm)	condition factor ($W/Ln^3 \times 100$)
Hatchery			
May 10	21.9 ± 1.4^a	13.1 ± 0.2^a	0.95 ± 0.01^a
May 19	23.3 ± 1.6^a	13.3 ± 0.2^a	0.98 ± 0.01^a
May 25	26.0 ± 1.7^a	13.8 ± 0.3^a	0.96 ± 0.01^a
Wild			
May 10	14.1 ± 0.6^b	11.4 ± 0.2^b	0.94 ± 0.01^a
May 19	10.6 ± 0.6^b	10.3 ± 0.2^b	0.97 ± 0.02^a
May 25	9.3 ± 0.6^b	9.8 ± 0.2^b	0.98 ± 0.01^a
Colonized*			
May 10 ($n=6$)	20.8 ± 1.7^a	13.0 ± 3.0^a	0.94 ± 0.02^a

note: Values with superscripted letters in common are not significantly different between groups for each variable ($p \geq 0.05$).

* In 1991, insufficient numbers of colonized fish were netted from the river to provide adequate samples for further experimentation.

Plasma cortisol determination

Plasma cortisol was determined by radioimmunoassay [^{125}I] (Baxter Healthcare Corporation, Cambridge Massachusetts. Clinical Assay No. 529) based on competitive binding principles (Foster and Dunn, 1974).

Hematology

Hematocrit values were determined as percent packed cell volume after centrifugation for 5 minutes at 11,500 revolutions per minute (RPM); Model MB microhematocrit centrifuge, International Equipment Co., Needham Heights, Ma., U.S.A.). The ratio of total leukocyte, lymphocyte, and thrombocyte to 1000 erythrocytes was manually determined from Wright-Giemsa stained blood smears by counting erythrocytes (average 637 per slide, range 475 to 802), lymphocytes and other leukocytes in 9 random fields per slide (i.e. per fish) using Yasutake and Wales (1983) as a reference.

Assay of specific immune response *in vitro*

To assess the ability of lymphocytes to generate specific antibodies, we established cell cultures of leukocytes as described by Tripp et al. (1987) and Maule et al. (1989). Duplicate 50 μl samples of anterior kidney tissue homogenate containing 2×10^7 leukocytes/ml were incubated for 7 d in 96

well microtitre plates with 50 μ l tissue culture media (TCM) containing the antigen trinitrophenalated (TNP)-lipopolysaccharide (LPS) or TCM only (negative controls). See Appendix B for recipes and a detailed protocol for this assay, and Appendix C for the conjugation technique of TNP to LPS. When immunized against TNP-LPS, the lymphocytes secreting anti-TNP antibody can be detected by the Cunningham plaque assay (Cunningham and Szenberg, 1968). At the time of the plaque assay, a 50 μ l aliquot of the cell culture suspension in each well was mixed with 10 μ l coho salmon serum complement and 10 μ l of TNP-tagged sheep red blood cells (SRBC) and put in sealed Cunningham chambers at 17°C for 2h. The antigen-antibody complexes activate the complement cascade and lyse the SRBC membranes, causing the appearance of holes in the lawn of TNP-tagged SRBC, each hole corresponding to an antibody producing cell (APC). The number of viable leukocytes at the time of the assay was determined using a hemacytometer and trypan blue dye exclusion. Results were expressed as number of APC per million viable leukocytes. (see Appendix B for details).

Corticosteroid receptor assay

This assay is based on competitive binding of a saturable, high affinity, low capacity binding of radio-labelled [3 H] triamcinolone acetonide (TA), a synthetic cortisol, and cold TA. The whole leukocyte binding study was carried out according to the procedure of Maule and Schreck (1990). Tissues were

harvested from the anterior kidney as previously described (Tripp et al., 1987). Tissue homogenates were obtained from up to 10 coho salmon smolts per sample in order to obtain enough cells to carry out the assay. The final concentration of leukocytes in a suspension of TCM (Appendix B) was $3.0-4.6 \times 10^7$ leukocytes/ml. To determine the number of high affinity cortisol receptors, samples were incubated in ^3H TA or 100-fold excess cold TA and radio-labelled TA. Total binding was determined by combining 0.1 ml of the leukocyte suspension, and 0.05 ml TCM, and 0.05 ml ^3H TA dissolved in TCM and incubating for 2 h. Non-specific binding was determined by incubating 0.01 ml of the leukocyte suspension in 0.05 ml excess cold TA for 1 h and then with ^3H TA for an additional 2 h. Specific binding was determined by subtracting non-specific binding from the total binding. The final incubation volume was 0.2 ml and contained $4-10 \times 10^6$ cells and 0.5-2.0 nM ^3H -TA. Samples were counted on a LKB rack beta scintillation counter, model 1214. A computer software program (McPherson, 1985) was used to analyze the binding data to obtain the maximum number of sites (N_{max}) per leukocyte based on Scatchard plot analyses (Scatchard, 1949).

Disease challenge test

Duplicate treatment groups of fish were exposed to *Vibrio anguillarum* (Strain RI, T.P.T. Evelyn, Pacific Biological Stn., Nanaimo, B.C.) by the bath immersion method (Gould et al., 1979) at concentrations of 1×10^7 colony forming units (cfu)/ml for

the chinook challenge (mean water temperature, 8°C) and 5×10^6 cfu/ml for the coho challenge (mean water temperature, 8°C). Control groups were treated similarly, but without the addition of *V. anguillarum* to the immersion bath. At the time of the challenge, the cell mass of *V. anguillarum* in culture was estimated by its optical density at 540 nm. A quantitative estimation of the concentration of bacteria in solution was determined by plate counts (Ramey, 1985). Mortalities were removed daily and deaths due to the pathogen were confirmed by isolation of *V. anguillarum* from the kidney on trypticase soy agar (TSA) plates (4% TSA (Difco), 0.5% NaCl). Data were expressed as percent total mortality and mean time to death.

Data analysis

Data were subjected to analysis of variance and where significant differences were found, the Tukey test (Steel and Torrie, 1990) was used to identify significantly different treatment means. Cortisol receptor values were compared with a Student's t test (Duncan et al., 1983). These analyses were performed with the SYSTAT statistical program (Wilkinson, 1988). As the mortality in the duplicate treatments of the disease challenges was not significantly different within groups, the results were pooled and then analyzed using contingency tables (chi-square analysis) to test for independence of the means (Duncan et al., 1983). Statistical significance was taken at the 5 % level in all tests.

Results

In 1990, migrating wild chinook salmon smolts had significantly higher plasma cortisol concentrations than their hatchery counterparts (Fig. 1.1). Migrating wild and colonized coho smolts had higher plasma cortisol concentrations than their hatchery counterparts but these differences were not significant (Fig. 1.2). In 1991, the elevation in concentration of plasma cortisol in the hatchery coho smolts was transient, and by May 19 and 25, concentrations were significantly lower than that of the wild smolts (Fig. 1.3).

Hematocrit values were significantly lower in hatchery-reared coho salmon smolts than the naturally-reared 1990 coho salmon smolts (Fig. 1.4). In 1991, the hatchery reared coho smolts had significantly lower hematocrit values by May 25, the last sampling period (Fig. 1.5) when cortisol concentrations were also lowered (Fig. 1.3). Hematocrit values were not significantly different between 1990 wild and hatchery chinook smolts (Fig. 1.6).

Ratios of lymphocyte and total leukocytes were higher in the wild chinook smolts than in their hatchery counterparts but these differences were not significant (Fig. 1.7). This trend of higher lymphocyte and total leukocytes ratios in wild fish was consistent in the 1990 coho smolts, but the colonized smolts had the lowest ratios of all coho groups (Fig. 1.8). In 1991, the trend of higher lymphocyte and total leukocyte ratios in wild fish was again consistent with those of the 1990 coho and chinook

smolts (Fig. 1.9). In 1990, hatchery coho smolts had significantly higher thrombocyte ratios than in the wild or colonized smolts (Fig. 1.8). This feature was not observed in the following year, or in the chinook smolts.

In 1991, tissues were taken to obtain a measure of specific immune response and cortisol receptor site number of anterior kidney leukocytes. Antibody producing cell (APC) number was not significantly different between wild and hatchery coho smolts, although numbers were consistently higher in the anterior kidney of the wild smolts (Fig. 1.10). The number of receptor sites (N_{\max}) for cortisol on leukocytes in wild smolts was almost two times that found in the hatchery smolts (Fig. 1.11).

Salmon stocks brought into a common environment at U.B.C. for one month showed no difference in total mortality in response to the disease challenge with *V. anguillarum* between the wild and hatchery chinook smolts (Fig. 1.12) or the wild, colonized or hatchery coho smolts (Fig. 1.13). This challenge was not used in 1991 because no discernible differences in survival were seen between hatchery-reared and naturally-reared smolts of either stock, and later challenges with the coho juveniles indicated that disease resistance in the wild and colonized fish were adversely affected by the rearing environment (Figure 2.7).

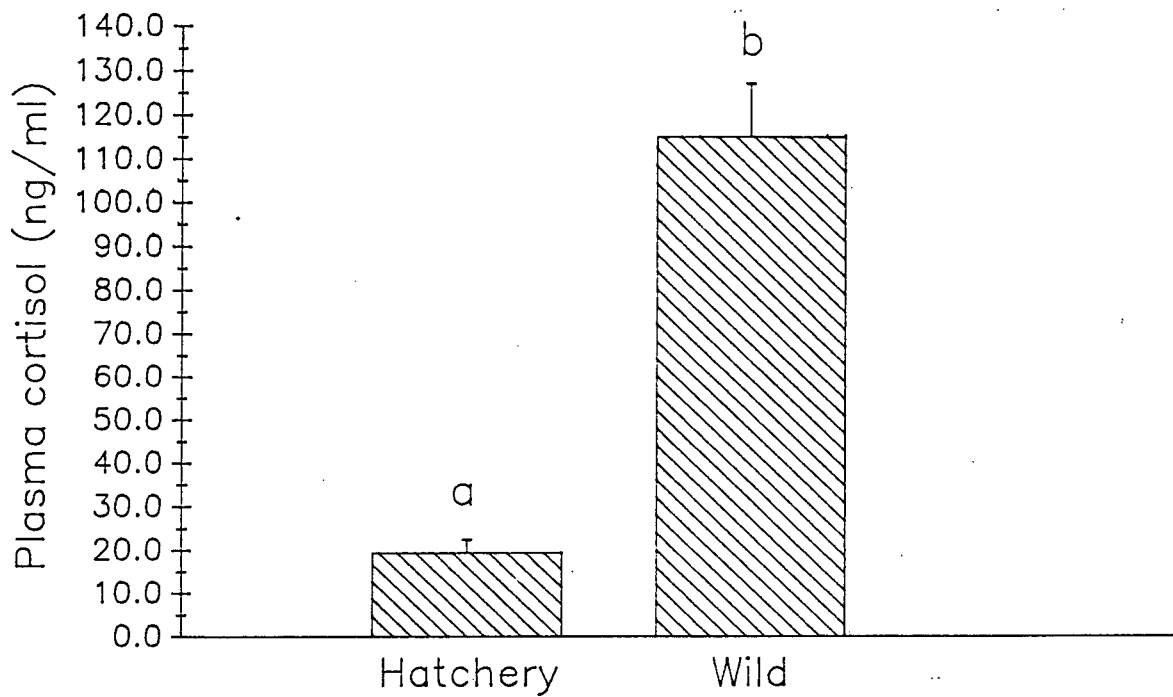


Figure 1.1. Mean plasma cortisol concentrations in May, 1990 of migrating wild chinook smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=32$.

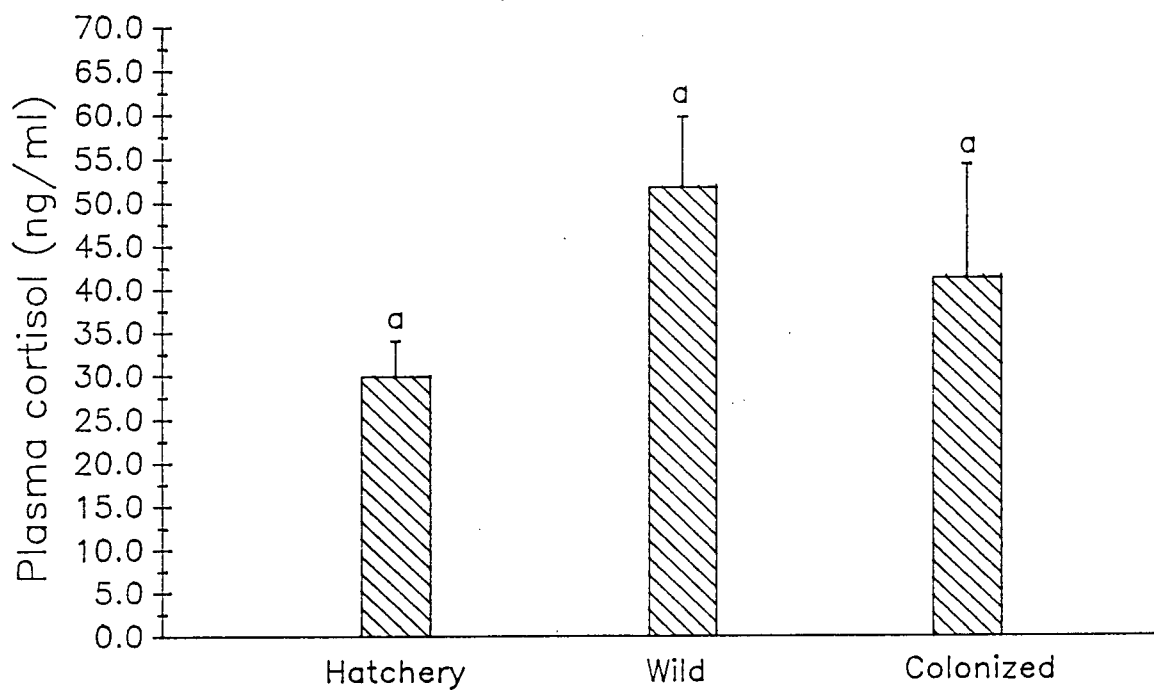


Figure 1.2. Mean plasma cortisol concentrations in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=32$.

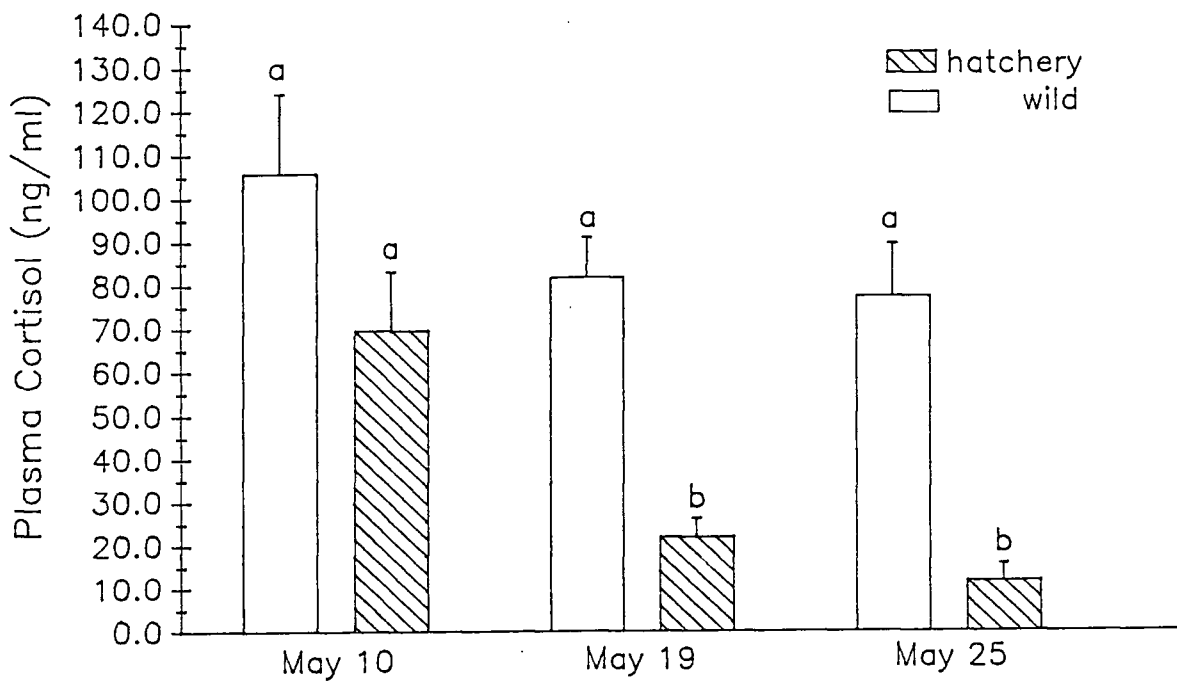


Figure 1.3. Mean plasma cortisol concentrations over the month of May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=10$.

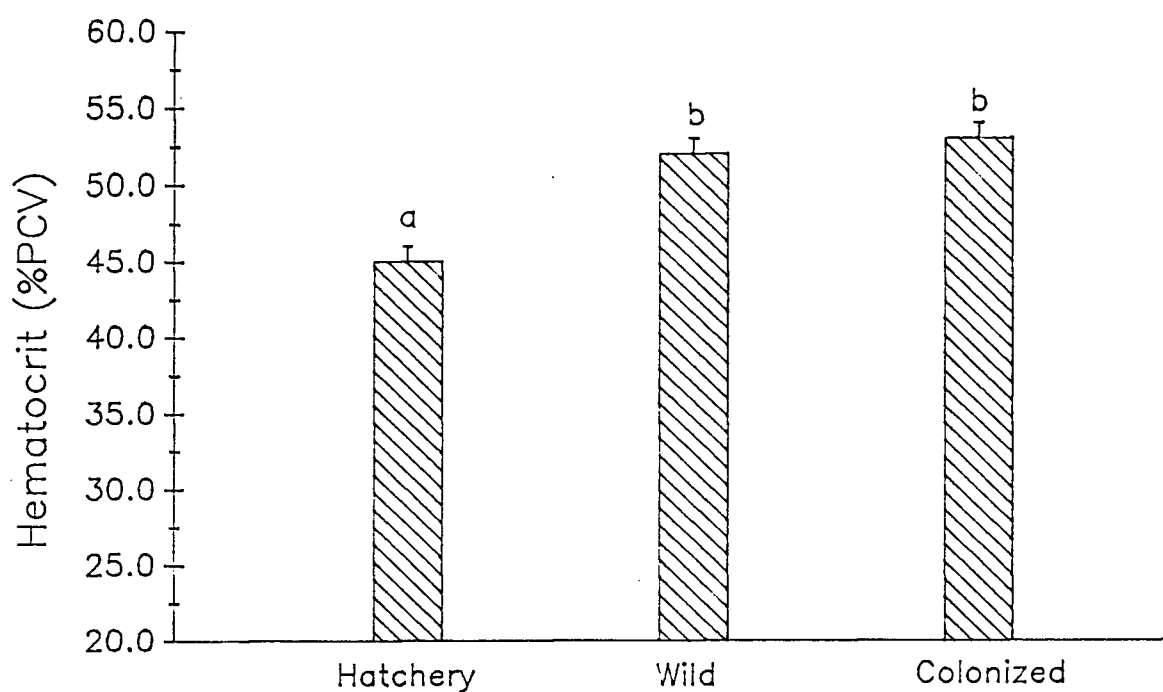


Figure 1.4. Mean hematocrit (% packed cell volume) in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=32$.

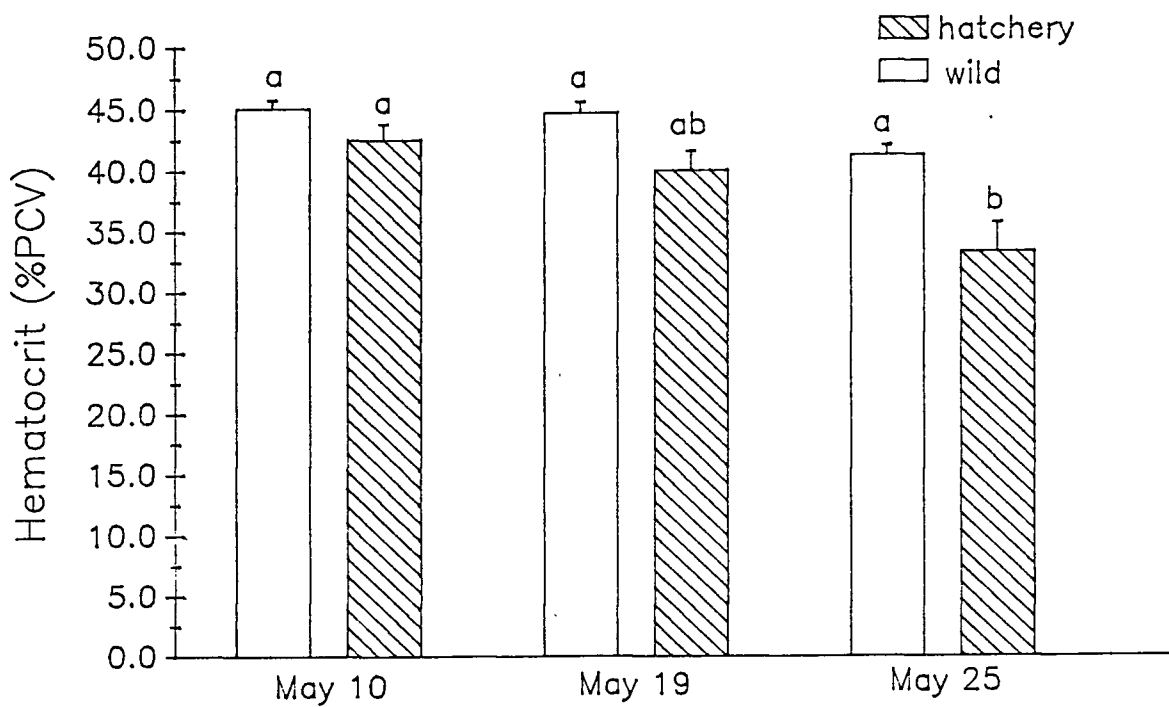


Figure 1.5. Mean hematocrit (% packed cell volume) over the month of May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=24$.

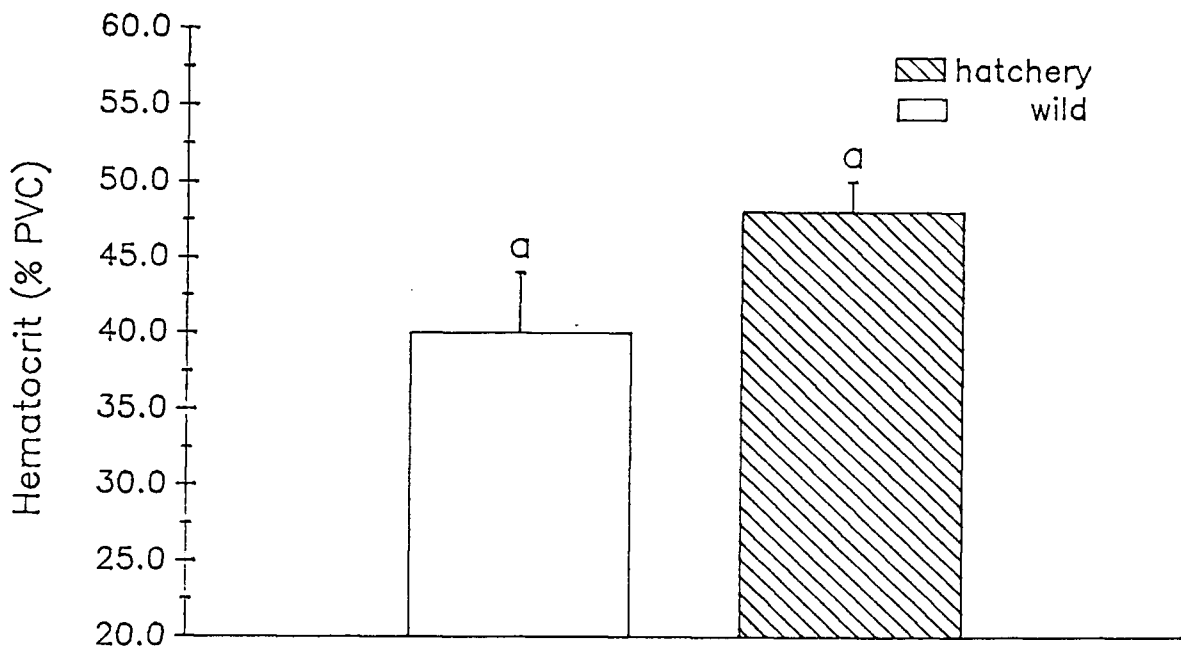


Figure 1.6. Mean hematocrit (% packed cell volume) in May, 1990 of migrating wild and chinook smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=32$.

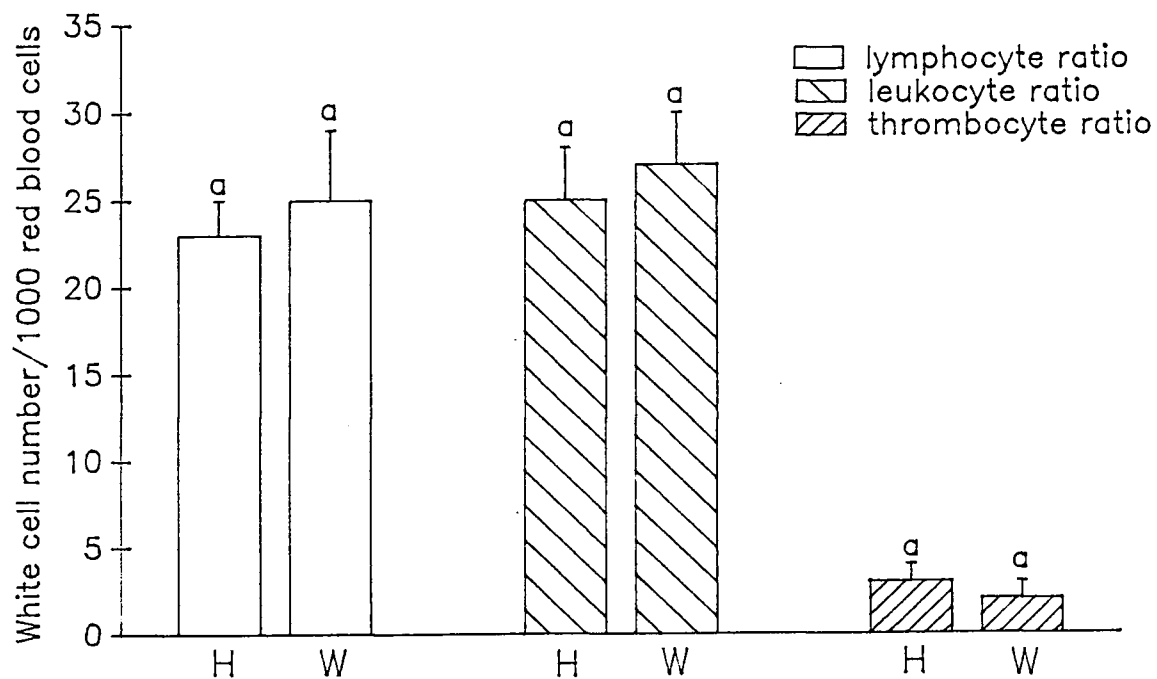


Figure 1.7. Mean lymphocyte, total leukocyte and thrombocyte ratios (no. cells per 10^3 erythrocytes) in May, 1990 of migrating wild chinook smolts and of their hatchery counterparts prior to release, \pm 1 s.e. Values with different superscripts are significantly different, $n=12$.

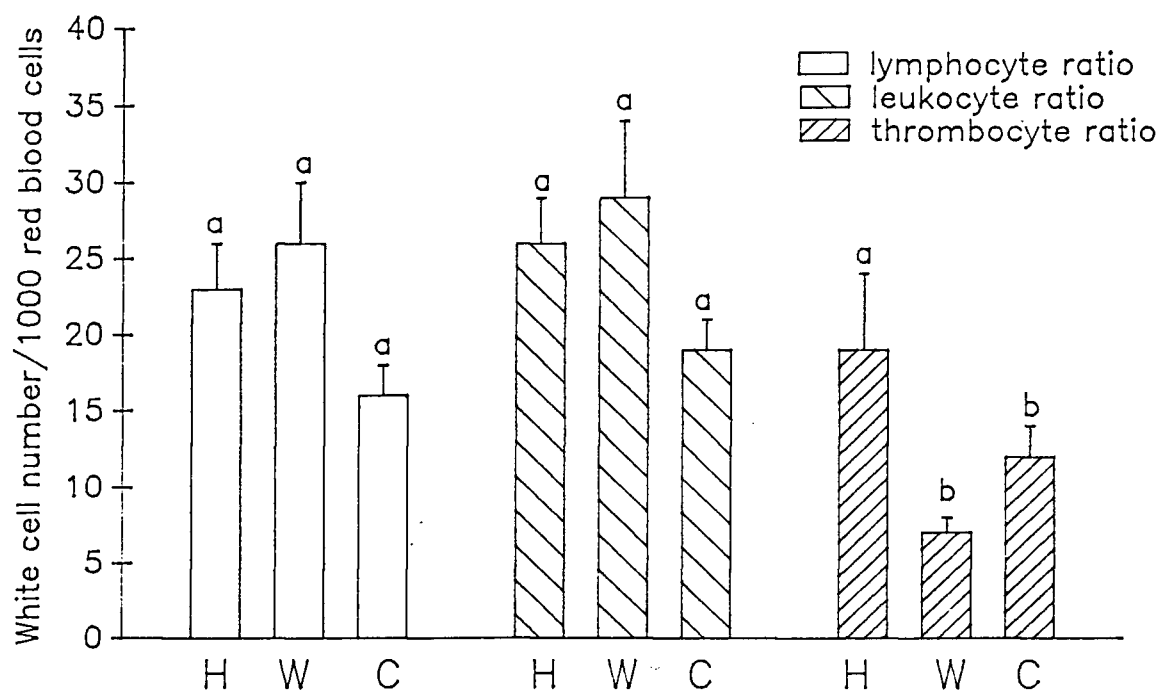


Figure 1.8. Mean lymphocyte, total leukocyte and thrombocyte ratios (no. cells per 10^3 erythrocytes) in May, 1990 of migrating wild and colonized coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=12$.

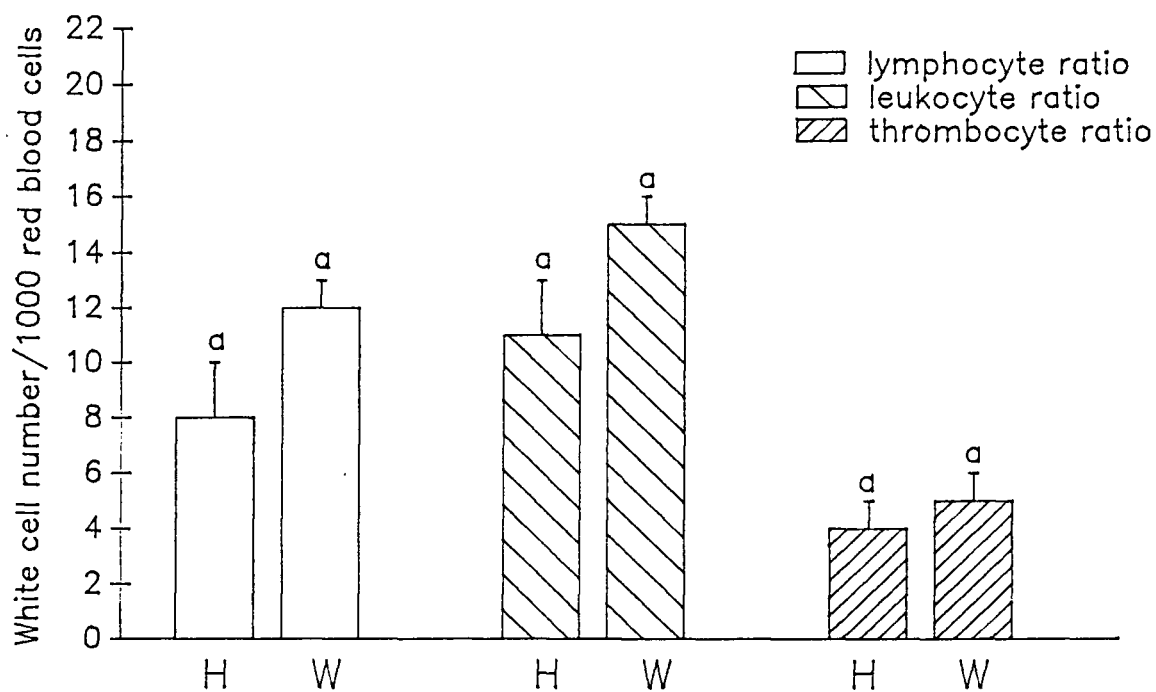


Figure 1.9. Mean lymphocyte, total leukocyte and thrombocyte ratios (no. cells per 10^3 erythrocytes) in May, 1991 of migrating wild coho smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=12$.

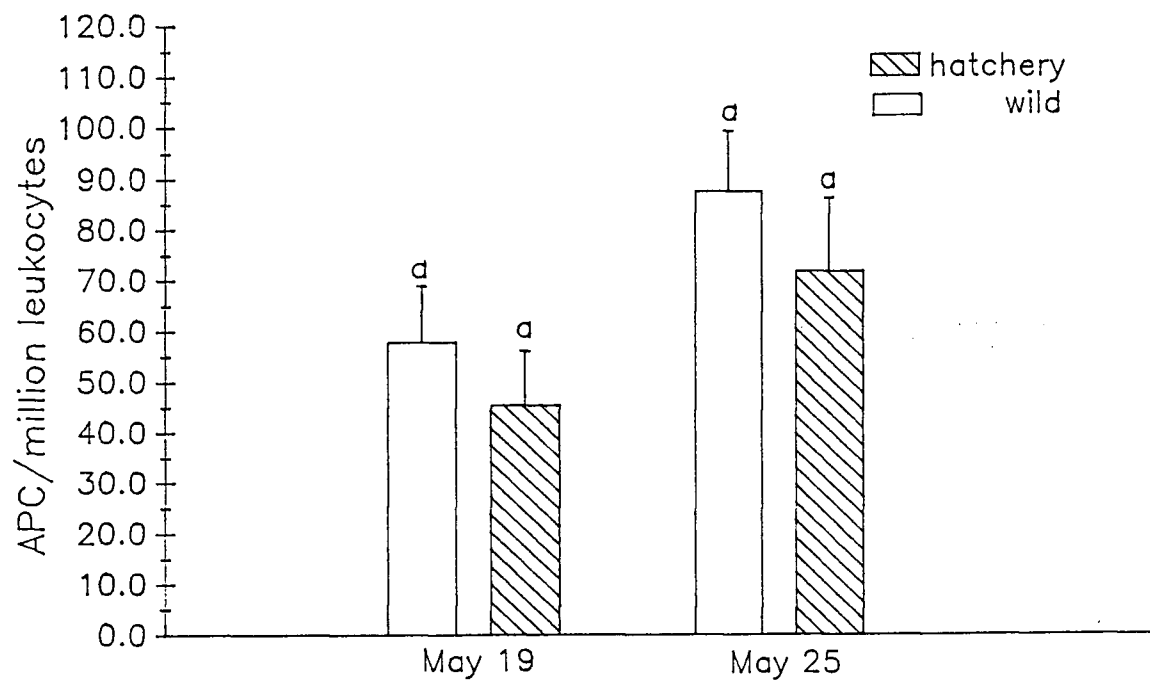


Figure 1.10. Mean number of antibody producing cells (APC)/ 10^6 leukocytes in May, 1991 of migrating wild smolts and of their hatchery counterparts prior to release, ± 1 s.e. Values with different superscripts are significantly different, $n=12$.

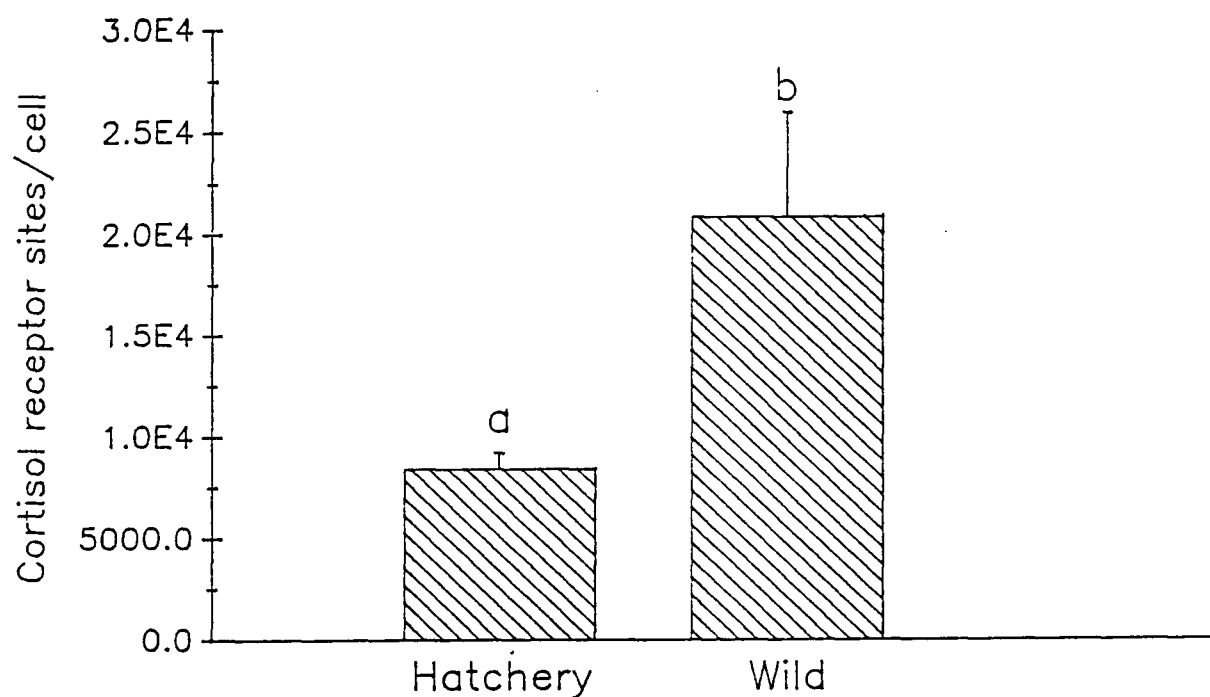


Figure 1.11. Mean number of receptor sites for cortisol in May, 1991 on leukocyte whole cell preparations of 7-10 anterior kidneys of migrating wild coho smolts (n=2) and of their hatchery counterparts (n=3) prior to release, ± 1 s.e. Values with different superscripts are significantly different.

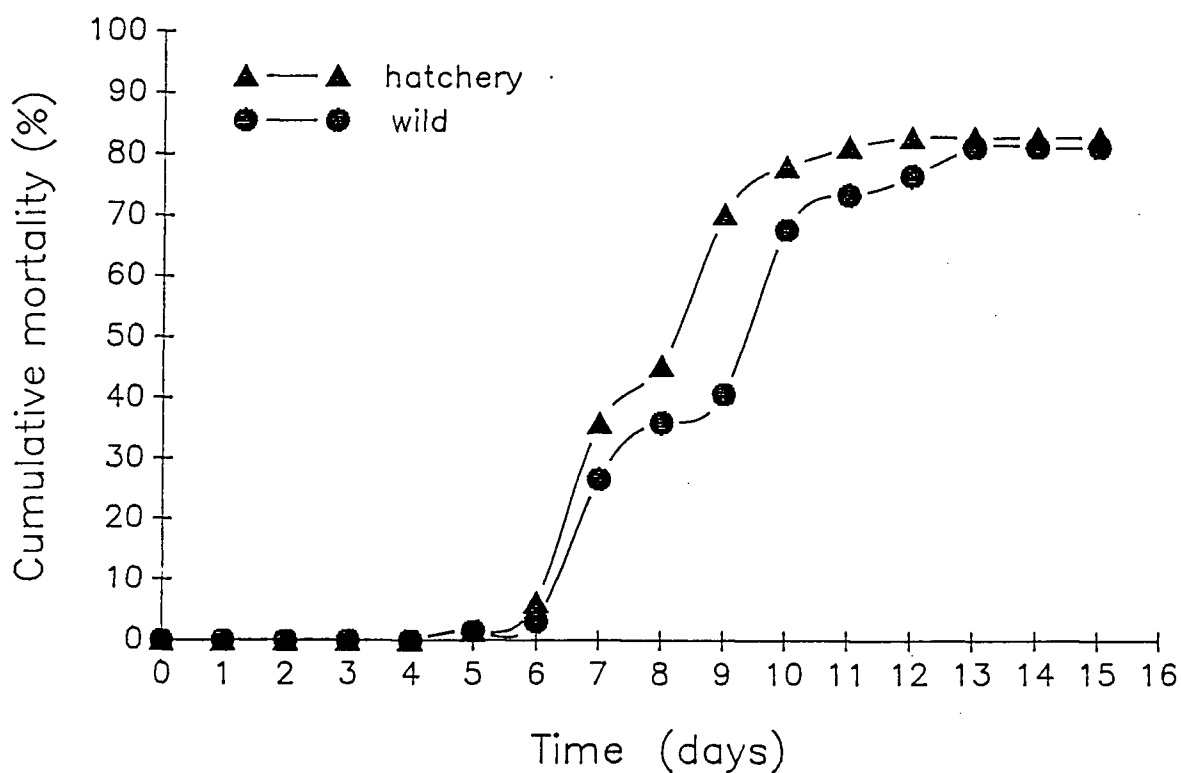


Figure 1.12. Total cumulative mortality (%) and time to death (d) for chinook salmon juveniles in response to a challenge by bath immersion with 1×10^7 cfu/ml of *Vibrio anguillarum*. Data represent means of pooled duplicate treatments, $n=64$. Values are not significantly different.

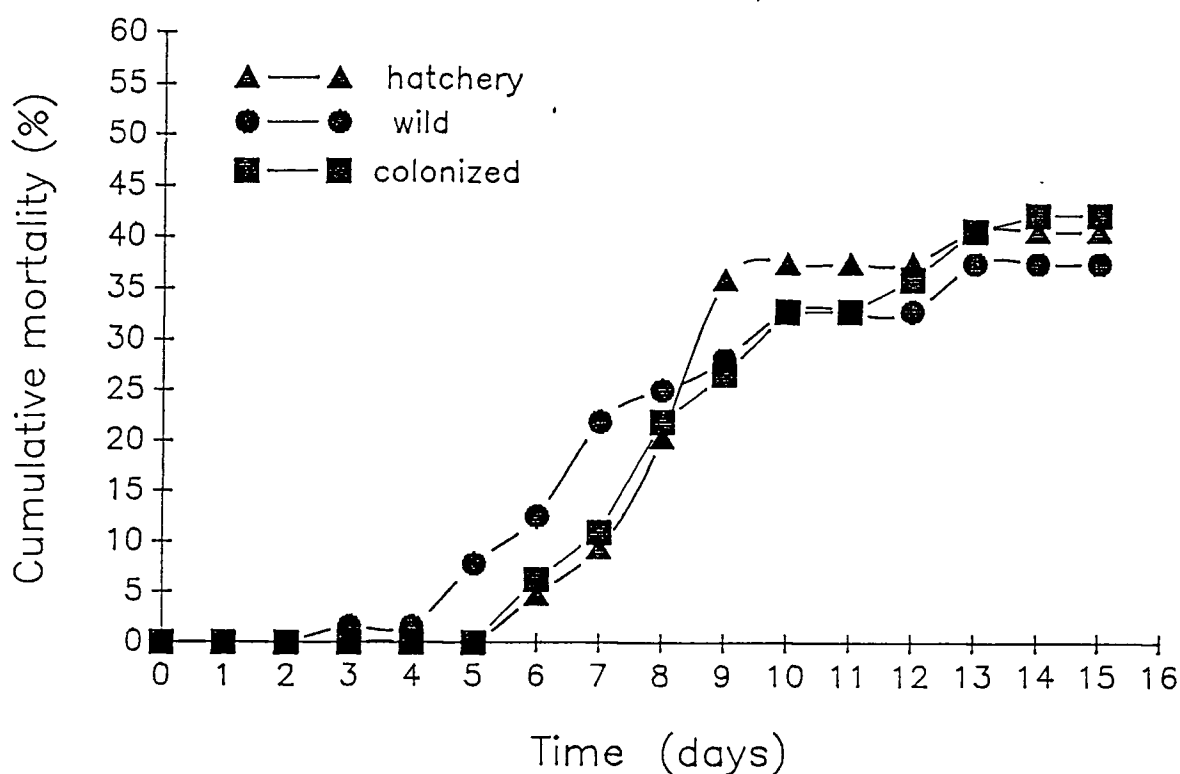


Figure 1.13. Total cumulative mortality (%) and time to death (d) for coho salmon juveniles in response to a challenge by bath immersion with 5×10^6 cfu/ml of *Vibrio anguillarum*. Data represent means of pooled duplicate treatments, $n=64$. Values are not significantly different.

Discussion

The significant differences in plasma cortisol concentrations between both wild and colonized smolts and the hatchery smolts are too large to assume that this change is precipitated by the difference in sampling time (wild and colonized fish migrated across the fish counting fence at night, necessitating the sampling at this time and hatchery fish were sampled the following day to keep the sampling time of these fish as close as that of the wild fish). It is more likely that higher plasma cortisol concentrations are attributable to physiological differences between the wild and colonized smolts and their hatchery-reared counterparts. Several possible explanations for differences seen in the levels of plasma cortisol between wild and colonized smolts and their hatchery-reared counterparts can be ruled out. First, differences cannot be attributed to differences in body weight of the fish. Other workers have found no correlation between individual fish weight, interrenal response, and plasma cortisol concentrations in salmonid smolts (Barton et al, 1985; Young, 1986; Mazur, 1991). Second, differences cannot be attributed to diurnal variations in plasma cortisol concentrations. Thorpe et al. (1986), found diel and seasonal changes in plasma cortisol concentrations in coho salmon. At certain times of the year, they found nocturnal resting plasma cortisol concentrations to be 2X higher than those measured during the day, but others have found little diurnal

difference in resting cortisol concentrations (Barton et al., 1986). Also, the seasonal elevation in plasma cortisol during smoltification has been shown to be 10-fold higher than those found at other times in the year (Young, 1989), therefore this would be more influential in causing plasma cortisol elevations. Third, differences cannot be attributed to higher plasma clearance rates of cortisol in hatchery fish. Shrimpton (pers. comm., Dept. Zoology, U.B.C.) found no difference in the metabolic clearance rates of cortisol in the same wild and hatchery fish used in this study to explain the observed divergence of plasma cortisol concentrations between the wild and hatchery fish. The difference in plasma cortisol concentration may be due to a decreased secretion of cortisol from interrenal cells of hatchery-reared fish. If hatchery practices induce stress, sustained stimulus of the interrenal cells in early rearing associated with this chronic stress may result in desensitization of the interrenal tissue, or tissue atrophy, mediated by the negative feedback inhibition of cortisol on these tissues (Ellis, 1981). This desensitization may also be affected by a reduced secretion of adrenocorticotrophic hormone from the pituitary or cortisol releasing factor from the hypothalamus (Barton et al., 1987).

The series of samples taken in the spring of 1991 from the coho salmon smolts indicated that levels of cortisol in the hatchery fish were highest on the first sampling date and were significantly lower than those of the wild and colonized fish on the last two sampling periods. Others have found that when the

migration inclination or natural state of readiness for seawater entry is ignored and fish are held back, they will lose there migratory response and revert to a parr-type physiology (Folmar and Dickoff, 1981; Schreck, 1981). Although cortisol is only one of many predictive indicators of the physiological state of smoltification, reversion may have occurred in the hatchery-reared coho smolts.

Wild smolts also had higher numbers of corticosteroid receptors on anterior kidney leukocytes than did the hatchery smolts. The levels of cortisol receptors were higher than those previously reported for other salmonids (Maule and Schreck, 1990) and this may have been because of the higher degree of non-specific binding in crude whole cell preparations. Higher non-specific binding could have elevated the absolute number of receptors present (A. Maule, pers. comm.), but would not change the relative differences in receptor numbers found between hatchery and wild fish. In coho salmon, the physiological changes associated with smoltification are thought to be mediated by increased cortisol receptor site number on the target tissues (Maule, 1989). It is interesting that both receptor site number and cortisol concentrations were higher in the wild coho smolts.

There was no significant difference found in the number of APC in the anterior kidney between wild and hatchery coho smolts. There appeared to be no relationship between increased cortisol concentration associated with the smolting response in the wild fish and decreased APC numbers in this experiment. Levels of circulating total leukocytes were also not significantly

different between the wild fish and the hatchery fish and, in fact, were slightly higher in the wild fish than those in the hatchery fish, despite the higher concentration of circulating cortisol in the wild fish. Maule and Schreck (1990), could not attribute the redistribution and number of leukocytes in lymphoid tissues solely to the action of cortisol and indicated that other factors may be involved in mediating immunological changes in response to cortisol at smoltification and following acute stress. The mechanism involved in causing immunosuppression may be different during the period of smoltification, or, alternatively, wild fish may initially have had higher numbers of these cells in circulation. Castillas and Smith (1977) also found higher initial levels of lymphocytes in populations of wild trout than those found in hatchery trout.

In order to perform disease challenges, fish were brought into and held in a common environment, necessitating the introduction of wild fish to artificial conditions. Disease challenges showed no difference in mortality between the wild and the hatchery fish. Wild fish may have been compromised in an artificial rearing environment and as such were predisposed to disease similar to the hatchery fish.

The problems of studying immune function during periods of smoltification are confounded by possible hormonal changes that accompany that process. It appears that hormonal effects could occur with differing magnitude between wild and hatchery fish. There is little known about the interactive effects of cortisol and other hormones that change during smoltification. It is

unclear if increases in plasma cortisol associated with the smolting process caused immunosuppression such as that which occurs in response to stress. It is clear, however, that there are lower levels of circulating cortisol concentrations and cortisol receptor site numbers in hatchery-reared fish as compared to fish reared in the wild. This may indicate a lower sensitivity of tissues to environmental cues resulting in a lowered response to stimuli in hatchery-reared smolts.

Chapter 2:

Effect of rearing history on the cortisol and specific immune responses to stress in juvenile chinook and coho salmon.

Introduction

Salmonid fish are sensitive to handling and respond with a stress-associated secretion of cortisol (Donaldson, 1981; Schreck, 1981; Barton and Iwama, 1991), the primary corticosteroid associated with stress in teleosts (Patiño et al., 1987). Elevation of blood cortisol has been shown to increase the susceptibility of some salmonids to infectious diseases (Wedemeyer et al., 1984; Pickering and Pottinger, 1989) and there is evidence to suggest that this effect may be mediated by suppression of the immune system by cortisol (Maule et al., 1987; Tripp et al., 1987). It has been shown that physiological levels of cortisol can cause a decrease in the levels of circulating lymphocytes (McLeay, 1975) and that the ability to generate antibody is reduced as early as 4 h after stress (Maule et al., 1989). Others have compared the stress response of wild and hatchery fish (Woodward and Strange, 1987; Iwama et al., 1991) and found marked differences.

The objective of this experiment was to examine the effect of early rearing history (ie. hatchery, wild, and colonized) on the magnitude of the stress response, as determined by changes in plasma cortisol concentration, and the secondary effects of cortisol on physiological features important to disease resistance in salmon.

A series of three experiments examined fish with different rearing histories. Experiment 1 examined the magnitude of the stress response, determined by increase in plasma cortisol

concentration of hatchery, wild and colonized juvenile coho salmon after 1 month in a common environment. Experiment 2 was similar to Experiment 1 but examined changes in hematological features in hatchery and wild chinook salmon juveniles following the stress. Experiment 3 examined the magnitude of the cortisol response and its effect on hematological parameters and specific immunological function of hatchery, wild, and colonized coho salmon juveniles after 6 months of rearing in a common environment.

Materials and Methods

Fish

In June, 1990, wild and colonized coho salmon fry, trapped from the Quinsam river, and their hatchery counterparts were transported from the Quinsam River Fish Hatchery, British Columbia and kept in 1000-L oval tanks continuously supplied with dechlorinated Vancouver tap water (pH 6.1, Total hardness 4.2 mg/L, [O₂] 10 ppm) at ambient temperatures ranging from 12°C in August to 4°C in December. Fish were fed a maintenance diet of EWOS grower pellets (EWOS Canada Ltd., Surrey, British Columbia) at 1 to 2 % body weight daily. Wild fish were started on freeze dried krill (Argent Laboratories, Richmond, British Columbia) and gradually introduced to pellets over a period of 2 weeks. In July, 1990 these fish were used in Experiment 1.

Also in June, 1990 juvenile wild chinook salmon, trapped from the Big Qualicum river, and their hatchery counterparts were transported from the Big Qualicum River Fish Hatchery and held similarly to fish in Experiment 1. After 1 month in this common environment, these fish were used in Experiment 2.

For Experiment 3, hatchery, wild, and colonized coho salmon juveniles were transferred to 200-L oval tanks with the same water source except that the temperature was maintained at a constant 9°C. Fish were allowed to acclimate to the new conditions for 2 weeks. The experiments were conducted in February, 1991, after 6 months in a common environment.

Experimental procedures and sampling

In each experiment the fish were acutely stressed by rapidly netting and holding them out of the water for 30-60 seconds and returned to their tanks. This approach has been demonstrated to evoke a large rise in plasma cortisol within a relatively short time in salmonids and other fishes (see Barton and Iwama, 1991). Fish were sampled at 8 and 30 h following the handling stress in Experiment 1. In Experiment 2, a third sampling period was added at 4 h following handling, in case elevations in plasma cortisol were being missed by sampling at 8 h post-stress. In Experiment 3, the primary objective of the experiment was to examine the effect of the rise in plasma cortisol concentration on the immune function and to see if the trend in response was similar in the wild, hatchery, and colonized fish after 6 months. For that reason only a 4 h sample was taken. At the time of sampling, fish were rapidly netted and transferred to a bucket containing a lethal dose (200 mg/l) of buffered tricaine methanesulfonate (MS222). This dose has been shown to inhibit a further rise in plasma cortisol concentration in salmon after anesthetization (Barton et al., 1986). Control fish were sampled similarly from their home tanks. After the fish were anesthetized, the caudal peduncle was severed and the blood was collected in heparinized capillary tubes. The plasma was separated by centrifugation and stored at -20°C for determination of cortisol concentration. Fork length (Ln) and weight (W) were recorded and were used to determine the condition factor (K) of

the fish by this equation ($K=W/Ln^3 \times 100$). The size of fish used in each experiment is presented in Tables 2.1, 2.2 and 2.3.

Table 2.1. Experiment 1: Mean \pm s.e. of weight, length, and condition factor of coho salmon juveniles reared one month in a common environment, n=36.

	weight (g)	length (cm)	condition factor
Hatchery	22.5 \pm 2.0 ^a	14.0 \pm 0.4 ^a	0.85 \pm 0.02 ^a
Wild	10.4 \pm 1.0 ^b	10.1 \pm 0.3 ^b	0.91 \pm 0.02 ^a
Colonized	18.6 \pm 1.4 ^a	12.5 \pm 0.3 ^c	0.95 \pm 0.03 ^a

note: Values with superscripts that are different indicates significant differences between groups, $p \leq 0.05$.

Table 2.2. Experiment 2: Mean \pm s.e. of weight, length, and condition factor of chinook salmon juveniles reared one month in a common environment, n=30.

	weight (g)	length (cm)	condition factor
Hatchery	6.9 \pm 0.3 ^a	8.8 \pm 0.1 ^a	1.0 \pm 0.01 ^a
Wild	4.8 \pm 0.2 ^b	7.8 \pm 0.1 ^b	1.0 \pm 0.02 ^a

note: Values with different superscripts indicates significant difference in that parameter between groups, $p \leq 0.05$.

Table 2.3. Experiment 3: Mean \pm s.e. of weight, length, and condition factor of juveniles reared 6 months in a common environment, n=36.

	weight (g)	length (cm)	condition factor
Hatchery	82.4 \pm 9.0 ^a	19.8 \pm 0.7 ^a	1.01 \pm 0.03 ^a
Wild	37.1 \pm 2.9 ^b	15.2 \pm 0.3 ^b	1.04 \pm 0.02 ^a
Colonized	55.3 \pm 7.7 ^b	17.2 \pm 0.8 ^b	1.02 \pm 0.02 ^a

note: Values with superscript that are different indicates significant differences between groups, $p \leq 0.05$.

Plasma cortisol determination

Plasma cortisol was determined by radioimmunoassay as previously described in Chapter 1: Materials and Methods.

Hematology

Hematocrit values and differential white blood cell counts were determined according to the methods described in Chapter 1: Materials and Methods.

Assay of specific immune response *in vitro*

In Experiment 3, the effect of the stress-related rise in plasma cortisol on the ability of lymphocytes to generate specific antibody was assessed. We established cell cultures of leukocytes as previously described (Tripp et al., 1989, Maule et al., 1989). Duplicate anterior kidney tissue samples were incubated for each fish in tissue culture media and treated as described in Chapter 1: Materials and Methods.

Disease challenge test

In Experiment 3, duplicate treatment groups of fish not subjected to the handling stress were exposed to *Vibrio anguillarum* at a concentration of 1.3×10^6 cfu/ml as described

in Chapter 1: Materials and Methods.

Data analysis

All data were subjected to analysis of variance and where significant differences were found the Tukey test (Steel and Torrie, 1980) was used to identify significant differences between treatment means. These analyses were performed with the SYSTAT statistical program (Wilkinson, 1988). As the mortality in the duplicate treatments of the disease challenge was not significantly different within groups, the results were pooled and analyzed using contingency tables (chi-square analysis) to test for independence of the means (Duncan et al., 1983). Statistical significance was taken at the 5 % level in all tests.

Results

In experiment 1, the plasma cortisol concentration of wild and colonized coho salmon was significantly elevated over control levels at 8 h. There was no significant elevation in plasma cortisol in the hatchery fish over control levels at 8 h. In all groups, except the wild group, the 30 hour post-stress cortisol concentration was not different from its control (Fig. 2.1). No significant changes were apparent in hematocrit values at 8 h following stress (Fig. 2.2).

In experiment 2, the plasma cortisol concentrations of wild chinook salmon were significantly elevated at both 4 and 8 h after the acute stress. There was no significant elevation in plasma cortisol concentration in the hatchery fish at any sampling time after the acute stress (Fig. 2.3).

The hematological profile of both the wild and hatchery chinook salmon showed a transient increase in lymphocyte and leukocyte ratios at the 4 h and 8 h post-stress sampling times. At 30 hours post-stress the lymphocyte and leukocyte ratios were below those of the control, but this decrease was not statistically significant (Fig. 2.4). Hematocrit values of the control fish were not significantly different from the post-stress hematocrit values at any sampling time (Fig. 2.5).

In experiment 3, wild and colonized coho salmon juveniles had significantly elevated plasma cortisol concentrations at 4 and 8 h following acute stress while the hatchery fish had no

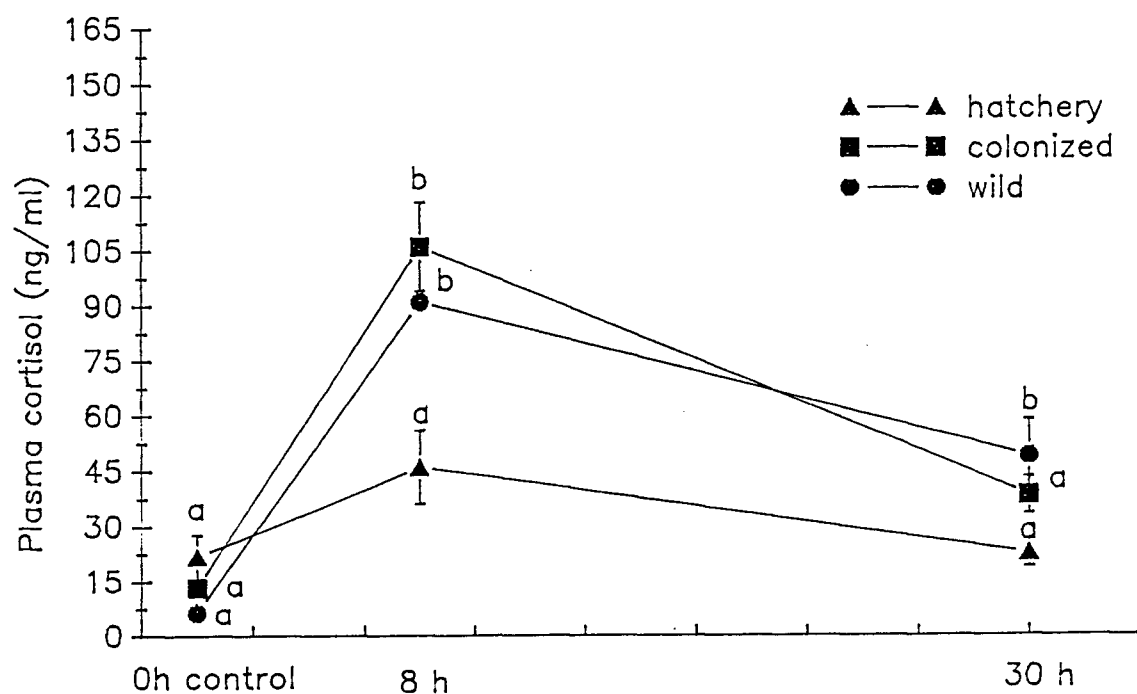


Figure 2.1. Means \pm s.e. of plasma cortisol concentration (ng/ml) of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress. Values with different superscripts are significantly different from their controls, $n=32$.

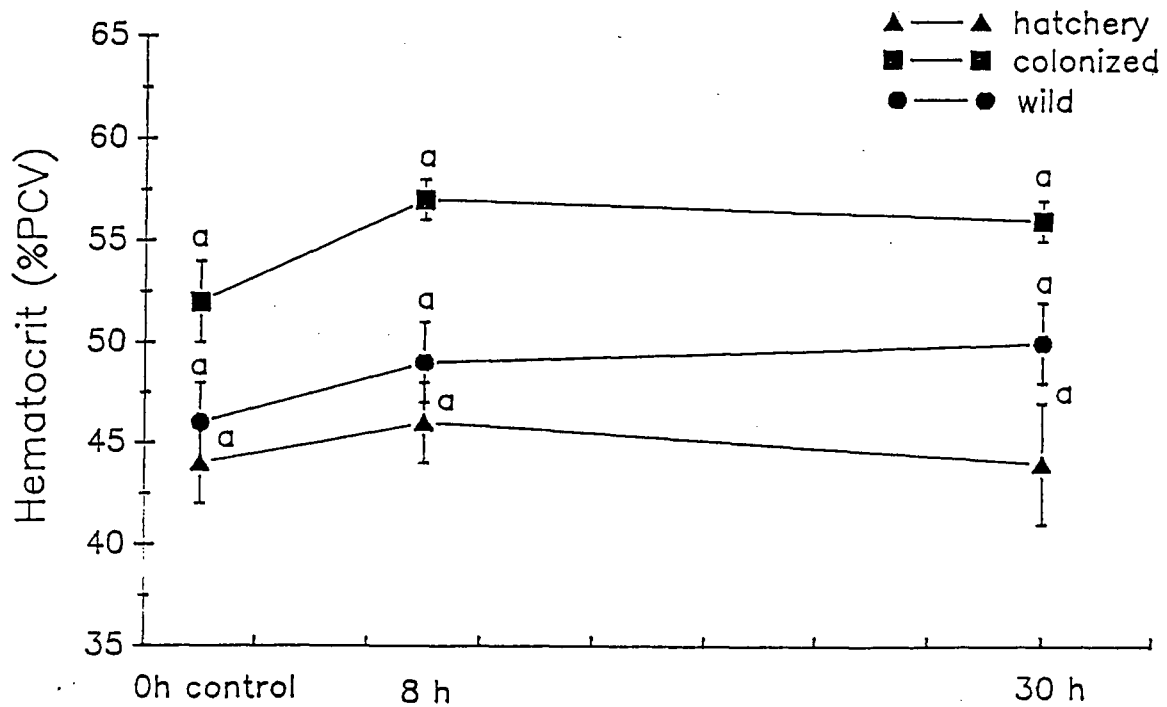


Figure 2.2. Means \pm s.e. of hematocrit values (% packed cell volume) of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress. Values with different superscripts are significantly different from their controls, $n=32$.

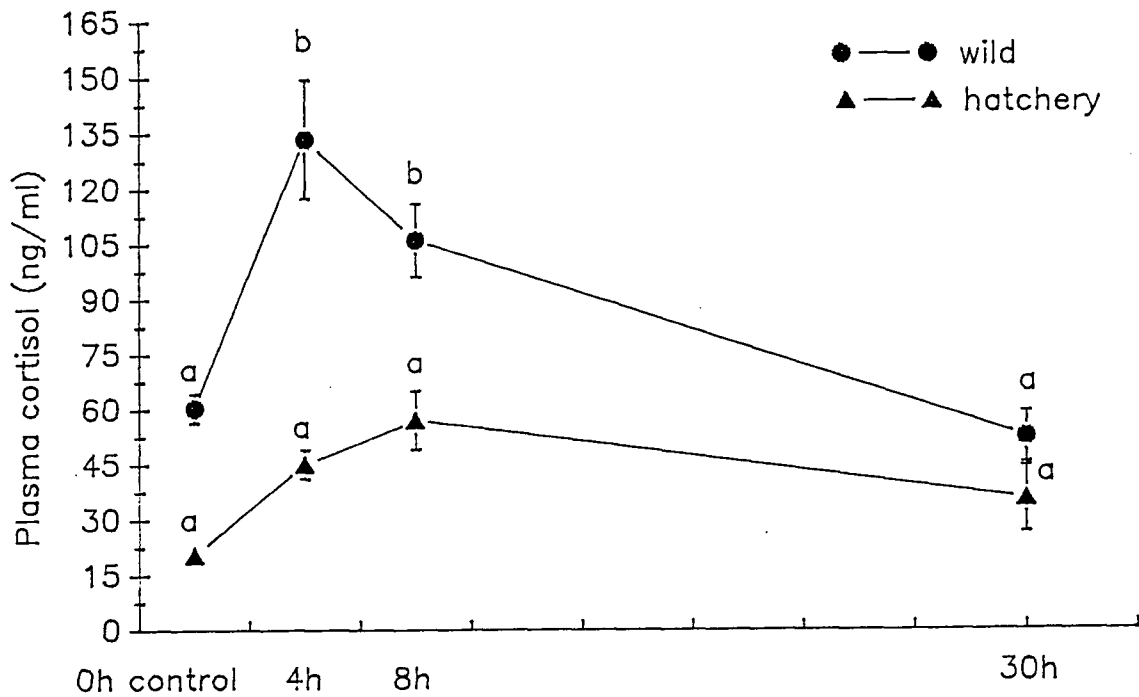


Figure 2.3. Means \pm s.e. of the plasma cortisol concentration (ng/ml) of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress. Values with different superscripts are significantly different from their controls, $n=32$.

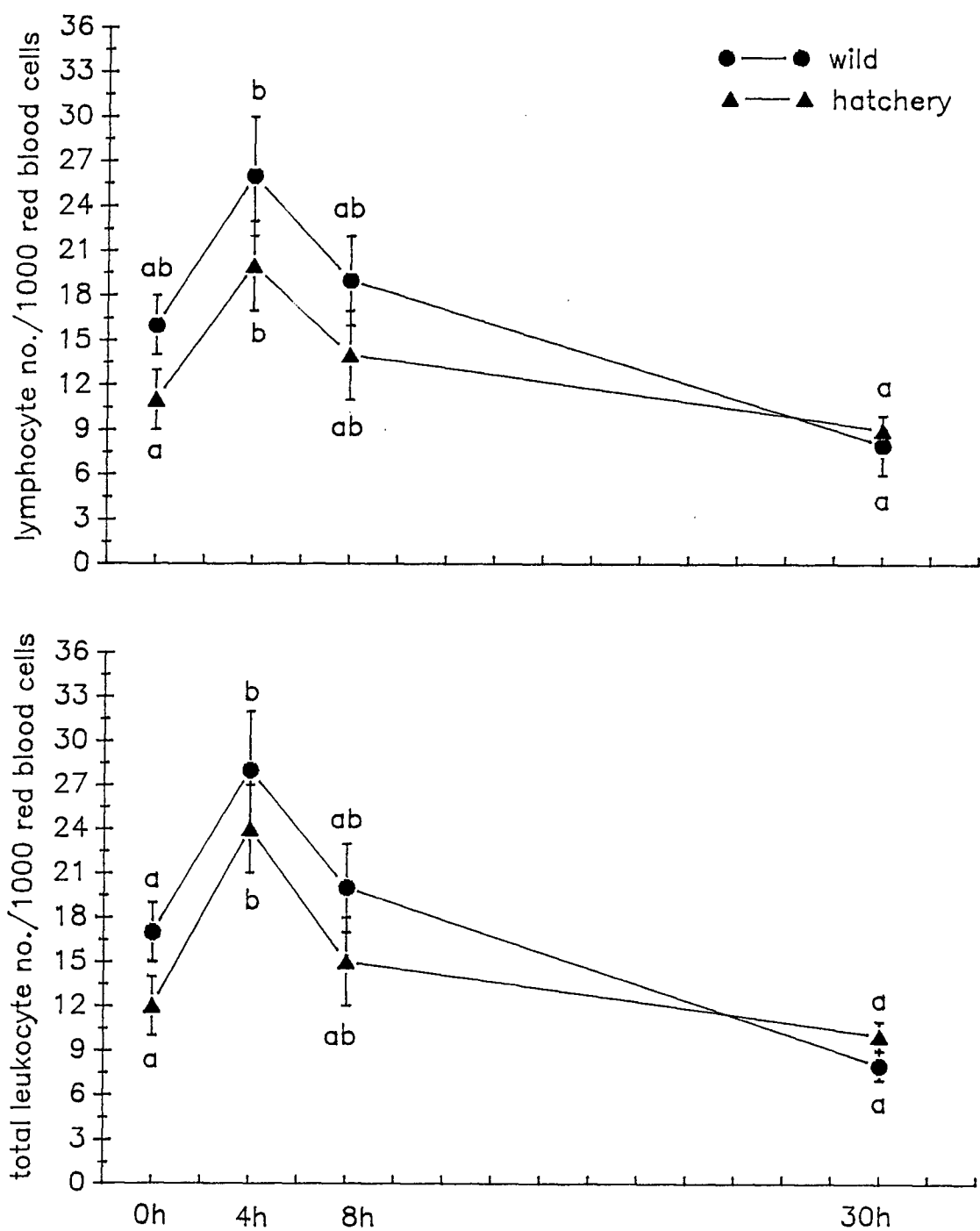


Figure 2.4. Means \pm s.e. of lymphocyte (top) and leukocyte (bottom) number per 10^3 red blood cells of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress. Values with different superscripts are significantly different from their controls, $n=12$.

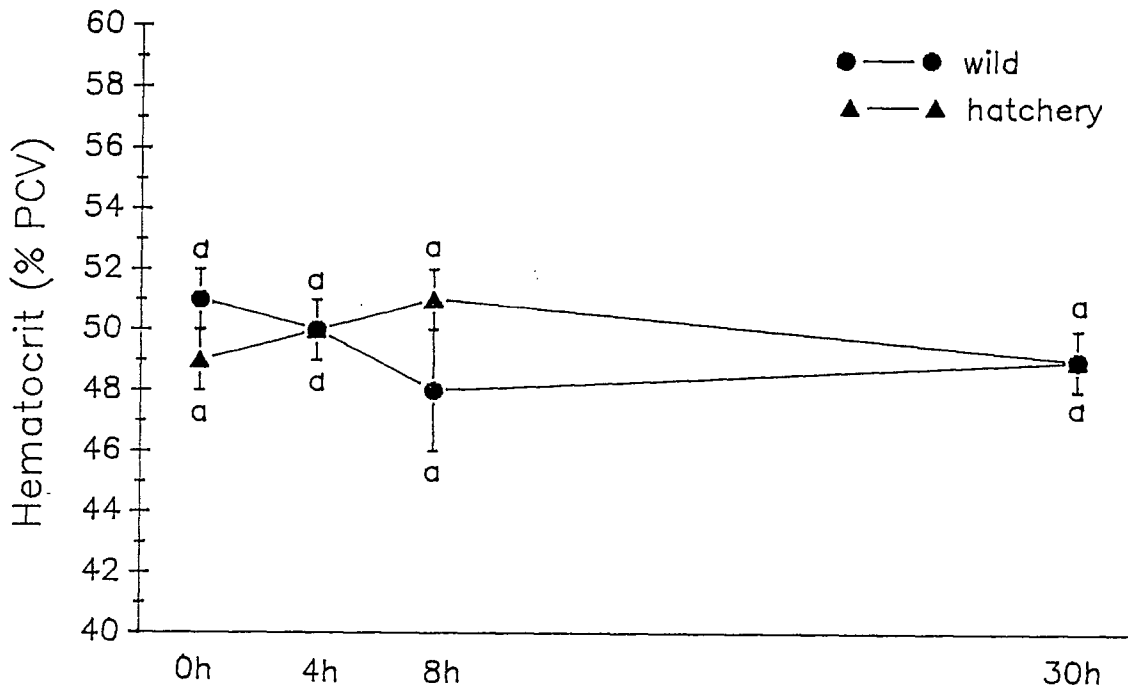


Figure 2.5. Means \pm s.e. of hematocrit values (% packed cell volume) of hatchery and wild chinook salmon juveniles in response to a 30-60 sec handling stress. Values with different superscripts are significantly different from their controls, $n=32$.

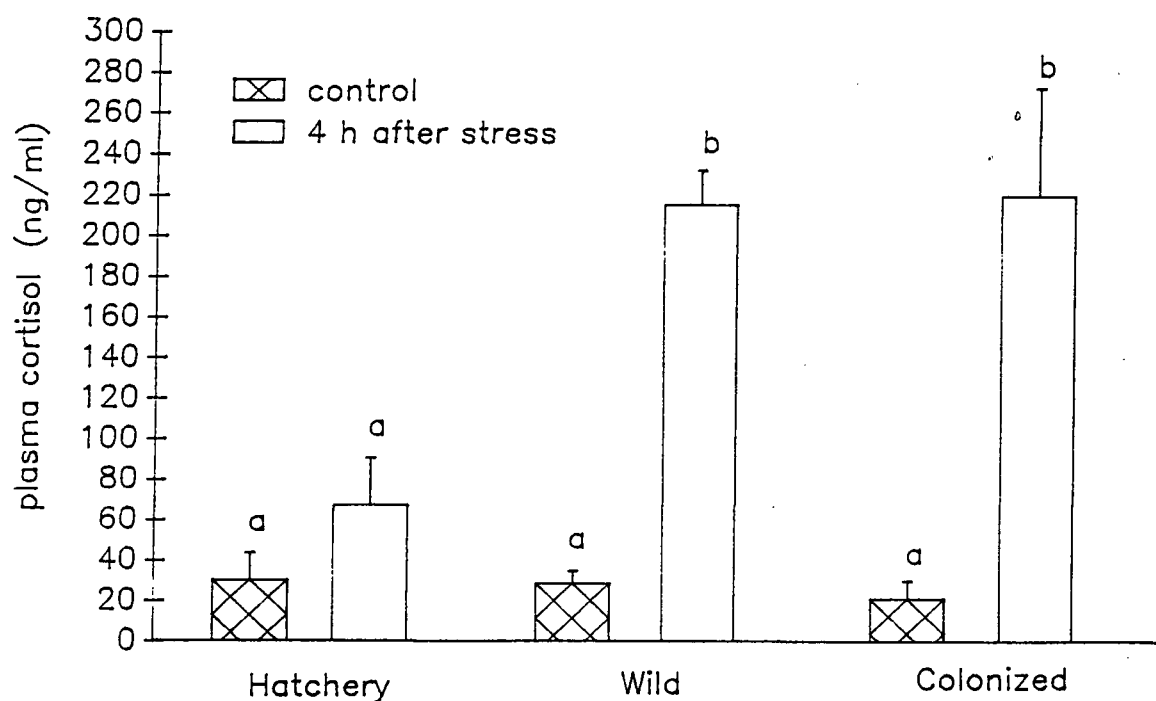


Figure 2.6. Means \pm s.e. of the plasma cortisol concentration (ng/ml) of hatchery, wild and colonized chinook salmon juveniles in response to a 30-60 sec handling stress after 6 months in a common rearing environment. Values with different superscripts are significantly different from their controls, $n=12$.

significant increase in plasma cortisol at these times (Fig. 2.6). No significant changes were evident in most of the hematological characteristics, including hematocrit values (Fig. 2.7), although lymphocyte and leukocyte ratios were decreased from the control values 4 hours following acute stress (Fig. 2.8). There was a significant decrease in APC number in the anterior kidney of the wild fish. This decrease was seen in the hatchery and colonized groups but was not statistically significant (Fig. 2.9). Initial levels of APC were highest in the wild than the other two groups (Fig. 2.9).

In response to a disease challenge of unstressed (0 h) fish, total cumulative mortality was significantly higher in the wild fish than in the hatchery-reared fish. Mortality in the colonized group was intermediate to that in the hatchery and wild groups, and not significantly different from either (Fig. 2.10). These results are different than those of 5 months earlier when there was no significant difference in mortality between the hatchery, wild, and colonized fish (Fig. 1.13).

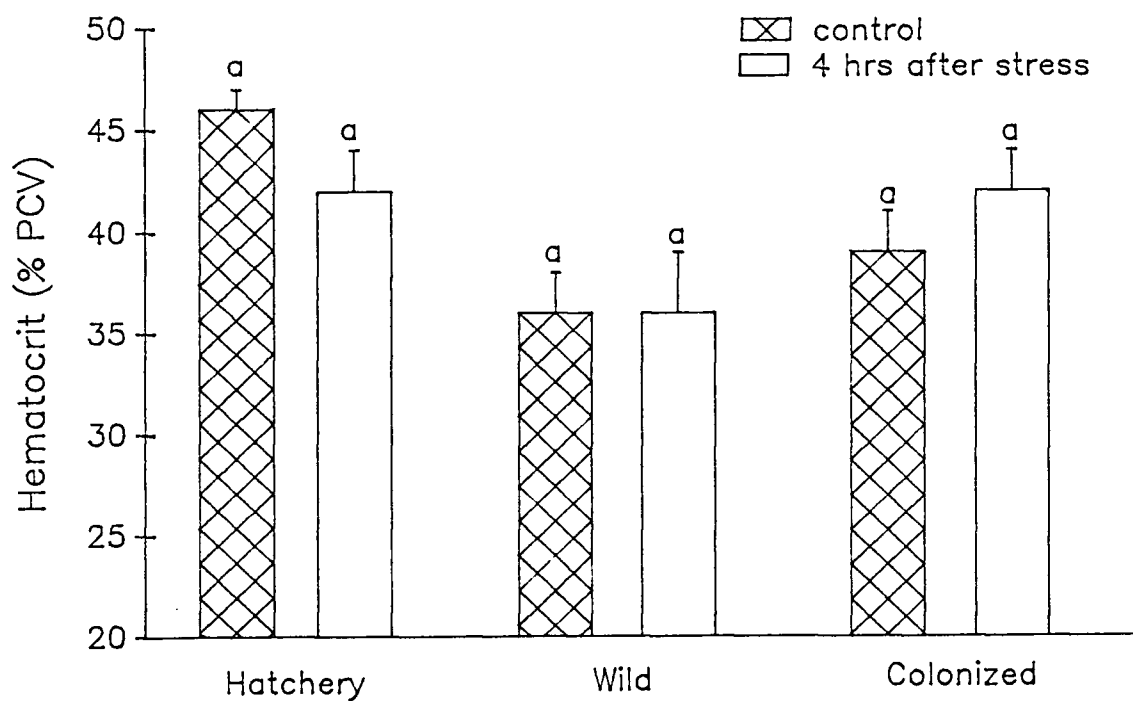


Figure 2.7. Means \pm s.e. of hematocrit values (% packed cell volume) of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment. Values with different superscripts are significantly different from their controls, $n=12$.

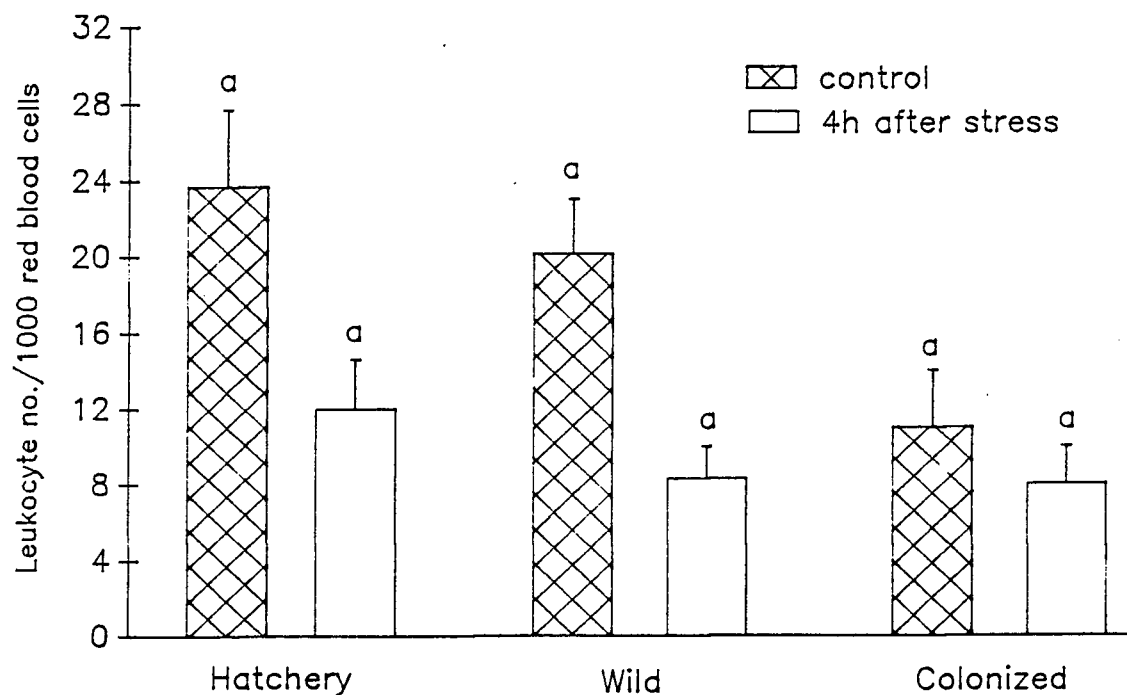


Figure 2.8. Means \pm s.e. of leukocyte number per 10^3 red blood cells of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment. Values with different superscripts are significantly different from their controls, $n=12$.

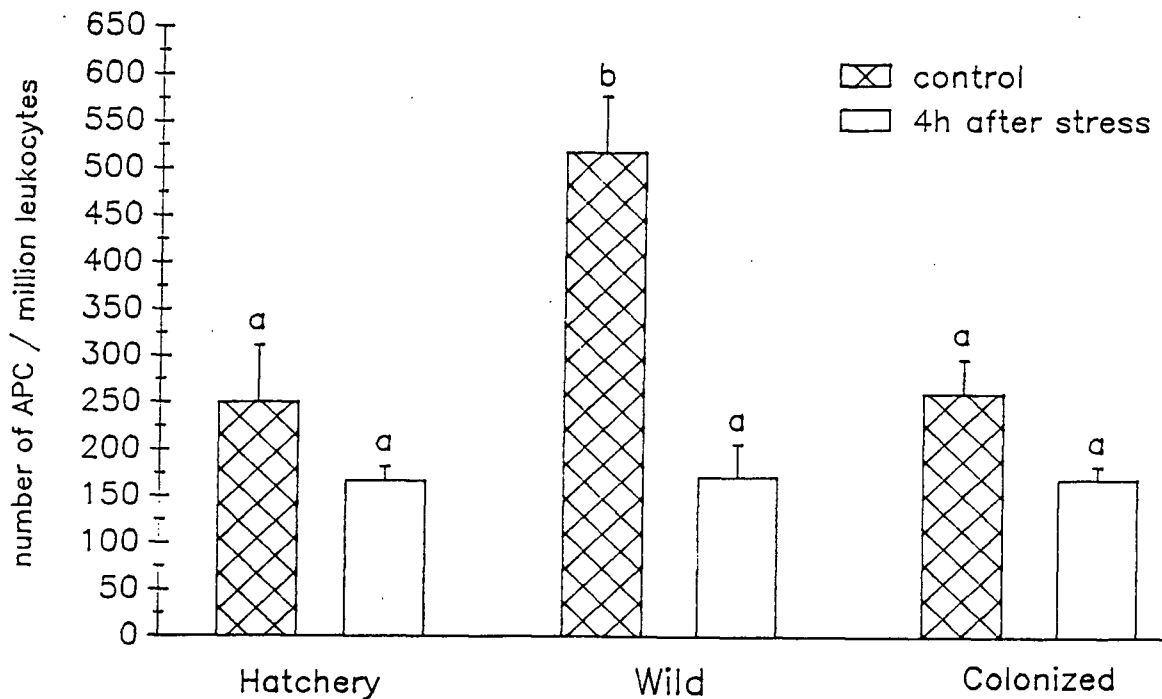


Figure 2. 9. Means \pm s.e. of antibody producing cell (APC) number per 10^6 leukocytes in the anterior kidney of hatchery, wild and colonized coho salmon juveniles in response to a 30-60 sec handling stress after 6 months rearing in a common environment. Values with different superscripts are significantly different from their controls, n=12.

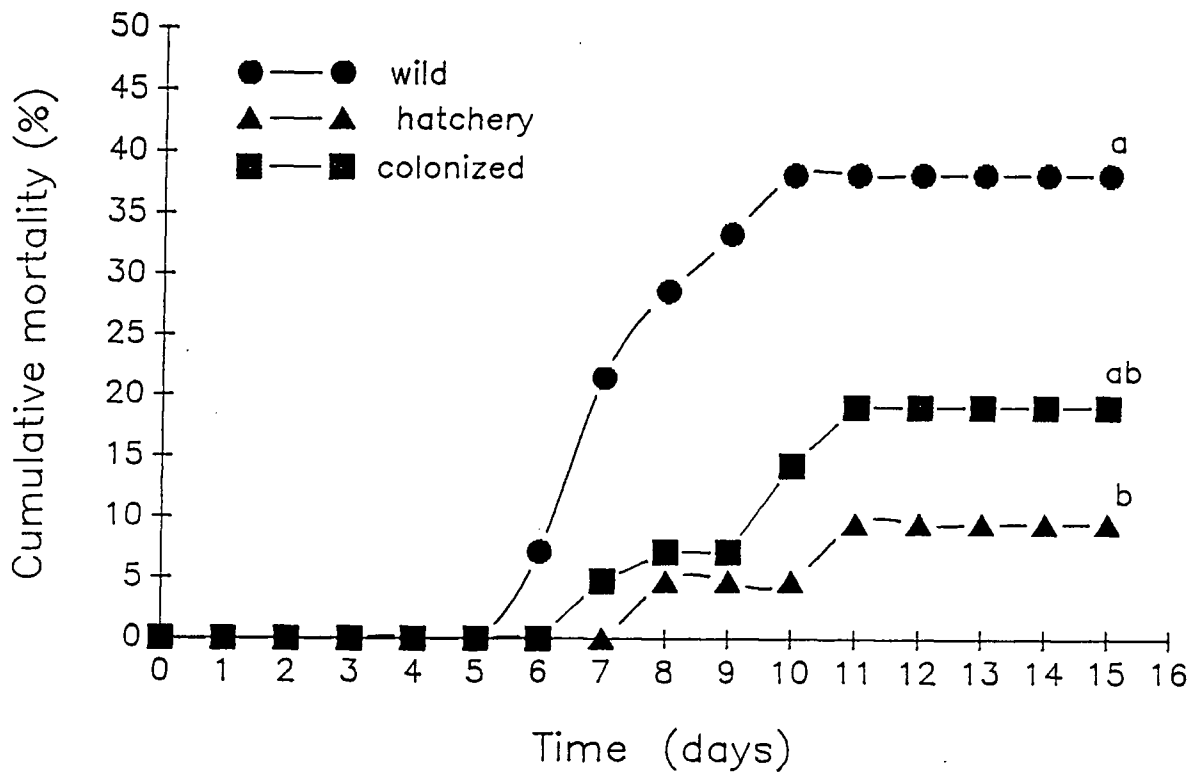


Figure 2.10. Cumulative mortality (%) and mean time to death (d) of hatchery, wild and colonized coho salmon juveniles in response to a bath immersion challenge with *V. anguillarum* at a concentration of 1.3×10^6 cfu/ml after 6 months rearing in a common environment, Values with different superscripts are significantly different from their controls, n=42.

Discussion

Certain results were common to all these experiments. At none of the sampling times did the chinook or coho hatchery juveniles respond to the handling stress with significant elevation in plasma cortisol concentration, a disturbance that consistently produced a significant increase in naturally-reared (wild chinook and coho salmon, and colonized coho salmon) juveniles. In coho salmon juveniles reared in a common environment for six months, the stress response of the fish reared in the natural environment (wild and colonized fish) was similar, indicating that longer rearing in a common environment did not affect the magnitude of the response to an acute stress.

Repetitive stress in salmonids can result in a negative feedback of cortisol on the hypothalamic-pituitary-interrenal axis and inhibit further interrenal response (Donaldson et al., 1981, Barton et al., 1987). Enhanced interrenal activity from stress in early freshwater rearing may have prolonged secondary effects on the function of both interrenal and lymphoid tissues of hatchery-reared salmonids. I speculate that this mechanism may be the cause of the lack of measured response of hatchery fish to stress, or that the duration of the elevation of plasma cortisol so brief in nature that it was missed by the sampling protocol.

Immunosuppression may occur despite the fact that plasma cortisol remains low in response to an acute stress, such as the one used in this study. Hatchery juveniles are subjected to daily disturbances in early rearing that I have shown evoke a

significant stress response in wild fish. The cumulative stresses to salmonids in early fresh water rearing may be responsible for a low cortisol stress response in hatchery fish that is lower than and not typical of their wild or colonized (in the case of the coho salmon) counterparts. However, assuming that these fish were of similar genetic stock, the possibility cannot be discounted that there was selection within the wild fish and within colonized hatchery releases for a high cortisol stress response. Adaptation to stress may have induced a low cortisol response in the hatchery-reared fish. There may be some selective advantage of a high cortisol stress response in terms of survival in the natural environment. Studies have recently been published where the cortisol stress response has been measured in order to obtain an indirect measure of disease resistance. Fevolden et al. (1991) hypothesized that, due to the immunosuppressive effects of cortisol, high cortisol response groups would have lower survival rates. However, they actually found that certain immune responses were higher in the high cortisol stress response groups. Maule et al. (1989) found both immunosuppressive and immunostimulatory effects of stress in chinook salmon. The tendency to equate a high stress response with a lowered immune capacity and resistance to disease may be erroneous. Indeed, in this study, resting numbers of APC were highest in the wild fish, a high cortisol response group. Although the relationship between an elevation in plasma cortisol and resultant immunosuppression (Tripp et al., 1987) holds in this study, the character of fish with the potential of high

interrenal activity may also be to have potentially elevated function of lymphoid cells and tissues. In the second experiment, the transitory increase in total leukocyte and lymphocyte ratios in the wild and hatchery chinook salmon may have been caused by increased plasma catecholamine concentrations at 4 h and 8 h following acute stress. Increases in the concentration of plasma epinephrine have been shown to cause splenic contraction and release of red blood cells and it may also increase the number of circulating white blood cells in the blood (Perry and Kincaid, 1989).

In the coho salmon stress experiment after 6 months of rearing in the artificial environment, wild fish had significantly higher mortality than the hatchery fish. Results of this challenge are comparable to a similar challenge on these same fish 5 months earlier that showed no difference in total mortality between the groups. The response to acute stress was unchanged over the 6 month period and it appears that chronic stress may have reduced the survivorship of wild fish to the disease challenge. Mortality in the colonized group was less than in the wild group, but the difference was not statistically significant. There was a change in relative mortality between hatchery and wild fish to the *Vibrio anguillarum* challenge. In other studies, mortality due to *V. anguillarum* has been shown to be stress-mediated and a reliable indicator of adverse conditions (Wedemeyer and McLeay, 1981). This may have been affecting the mortality of the naturally-reared fish in the artificial environment.

In these experiments, naturally-reared salmon juveniles were shown to have different physiological responses to acute stress than their hatchery counterparts. It appears that the high cortisol response has been retained in the naturally reared fish while this response was lost in the fish reared in the hatchery. In view of the possibility that the natural environment seems to select for this type of response, the strategy of enhancement by colonization appears to be a viable and economical way to supplement wild populations and increase survival rates in hatchery releases from the time of seaward migration onward.

Chapter 3:

The effect of diet on immune function at first feeding in
developing chinook salmon.

Introduction:

The diet of intensively cultured fish plays a vital part in determining their health and well being. Present commercial diet formulations of salmonid feeds are generally considered to be nutritionally sound. Indeed, the standard diet used to initiate feeding in SEP hatcheries (Plotnikoff et al., 1985) and the commercial diet examined in this study have been shown to contain adequate levels of vitamins and minerals (Felton et al., 1990), as well as adequate levels of less labile components of fish diets. Given this, however, the feeding of commercial diets has been shown to cause decreased immune function in salmonids (Blazer and Wolke, 1984). One absolute difference between the feeding of a live diet and a formulated diet is the degree of rancidity present in commercially prepared diets, an aspect of the diet that is absent from a live diet.

Fish feeds and fish tissues contain relatively high concentrations of highly unsaturated fatty acids. These are important components of cell membranes and are vulnerable to lipid peroxidation and resultant tissue damage (Tacon, 1985; Lall, 1990). The autoxidation of lipids is one of the most deleterious changes that may occur to fish diets. Oxidized lipid may have adverse effects on fish health due to the destruction of vitamin A and E and other essential nutrients (Hung et al., 1980). A review of various adverse effects of nutritional toxicities and deficiencies on the immune function in fish was recently written by Landolt (1989).

A second type of study that helps to clarify the effect of oxidized dietary lipids on the immune system are those that examine the moderate depletion of vitamin E, vitamin C and selenium from the diet. The importance of these factors as antioxidants has been well studied. Vitamin E terminates the free radical reaction because it is an efficient scavenger of free radicals. Selenium is a necessary component of glutathione (GSH) peroxidase, which is able to convert fatty acid hydroperoxides to innocuous hydroxy fatty acids (Bell and Cowey, 1985). One the most important functions of vitamin C is that it quenches oxygen radicals arising from cellular respiration (Hardie et al., 1991).

Studies have shown that feeding diets deficient in selenium have resulted in an impairment of the antigenic response in rodents (Purnham et al., 1983). Diets deficient in both vitamin E and selenium in Atlantic salmon during the first four weeks of feeding resulted in twice the mortality of the controls with adequate levels of these components (Poston et al., 1976). Diets with moderate depletion of vitamin C, vitamin E and selenium did not cause gross or histologic pathology in Atlantic salmon or rainbow trout (*O. mykiss*), yet had more subtle effects on disease resistance and immune function (Blazer and Wolke, 1984; Hardie et al., 1991). Earlier studies with coho salmon, where there was inclusion of rancid lipid into the diet without causing gross or histologic change, found no effect of the rancidity factor on the immune response (Forster et al., 1988).

The majority of studies on the effects of rancidity on fish

health, with the noted exception of Poston et al. (1976), involved experimenting with fish that had reached immunological maturity, known to occur soon after the onset of first feeding in rainbow trout (Tatner, 1986) and masu salmon (*O. masou*) (Fuda et al., 1991). The study described herein attempted to examine the effect of a commercial diet compared to a live or fresh frozen diet of krill on the immune response at this critical period in the development of lymphoid organs and ontogeny of immunological responsiveness.

The period following swim-up in salmonid fishes is one characterized by a rapid rate of growth (Brett, 1979). During the normal course of metabolism, the autoxidation of the highly unsaturated fatty acids in formulated commercial feeds gives rise to free radicals that are toxic or potentially toxic, highly reactive metabolites of oxygen (Bell and Cowey, 1985). In fish, damage from these metabolites is ameliorated by a multilevel defense system including GSH, previously mentioned, and superoxide dismutase (SOD).

The glutathione peroxidase systems that protect fish tissues from damage by hydroperoxides are one to two orders of magnitude lower in salmonid liver and kidney tissues than in mammals and are nearly absent in the gut (Tappel et al., 1982). Autoxidized dietary lipids would be necessarily absent in the diet of wild salmon as they consume live prey (Marliave, 1989). During the period of accelerated growth and increased food consumption following swim-up in hatchery-reared salmonids, production of free radicals from oxidized foodstuffs may override

the capacity of the natural defense systems to cause damage to developing lymphoid tissues. There is some evidence that feeding chum salmon (*O. keta*) a live diet from swim-up has lowered mortality in response to a bacterial challenge, despite the fact that the swim-up fry fed the live diet were on a lower ration level (Marliave, 1989).

Because of the dependence of young salmonids on the non-specific immune system (NSIS) during the period before the immunological maturation of non-specific immune defenses (Grace et al., 1980), lysozyme activity was examined in the chinook salmon fry in each of the diet treatments as well the specific immune function of the anterior kidney leukocytes. Lysozyme is an enzymatic component of the NSIS, located in the lysosomes of neutrophils and macrophages, and is secreted into the blood by these cells (Mock and Peters, 1990). Lysozyme is active in breaking down the peptidoglycan layer of bacterial cell walls (Murray and Fletcher, 1976). It is also thought to be effective against gram-negative organisms through the synergistic action with complement and other enzymes (Hjelmeland et al., 1983). Lysozyme activity has been found to be higher in leukocyte-rich tissues at points where the risk of bacterial infection is high, such as in mucus and the gills (Lindsay, 1986).

The haemolytic plaque assay determines the number of leukocytes in the lymphoid tissue of the kidney capable of an antibody response to a hapten-carrier stimulus and is a standard technique for quantifying antibody producing cell numbers in mammalian immunology (Roitt et al., 1985). It has been

characterized for use in fish as an assay with a narrow statistical margin and a high sensitivity (Anderson, 1990) and was used in the present study as a tool to determine immunocompetence.

The objective of this study was to compare the effect on the immune response of juvenile chinook salmon of initiating feeding with either a commercial diet or a wild-type diet of live or fresh frozen krill, without specific regard for ration level or standardization of nutritional composition of these diets.

Materials and Methods

Fish and Diets

Juvenile chinook salmon were hatched and reared to swim-up at the Capilano Hatchery, North Vancouver, at which time feeding was initiated on one of three treatments; Oregon Moist Pellet (OMP) commercial starter, live ocean euphausiids (selective gear was used that fished almost exclusively for *Euphausia pacifica*), or commercially prepared, fresh frozen, bulk krill (Murex brand (*Euphausia pacifica*), Aquafoods, Langley, B.C.). The live diet and frozen krill treatments were initiated on day 46 post-hatch, 3 and 11 days, respectively, after the OMP treatment. After 11 days, the live diet treatment was replaced with fresh frozen krill. Practical considerations limited the feeding of live diet for more than 11 days. Fish were held in Capilano Hatchery troughs with 45,000-50,000 fish per treatment. Fry were held from hatch in heated spring water maintained at a constant temperature of 10°C. During the course of feeding the live diet, the euphausiids were introduced into the water where they were actively captured by the fry. The frozen krill were supplied to the fry in netted bags, hung every 0.6 m in the trough. The three treatment groups were sampled on three consecutive days; groups were OMP-91 days post-hatch, live diet-88 days post-hatch, and frozen krill-80 days post-hatch (fish mean wt \pm S.E.: OMP diet; 3.06 ± 0.09 g, live diet; 2.03 ± 0.07 g, frozen krill; 1.56 ± 0.05 g).

Experimental procedures and sampling

Fish (n=30) from each treatment were rapidly netted and transferred to a bucket containing a lethal dose (200 mg/l) of buffered tricaine methanesulfonate (MS222). After the fish were anesthetized, the caudal peduncle was severed and the blood was collected in heparinized capillary tubes. The plasma was separated by centrifugation for 5 minutes at 11,500 RPM (Model MB microhematocrit centrifuge, International Equipment Co., MA, U.S.A.) and stored at -20°C prior to determination of lysozyme activity. Fork length and weight were measured and used to determine condition factor (K) (Pickering and Duston, 1983).

Hematology

Hematocrit and differential white blood cell counts were made as previously described in Chapter 1: Materials and Methods.

Specific immune response

To assess the ability of lymphocytes to generate specific antibody, cell cultures of leukocytes were established as previously described in Chapter 1, according to Tripp et al. (1987) and Maule et al. (1989). In order to achieve a concentration of 2×10^7 leukocytes/ml, anterior kidney tissue homogenates of five fish were pooled. Cultures were treated as in Chapter 1: Materials and Methods.

Plasma lysozyme determination

Lysozyme activity was determined using the modified lysoplate assay of Ossermann and Lawlor (1966). This assay is based on the lysis of the lyophilized, lysozyme-sensitive, Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma) in agarose gel. Agar plates were prepared by suspending 0.6 g/L *M. lysodeikticus*, 1.17 g/L NaCl, and 5 g/L agarose (Sigma) in 1 L Phosphate Buffer (see Appendix B). The agarose was dissolved by heating and 25 ml was poured into Petri plates and allowed to solidify. Ten- μ l samples of plasma or standard were put into wells (diameter about 3 mm) punched in the agar. The diameter of the zone of clearing around the well was measured after incubation in a moist chamber at 22-25°C for 17 h. The lysozyme activity of each plasma sample was calculated using logarithmic regression analysis, employing the formula $Y=3.97\log X-2.87$ ($r^2=0.921$), where Y= the diameter of the area cleared by the lysis (mm), and X= the lysozyme activity (U) based on a external standard curve using hen egg white lysozyme (HEWL, Sigma).

The activity of HEWL was determined using a turbimetric method (Grinde, 1989) at pH 6.0. The specific activity of HEWL varies with pH and must be determined at the pH of the assay. By this method, one unit of lysozyme activity (U) is defined as the amount of enzyme causing a decrease in the absorbance of a suspension of *M. lysodeikticus* (0.2 mg/ml 0.06M phosphate buffer, pH 6.0) of 0.001/min when the reaction is carried out at 23°C and an absorbance of 530 nm. It was found that 1 lysozyme unit (U)/ml

corresponded to 2.2 μg of HEWL. Although the corresponding lysozyme concentrations ($\mu\text{g/ml}$) of the plasma samples were calculated and reported in the results, there has not yet been an international standardization of these methods. Although the use of the commercially available HEWL is universal as a standard in assays for fish lysozyme, the pH and tonicity of the buffers used are variable. Thus the concentrations of lysozyme determined in this study are relative and may not be comparable to those of other investigators.

Data analysis

All data were subjected to analysis of variance and where significant differences were found, Tukey's test (Steel and Torrie, 1980) was used to identify significant differences between treatment means. These analyses were performed with the SYSTAT statistical program (Wilkinson, 1988). Statistical significance was taken at the 5 % level in all tests.

Results

Specific immunological function of anterior kidney leukocytes was higher in fish from the OMP treatment than in those from either the live or frozen krill treatments (Fig. 3.1). Although the use of pooled anterior kidney samples as cell suspensions precluded the regression of APC number against individual fish weights. When the mean APC number was regressed against the average weights of the fish used across all diet treatments, there was a significant correlation between APC number in the anterior kidney and fish weight ($r^2 = 0.941$).

Non-specific immunological function as measured by lysozyme activity, was higher in fish from the live diet treatment than in those from the frozen krill treatment but not significantly higher than that seen in the OMP treatment group (Fig. 3.2). There was no correlation between weight of the fish and lysozyme activity. There was no difference in the levels of lysozyme activity found in this study and those reported for adult salmonids and where reported, the plasma lysozyme concentrations in the literature were comparable to values obtained in this study (Lindsay, 1986; Mock and Peters, 1990).

The hematological profiles of the fish from the three diet treatments were not significantly different from each other, yet in all of the wbc/rbc ratios measured (lymphocyte, leukocyte, and thrombocyte) the live and frozen krill treated fish had higher ratios of these cells than the OMP fed fish with the highest values in the live krill-fed fish (Fig. 3.3).

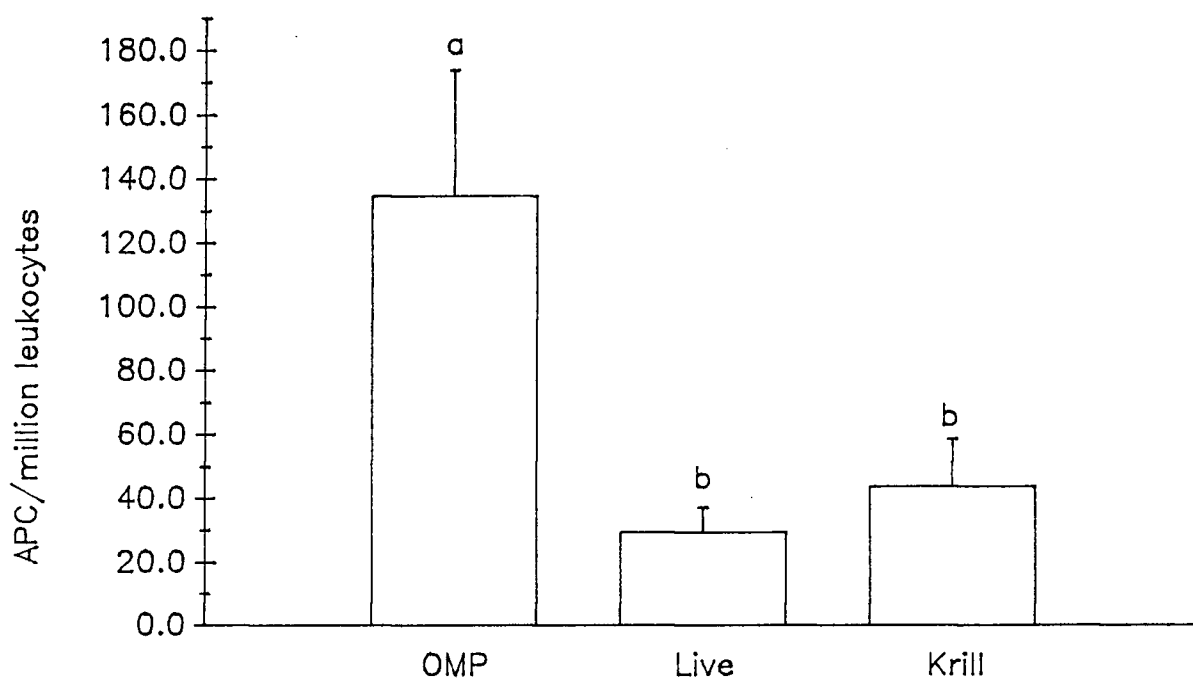


Figure 3.1. Means \pm 1 s.e. of the number of antibody producing cells (APC)/million viable leukocytes in the head kidney of chinook salmon fry fed either live or frozen krill (*E.pacifica*), or a commercially prepared diet (OMP). Values with superscripts that are different are significantly different, $p < 0.05$. $n = 12$.

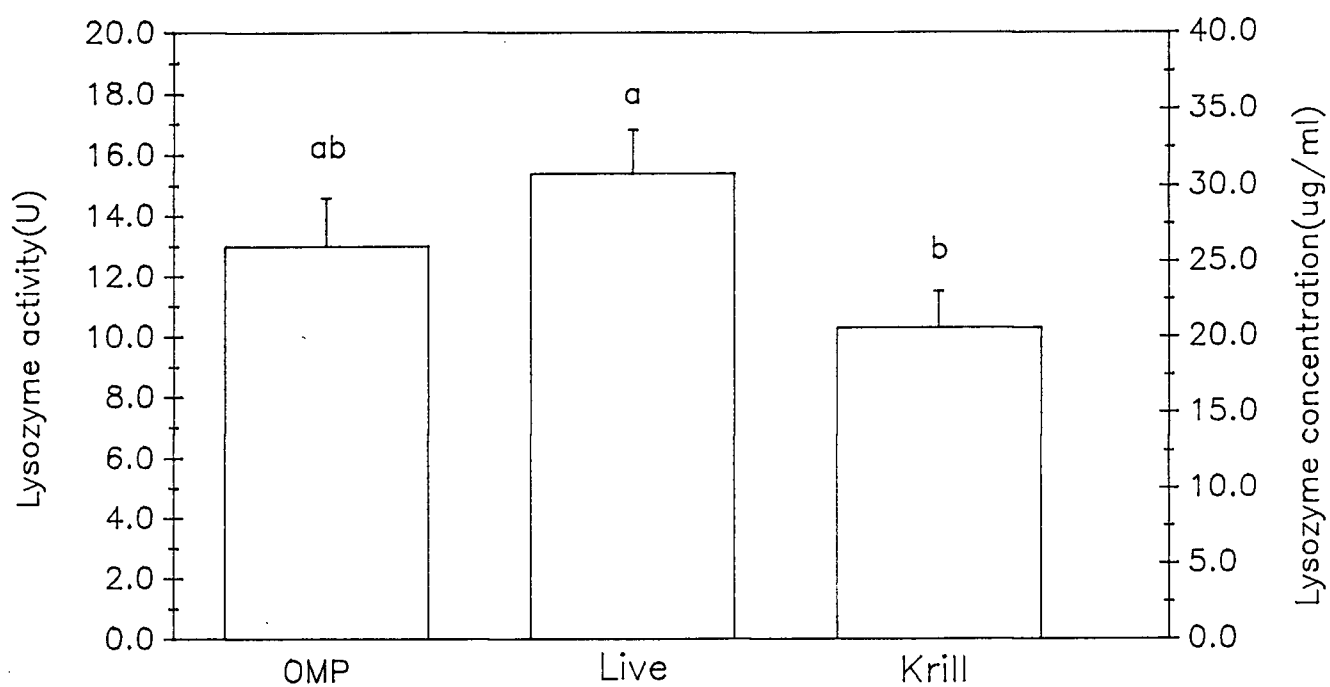


Figure 3.2 Means \pm 1 s.e. of lysozyme activity (U) and concentration ($\mu\text{g}/\text{ml}$) in the plasma of chinook salmon fry fed either live or frozen krill (*E. pacifica*), or a commercially prepared diet (OMP). Values with superscripts that are different are significantly different, $p < 0.05$. $n=12$.

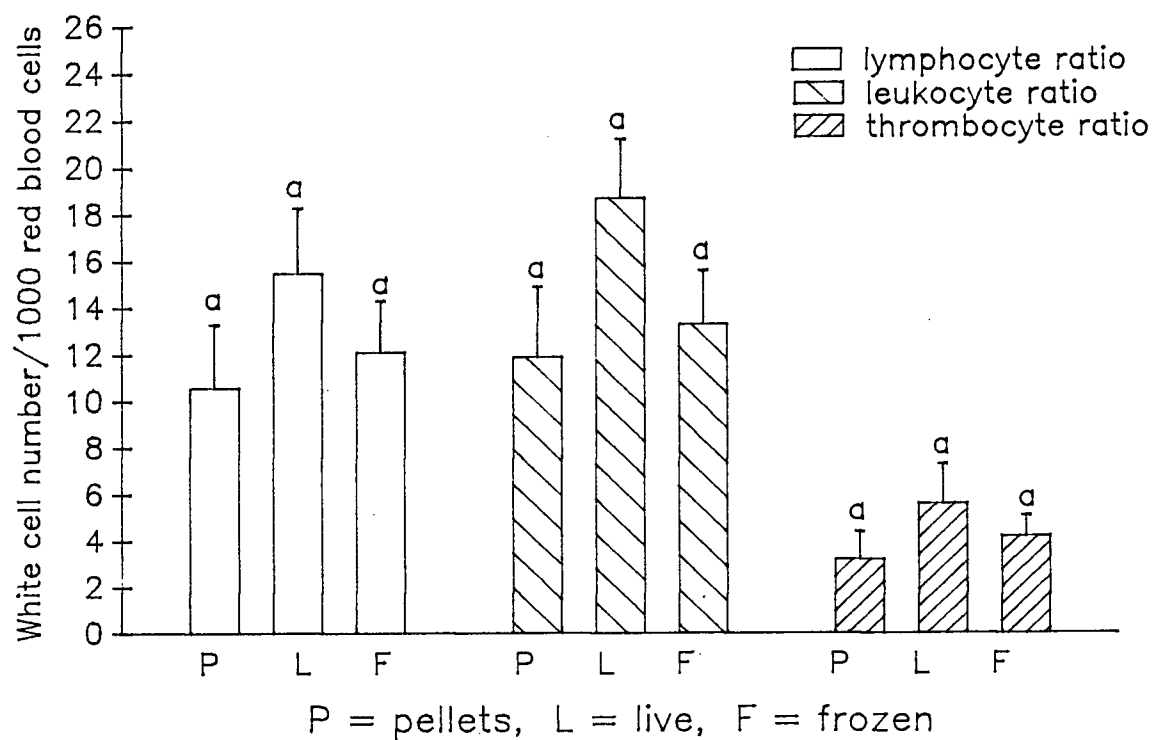


Figure 3.3 Hematological profile of chinook salmon fry fed either live or frozen krill (*E. pacifica*), or a commercially prepared diet (OMP). Values with superscripts that are different are significantly different, $p < 0.05$. $n=6$.

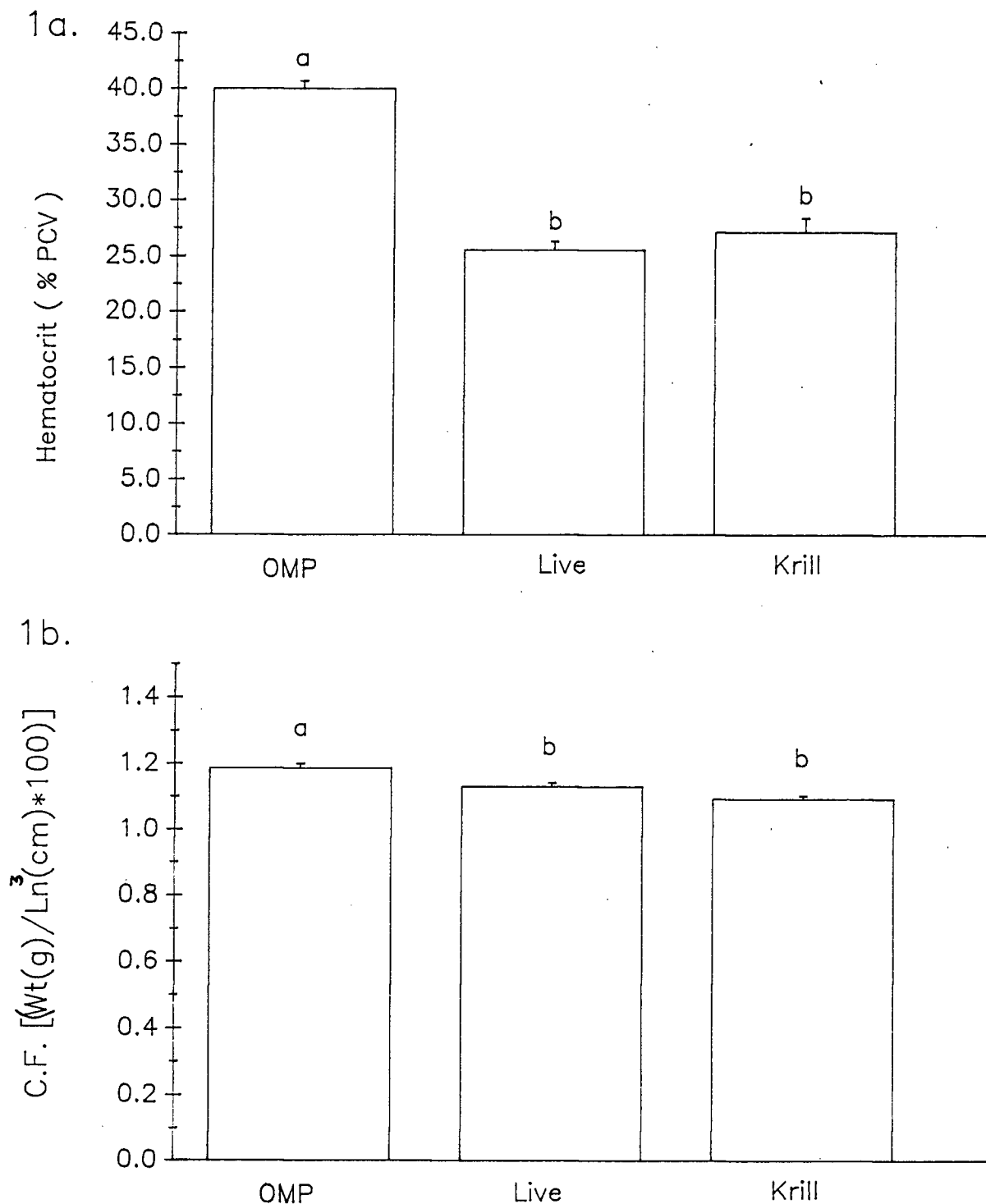


Figure 3.4. a; Means \pm 1 s.e. of hematocrit (% packed cell volume) and b; condition factor (C.F.) of chinook salmon fry fed either live or frozen krill (*E. pacifica*), or a commercially prepared diet (OMP). Values with letters in common are not significantly different, $p < 0.05$. $n = 30$.

The fish of the OMP treatment had a higher mean hematocrit than either the live or frozen krill-treated fish (Fig. 3.4a). Hematocrit values from the two latter treatments were not significantly different in this respect.

The mean weight of the fish in the OMP treatment (3.06 ± 0.09) was significantly higher than that of the live fed (2.03 ± 0.07) or the frozen krill-fed (1.56 ± 0.05) fish. Fish of the latter two treatments were also significantly different in mean weight, but not in condition factor (Fig. 3.4b). The OMP treated fish had a significantly higher condition factor than both the live fed diet fish or the frozen krill fed fish (Fig. 3.4b).

Discussion

As the fish in each diet treatment were ponded on different days, the OMP fed fry initiated feeding 11 days before the frozen krill fed fry and 4 days before the live fed fry. As a result, the physiological and immunological state of development in the OMP group appeared to be more advanced than that of the other groups.

The effect of feeding a diet free of rancidity on the specific immune system cannot be discerned from the levels of APC determined in this study. The specific immunological response appeared to be closely correlated with weight at the early stages of development. As this study shows, the presence of lymphocytes and lymphoid tissue may not herald immunological maturity in terms of the specific immune response. Tatner (1986) suggested that a critical mass of immunocompetent cells must be reached before a fish can mount a full antibody response to a stimulus. Indeed, fry have been shown to be refractory to vaccination with *V. anguillarum* in each of two separate studies, where a different chronological age was determined as the time of the appearance of immunological response (Manning et al., 1982). Manning et al. (1982) found in both of their studies that the transition to a immunocompetent state coincided with the period of transition from a relatively low growth rate to a higher growth rate, at the onset of first feeding.

The number of APC in the head kidney of the OMP fed fry indicated they had reached an immunocompetent level at 91 days

post-hatch, yet the number of APC in the live fed fry, although only 3 days behind, did not indicate immunocompetence. The high correlation of APC number with weight indicates that specific immune response is dependent on the attainment of a critical size and less on the specific age in these fish. Other studies have also shown this to be true (Tatner, 1986; Fuda et al., 1991).

In light of the apparent dependence of the development of specific immunological maturity on fish weight, non-specific immune defenses may be relatively more important during early ontogeny of the specific immune system. Indeed, for a period of time in immunological development, salmonid fry are entirely dependent on nonspecific defense mechanisms for protection against invading pathogens (Grace et al., 1980; Fletcher, 1982; Manning et al., 1982). Yousif et al. (1991) have shown that the nonspecific factor examined in this study, lysozyme, is present in salmonid eggs. The NSIS in the chinook salmon fry in this study was fully active at 80-91 days post-hatch and, thus, independent of the fish weights. A slight advantage of feeding a live diet on the level of lysozyme activity in the chinook salmon fry was apparent. Higher ratios of white blood cell/red blood cell (WBC/RBC) in the live fed fry also may indicate a slight advantage in terms of disease resistance of using this diet type to initiate feeding in salmonids.

The differences in mean weights of the fish in each diet treatment resulted, in part, from the staggered swim-up times, but it is also possible that ration was restricted in the live krill-fed and frozen krill-fed fish. The delivery system for the

frozen krill may have allowed only the more aggressive fry to become satiated. Also, on two occasions, the trawler was unable to fish the live krill and, thus, the fish remained unfed for two of the eleven days on live diet. The mean condition factors of the fish in the live and fresh frozen krill treatment groups does not indicate that these fish were limited nutritionally, although ration level may have contributed to their lower weights relative to the OMP fed fry. Control of the factors such as swim-up time and ration level would be better achieved in a laboratory situation and further studies should incorporate this into the design.

Although this study failed to demonstrate that a live diet is beneficial in terms of specific immune response, or that possible rancidity was detrimental to immunological development it does show that feeding of a live diet to salmonids compares favorably to feeding a prepared diet with respect to circulating leukocyte numbers and lysozyme activity in plasma, even at a lower ration level. It also supports the suggestion that the time of first feeding in chinook salmon fry is an important period of immunological maturation, a period also characterized by rapid growth. If there is a period during physiological maturation where even a small degree of rancidity in prepared diets would cause sublethal effects such as immunosuppression, it is probably at this time. The high growth rate (brought about by the onset of feeding) could cause an increase in the amount of autoxidated lipids in the gut as a natural result of metabolizing the unsaturated fatty acids in prepared diets and overwhelm the

natural antioxidant systems, thereby allowing damage to occur to the developing lymphoid tissues.

General Conclusions and Recommendations

The findings of these experiments suggest that the plasma cortisol increase in response to stress and environmental stimuli are different between both wild and colonized salmonid fish and their hatchery counterparts. The mechanism behind the lower cortisol response of hatchery fish may be down regulation of the cortisol production by the interrenal tissue brought about by chronic stress in the hatchery rearing environment.

There is an indication that wild and colonized fish may have a better intrinsic ability to mount an immune response. In wild and colonized fish however, the immune response may be affected by cortisol mediated immunosuppression during smoltification and during chronic stress in a common artificial rearing environment. Cortisol may affect immune function differently at different physiological stages, such as smoltification.

Wild and colonized juvenile fish have a different physiological character than their hatchery-reared counterparts with regard to cortisol response. This character appears to be determined by the nature of the early rearing environment and remains unchanged after an extended period in a common environment. Disease resistance of wild and colonized fish decreases over the course of holding them in an artificial environmental rearing environment, possibly as a direct result of cortisol mediated immunosuppression.

In view of the negative effects of cumulative stressors in

intensive culture on developing interrenal and lymphoid tissue, further work to find ways of mitigating stress in the hatchery environment may produce hatchery fish with a higher capacity to survive the rigors of the natural environment.

Feeding a live diet to fish was found to compare favorably with feeding a commercial diet, even at a lower ration level. This may be one modification that could be incorporated into hatchery rearing of salmonids to achieve a more "wild-type" fish. Other modifications of the hatchery environment might include using natural water temperature profiles, providing exercise by varying flow rates, and by allowing the aggressive capture of live prey. A portion of the diet fed to swim-up fry could include marine euphausiids, which may prevent any possible deleterious effects of rancidity in prepared feed on the development of the immune system. This approach would be an economically viable prospect to undertake for coastal British Columbia salmon enhancement facilities that are close to a seawater source.

Physiologically different wild fish are presumably the result of selection in the natural environment and might be considered as a model by which to measure the quality of hatchery smolts destined for release. In intensive culture of salmonids reared to supplement natural populations, it may be necessary for hatcheries to adapt new policies to improve the survival of their hatchery releases and produce fish that are more like their wild counterparts.

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Appendix A: Known physical parameters of the hatchery and river rearing environments.

Big Qualicum fish hatchery, Qualicum Beach, B.C., 1990

Chinook salmon from the Big Qualicum fish hatchery had been reared at densities of $0.9 \text{ kg}\cdot\text{m}^{-3}$ at ponding in January to $10.5 \text{ kg}\cdot\text{m}^{-3}$ just prior to release. Fish were ponded in concrete raceways. Water temperature was 5°C at ponding time and increased to 11°C just prior to release. Fish were maintained during this period on Oregon Moist Pellet (OMP) at the rate of 0.82 % to 2.93% of body weight per day as they increased in size. Fish were not vaccinated and received no prophylactic treatments. No measurements were taken at the river or were available from hatchery staff.

Quinsam fish hatchery, near Campbell River, B.C., 1990/91

Coho salmon from the Quinsam River fish hatchery were ponded in earthen ponds and fed OMP at a average ration level of 1-2 % body weight per day. Parameters are available from March until fish were released in June. Flow loading, or biomass per flow rate, was 0.13 kg/L per min at the beginning of this period and 0.25 kg/L per min at time of release. Dissolved oxygen was an average of 9 ppm at the outflow in March and decreased to 7-8 in May. The water source was ground water and remained a constant 10°C throughout this period. Non-filterable residue averaged 0.2 mg/L at the inflow and increased steadily from 0.6 mg/L in March to 2.0 mg/L in May at the outflow. Hatchery fish were not

vaccinated and received no prophylactic treatment for 3 months prior to release.

Water in the river was considered saturated at all times; [O₂] 11-12 ppm. Temperature increased steadily from a March low of 3°C to a high of 15°C in June. Conditions in the hatchery in 1991 were similar to those in 1990 except in March, 1991, [O₂] in the ponds was 8.35 ppm and declined further to 7.47 ppm at the end of May.

Appendix B Part 1: Protocol for the Hemolytic Plaque Assay

Recipes for media, buffers, and solutions are given in Part 2 of this Appendix. All chemicals are from Sigma Chemical Co, St. Lewis, Mo, U.S.A., unless otherwise noted.

a. Tissue extraction.

Head kidney tissue was excised aseptically and placed in 12 X 75 mm sterile culture medium (TCM) in a volume dependent on the size of the fish tissue (usually 0.5-2.0 ml for smolts). This was done while working in a laminar flow hood (or with a UV hood in the field). All surfaces were wiped with 70 % EtOH including the skin surface of the fish. Tissues were excised on a bed of paper towel soaked with EtOH. With a flamed scalpel, a ventral incision was made, extending from the pectoral fins to the pelvic fins. With a re-flamed scalpel, a dorsal second incision was made behind the operculum extending to just below the backbone. The head was grasped and stripped away head with the attached organs to expose the triangular head kidney. The head kidney was removed from the back musculature with sterile flamed instruments. This was best done with tweezers in smaller fish and pinching it out or in larger fish (> 30 g) by using a scalpel and scraping the tissue out to break the connective ties to the back musculature. Fish were kept on ice until sampled and all samples were kept in sealed culture tubes on ice before processing.

Cells were dissociated from tissue by aspirating the tissue

with a sterile 1 cc tuberculin syringe (Benton Dickinson and Co., Rutherford, New Jersey). After the connective tissue and other tissue debris settled out, the cell suspension was transferred to a new tube and, if practical, ie. if the sample is large enough that some cell loss is acceptable, the tube is centrifuged at 500g X 10 min (Beckman Model TJ6, Beckman Instruments, Palo Alto, Ca, U.S.A.) and the supernatant poured off and replaced with fresh TCM. Tubes were vortexed to resuspend cells.

b. Establishment of cell cultures.

Approximate cell numbers were established by manual cell counts by the trypan blue exclusion method and a hemacytometer. A fixed volume eppendorf and sterile yellow tips were used to remove 10 μ l from the culture tubes and were put to a well of a 96 well culture plate containing 90 μ l trypan blue (0.4% in PBS) and 100 μ l PBS. Contents were mixed with a Pasteur pipet and added to a hemacytometer chamber. Live WBC's were counted and the concentration of cells was calculated as follows; # cells/ml = 100 (# of cells in 16 squares) X 20 (dilution factor) X 10^6 . A final concentration of 2×10^7 wbc/ml was desired. At this point if the concentration of cells was too high a dilution was made with TCM. 50 μ l of the cell suspension was added to wells of a 96 well culture plate (Falcon) containing either 50 μ l of TCM/TNP-LPS (test wells) or TCM (background controls). Cultures were incubated in a gas box (American Scientific Products) purged with blood gas (10% CO₂, 10% O₂ , 80% N₂) for 7 days at 17°C. Cells

were fed on alternate days with feeding cocktail (10-20 μ l/well). The gas box was purged with blood gas after each feeding.

c. Haptenation of sheep red blood cells (SRBC).

SRBC's were obtained from an adult ewe (Sheep Unit, Department of Animal Science, U.B.C.). SRBC were stored in a 1:1.2 ratio of blood to Alsever's buffer and were successfully maintained up to 1 month in the refrigerator. For haptenation, approximately 2.5 ml of this mixture is pipetted into a centrifuge tube and centrifuged for 5 min X 1200g X 4^o C. The supernatant was discarded leaving 0.5 ml packed cells. The packed cells were then washed 3X with modified barbitol buffer (MBB), brought up to a 10 ml volume and centrifuged as before. After the final wash and spin, the TNP solution (200 μ l aqueous TNP in 3.5 ml cacodylate buffer) was added to the SRBC. The light sensitive TNP must be kept foil-covered. The mixture was vortexed and mixed on a nutator (Clay-Adams) at room temperature for 20 minutes. Immediately after mixing, the tube was centrifuged as before and the supernatant discarded (should be orange in colour) and cells resuspended in a glycylglycine solution (3.7 mg glycylglycine in 5.83 ml MBB). The tube was centrifuged as before and the supernatant aspirated (this time it should be yellow). The cells were washed with MBB twice and resuspended in MBB for storage in the refrigerator until use. Haptenated SRBC have been used with success for up to 4 days following haptenation.

d. Harvest assay

1. SRBC's were washed twice with MBB as described above in the haptenation protocol and brought up to 3.5 ml volume in MBB after the final wash and spin.

2. Dilute complement was prepared from frozen (-80°C) adult coho serum obtained in November, 1989, from Capilano Hatchery, North Vancouver. Based on the results of serial dilutions of the complement with MBB it was found that a 1 in 10 dilution produced the desired level of activity in a two hour time frame when combined with the haptenated SRBC. Complement was kept on ice throughout the assay.

3. Cell cultures were prepared for the assay as follows; Tissue culture plates were removed from the gas box and centrifuged at $500g \times 10 \text{ min}$ at 4°C . The supernatant was discarded and fresh TCM was added back. Culture plate sealers (Dynatech Inc., Chantilly, Va., U.S.A.) were placed over the wells and the plate was vortexed to resuspend the cells. The volume added back depended on the number of cells in the culture and the number of plaques one wanted to count.

4. Cunningham chambers were filled as follows; Working with 10-15 wells at a time, 50 μl of one well containing the cell suspension, 10 μl TNP-SRBC, and 10 μl dilute complement was added to a new tissue culture well. The contents of the well were

gently mixed with a Pasteur pipet and added to one side of a Cunningham chamber. After the second chamber was similarly filled, the edges were then sealed with melted paraffin by carefully dipping the sides of the chamber in wax. Chambers were incubated at 17°C X 1.5-2 hours.

5. Plaque counts were made using dark field microscopy at 2.5X and questionable plaques checked for the presence of a lymphocyte in the center at 10X. Results were expressed as number of plaques (APC)/10⁶ viable WBC (the concentration of viable WBC must be determined at the time of the assay). There can be a 100 fold reduction in viable cell numbers over the course of the seven day culture incubation.

Appendix B Part 2: Recipes

Tissue Culture Medium

Make up aseptically in a sterile 75 cm² tissue culture flask (Corning):

- 172.0 ml RPMI 1640 Medium (without L-Glutamine)
- 20.0 ml Fetal Bovine Serum (Gibco Laboratories, Long Island, New York)
- 2.0 ml Non-Essential Amino Acids (Whittaker, Bioproducts, Walkersville, Ma)
- 2.0 ml Sodium Pyruvate (Whittaker)
- 2.0 ml L-Glutamine (Whittaker)
- 2.0 ml AUC Supplement (see below)
- 2.0 ml G Supplement (see below)
- 0.1 ml 2-Mercaptoethanol 0.1 M
- 0.2 ml Gentamicin Sulphate (Whittaker)

Store ingredients in the refrigerator, keep L-Glutamine frozen in sterile aliquots and only unthaw as much as needed.

AUC Supplement

Dissolve in 100 ml Minimal Essential Medium (M.E.M, Gibco)

- 0.1 g Adenosine
- 0.1 g Uridine
- 0.1 g Cytosine

Filter sterilize using a 0.45 u filter unit (Nalgene Brand, Nalge Co., Rochester, N.Y.) or equivalent and refrigerate in 10-20 ml aliquots.

G Supplement

0.1 g Guanosine in 100 ml M.E.M.. Warm in water bath at 37°C to dissolve guanosine and then filter sterilize.

Feeding Cocktail

- 7.50 ml feeding "stocktail" (see below)
- 3.75 ml Fetal Bovine Serum
- 0.50 ml AUC Supplement
- 0.50 ml G Supplement

Thaw frozen feeding "stocktail" and combine aseptically with the other ingredients.
Store refrigerated.

Feeding "Stocktail"

70 ml RPMI 1640
10 ml Essential Amino Acids (Whittaker)
5 ml Non-essential Amino Acids
5 ml Dextrose solution (200 mg/ml maniculture H₂O)
5 ml L-Glutamine

Combine the ingredients together and adjust the pH to 7.2 using 10 N NaOH. Filter sterilize and freeze in 15 ml aliquots.

Phosphate Buffered Saline (PBS)

Dissolve the following in 1 L nanopure H₂O:

1.00 g KH₂PO₄
8.50 g NaCl
9.25 g Na₂HPO₄

adjust pH to 7.3-7.4.

Phosphatate Buffered Saline (pH 6.0, 0.06 M)

Stock A:

8.28 g NaH₂PO₄·H₂O in 1 L nanopure H₂O

Stock B:

8.52 g Na₂HPO₄ in 1 L nanopure H₂O

Use 438.5 ml of Stock A and 61.5 ml Stock B make up to 1 L with nanopure H₂O.

Modified Barbitol Buffer (MBB)

To make one 1 L of 5X stock;

1 vial of Barbitol Buffer,
add 1 part MBB 5X stock to 4 parts PBS.

pH should be 7.3-7.4.

Alsever's Solution (Anticoagulant)

Dissolve the following in 100 ml H₂O;

2.05 g Glucose
0.80 g Tri-sodium citrate (anhyd.)
0.42 g Sodium chloride

Adjust the pH to 6.1 with 10 % citric acid solution.
Sterilize by filtration.
Collect blood into 1.2 volumes of Alsever's solution.

Cacodylate Buffer

To prepare a 0.28 M solution of $(\text{CH}_3)_2\text{As}(\text{O})\text{ONa}\cdot\text{H}_2\text{O}$ (sodium cacodylate); use 2.24 grams in 50 ml of H_2O , adjust the pH to 6.9-7.1 with 0.2 M HCl.

Appendix C: Conjugation of TNP to LPS.

Solution A:

100 mg LPS (w) in 5.0 ml cacodylate buffer (pH 6.9, 0.28M)

A 5ml syringe was used to inject the cacodylate buffer into the bottle containing lysophilized powder. Adjust pH to 11.5 with 5 N NaOH, fine tune with 1N HCl if needed.

Solution B:

60 mg of picrylsulfonic acid (TNBS) in 5.0 ml cacodylate buffer and mix on a nutator (Clay-Adams) in a foil wrapped container. Add solution B dropwise to solution A while shaking.

Place on a nutator for 2 h. 15 cm of dialysis tubing (6-8 Kd cutoff) was soaked in PBS. One end was clamped with dialysis tubing and the TNP-LPS mixture was poured in the tube. The other end was then clamped and placed in 1 L of PBS in a foil wrapped erlenmeyer flask with a stir bar. This was dialyzed for 4 hours against PBS. The PBS was then renewed and the solution was again dialyzed overnight. The PBS was replaced with RPMI and left to dialyze for 24 hours. The contents of the dialysis tubing were

transferred into a foil wrapped centrifuge tube and pasteurized at 70°C for 40 min. in a water bath. The TNP-LPS was transferred aseptically into a foil wrapped serum bottle with a septum and clamped metal top and stored in the refrigerator. The concentration of this stock bottle was 10 mg/ml. The working concentration was 1.0 µg/ml in TCM (cells were plated at 0.5 µg/ml).