AN ABSTRACT OF THE THESIS OF

Crystal R. Hackmann for the degree of Master of Science in Fisheries Science presented on January 6, 2005.

Title: Physiological Ecology of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) Rearing in Fluctuating Salinity Environments.

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Carl B. Schreck and Selina S. Heppell

Estuaries provide juvenile salmonids with highly productive feeding grounds, refugia from tidal fluctuations and predators, and acclimation areas for smoltification. However, these dynamic, fluctuating salinity environments may also be physiologically stressful to growing juvenile fish. In order to evaluate the costs and benefits of estuarine marshes to juvenile Chinook salmon, I observed habitat use, diet, and growth of fish in the Nehalem Estuary on the Oregon coast. I also examined physiological costs associated with salmon living in fluctuating salinities and growth rates in laboratory experiments.

I collected growth, diet and osmoregulation information from juvenile Chinook salmon in three tidal marsh sites in the Nehalem Bay and from juveniles in the Nehalem River. Stomach contents indicated that a high proportion of the diet is derived from terrestrial prey. These allochthonous prey resources likely become available during the flood stages of tidal cycles when drift, emergent and terrestrial insects would become available from the grasses surrounding the water. This field study confirmed that juvenile Chinook salmon utilized fluctuating salinity habitats to feed on a wide range of items including terrestrial-derived resources.

Although field studies indicate that fish in estuarine habitats grow well and have access to quality prey resources, experimental manipulations of salinities were used to

quantify the physiological costs of residing in the freshwater-saltwater transitional zone. In the laboratory, I designed an experiment to investigate the physiological responses to fluctuating salinities. Experimental treatments consisted of freshwater (FW), saltwater (SW) (22-25‰); and a fluctuating salinity (SW/FW) (2 - 25‰). These treatments were based on typical salinity fluctuations found in estuarine habitats. I measured length, weight, plasma electrolytes and cortisol concentrations for indications of growth and osmoregulatory function. The fluctuating salinity treatment had a negative effect on growth rate and initial osmoregulatory ability when compared with constant freshwater and saltwater treatments. The results indicated that fluctuating salinities had a small but marginally significant reduction in growth rate, possibly due to the additional energetic requirements of switching between hyper- and hypo-osmoregulation. However, 24-hour saltwater challenge results indicated that all fish were capable of osmoregulating in full-strength seawater.

In a second experiment, I manipulated feed consumption rates of juvenile spring Chinook salmon to investigate the effects of variable growth rates on osmoregulatory ability and to test the validity of RNA:DNA ratios as indication of recent growth. The treatments consisted of three different feeding rates: three tanks of fish fed 0.75% (LOW) body weight; three tanks fed 3% (HIGH) body weight; and three tanks were fasted (NONE) during the experiment. These laboratory results showed a significant difference in the osmoregulatory ability of the NONE treatment compared to the LOW and HIGH treatments which indicates that a reduction in caloric intake significantly effected osmoregulatory capabilities during a 24 hour saltwater challenge. Furthermore, this suggests that there is a minimum energetic requirement in order to maintain proper ion- and osmoregulation in marine conditions.

Estuarine marshes have the potential to provide productive feeding grounds with sufficient prey input from terrestrial systems. However, utilization of these marshes in sub-optimal conditions could alter behavior or impair physiological condition of juvenile Chinook salmon prior to their seaward migration by providing insufficient prey resources in a potentially stressful, fluctuating environment. Therefore, the physiological costs

associated with estuarine habitat use should be well understood in order to aid future restoration planning.

Physiological Ecology of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) Rearing in Fluctuating Salinity Environments

by

Crystal R. Hackmann

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CONTRIBUTION OF AUTHORS

Carl Schreck and Selina Heppell assisted with field research and experimental designs, statistical analyses, data interpretation, and writing of manuscripts. Dan Bottom assisted with interpretation of data and provided editorial comments on all manuscripts. Dave Herring also contributed with the development of the RNA:DNA method and sample-data analysis in Appendix 3.

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Physiological Ecology of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) Rearing In Fluctuating Salinity Environments

CHAPTER 1

GENERAL INTRODUCTION

Chinook salmon (*Oncorhynchus tshawytscha*) exhibit a wide variety of juvenile and adult migratory patterns. Two basic life history types exist: ocean-type Chinook and stream-type Chinook salmon. Ocean-type Chinook move to the estuary to rear within several weeks of emergence. Stream-type Chinook remain in the rivers for 1 or 2 years before migrating to the sea (Healey 1991). Within these two life history types there is a great deal of individual variation as well. Individuals emerge and migrate to the estuary at slightly different times. The estuarine residency may also vary from a period of a few days up to a few months (Shreffler et al. 1990; Healey 1982). Individuals that rear in estuarine habitats for an extended period of time are potentially more sensitive to losses and changes in the estuarine environment than are the individuals which utilize these habitats for much shorter periods of time. Magnusson and Hilborn (2003) found that juvenile fall Chinook salmon residing in severely altered estuarine habitats had lower survival rates than those residing in natural, pristine habitats. Therefore, information on fish habitat use and physiological effects of estuarine tidal marshes on juvenile Chinook health is important for focusing habitat restoration priorities.

It has been recognized that estuarine habitats provide numerous functions for the juvenile fishes that utilize them. Some of these functions include: nursery areas, feeding grounds (Healey 1980; Levy and Northcote 1982; Shreffler et al. 1990), and refugia from tidal fluctuations and predation (Macdonald et al.1987). For juvenile salmon, estuaries are also a transition zone wherein fish gradually adapt to the environmental differences between freshwater and marine environments (Healey 1982; Iawata and Komatsu 1984; Zaugg et al. 1985). This process of adapting to a marine environment ("smoltification") requires numerous physiological, morphological, and behavioral changes (Clarke and

Hirano 1995). These changes take some time to complete and those fish that are allowed to have a more gradual acclimation to seawater have shown increased survival to adulthood (Iwata and Komatsu 1984; Macdonald et al. 1988).

A change in environmental conditions requires different physiological processes in order to cope with environmental changes. In freshwater hypo-osmotic environments, juvenile salmon are actively preventing the inflow of water into the body tissues. Upon entry into the saltwater environment, these juvenile fish are faced with new sorts of environmental assaults. Juveniles must adjust to new physiological functions, such as preventing the loss of water in tissues and the accumulation of ions in the blood plasma. It is also energetically costly to maintain internal homeostasis under such conditions, particularly within dynamic environments. Fish unprepared for the saltwater environment suffer physiological stress due to osmoregulatory constraints. Initially, stress responses can be adaptive to changes in environmental conditions; under chronic stress these responses could become maladaptive and threaten the individual's health (Wedemeyer et al. 1990). However, if the effects of these stressors exceed toleration limits, the result may be physiological dysfunction, such as impaired fish health, growth, reproductive potential, or survival.

Environmental effects on the ability to grow and develop normally in estuaries are biological points of interest when studying juvenile Chinook salmon. Growth provides an integrated assessment of the environmental conditions influencing fish (Wooten 1990), including temperature (Brett 1979), water quality (Sadler and Lynam 1986), and biological conditions (Gjerde 1986). Traditionally, smoltification has been related to the attainment of a threshold or critical size (Skilbrei 1990). Research has shown that juvenile pre-smolts grew more slowly than smolts in saltwater environments (Clarke et al. 1981) and that the approximate minimum size for smoltification of fall Chinook is 4-5g (Clarke and Blackburn 1977, 1987). However, some evidence also suggests that onset and plasticity of smolting is related to higher growth rates (Beckman et al. 2003). Therefore, growth can potentially be a valuable technique to evaluate fish population health, habitat quality, prey availability, and management activities.

Currently there is a general lack of information concerning estuarine residency and attributes of the tidal freshwater and oligohaline transition zones needed to support juvenile salmonids. In Chapter 2, we report results of laboratory studies of juvenile spring (stream-type) Chinook salmon. Our objectives were to determine the effects of fluctuating salinities, such as those encountered in estuarine habitats, on the osmoregulatory abilities and growth rates of juvenile Chinook. In Chapter 3, we studied juvenile fall (ocean-type) Chinook salmon in estuarine tidal marshes located in the Nehalem Bay along the Oregon coast and juvenile spring (stream-type) Chinook salmon in the laboratory. The objectives were to determine abundance and diet patterns of juvenile Chinook using tidal marshes in the freshwater-saltwater transition zone and to determine the effects of feeding condition on future osmoregulatory abilities in saltwater.

The majority of the Pacific Northwest's estuaries have been modified from their historic conditions (Simenstad et al. 1982), thereby making it important to understand the roles that these estuarine habitats play in salmonid physiology and health. This research provides important information for restoration efforts, and conservation prioritization for critical habitats. Estuarine tidal marshes play an important role in the diet and growth of juvenile Chinook during their estuarine residency. It is our belief that the quality of these habitats can also affect the health and survival of juvenile Chinook during the seaward migration.

CHAPTER 2

Osmoregulation and Growth of Juvenile Spring Chinook Salmon (*Oncorhynchus tshawytscha*) in Fluctuating Salinities

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ABSTRACT

Estuarine habitats are dynamic environments that are utilized by juvenile Chinook salmon (*Oncorhynchus tshawytscha*) during their seaward migration. Fluctuating salinities encountered in these environments may be physiologically stressful to growing juvenile fish. We ran a laboratory experiment to determine the physiological effects associated with fluctuating salinities. We measured length, weight, plasma electrolytes and cortisol of juvenile Chinook salmon reared in freshwater, saltwater, and fluctuating salinity over a 28-day period to assess growth and changes in osmoregulatory function. Fish thrived in all treatments, suggesting highly adaptable osmoregulatory abilities. However, the fish from the fluctuating salinity treatment negatively showed reduced growth rates and initial osmoregulatory abilities when compared with fish from the other treatments. Our results suggest that although juvenile Chinook salmon can quickly adapt to saltwater, the fluctuating salinities common in estuarine environments may impose energetic costs.

INTRODUCTION

During their seaward migration, juvenile salmonids utilize estuarine habitats as nursery areas, feeding grounds and refugia from tidal fluctuations and predators (Healey 1980; Macdonald et al.1987; Shreffler et al. 1990; Levy and Northcote 1992). The estuary also provides a transition zone for gradual adaptation from the freshwater to marine environments (Healey 1982; Iawata and Komatsu 1984; Zaugg et al. 1985). Typically, estuarine tidal marshes are marked by variable salinity gradients created by inputs of freshwater from rivers, runoff, and groundwater that mix with saltwater from tidal cycles (Seliskar and Gallagher 1983). During the process of adaptation to marine life, or smoltification, a number of behavioral, morphological, and physiological changes occur that affect numerous organs, hormones, and processes associated with metabolism and osmoregulation (Hoar 1976; Hoar 1988). Exposure to the increasing salinity gradient

of the estuary allows juvenile salmon to adapt gradually to saline water minimizing stress and improving physiological condition (Macdonald et al. 1987). This gradual exposure also has been shown to improve the ability of juveniles to tolerate the higher salinities experienced in the ocean (Iwata and Komatsu 1984; Macdonald et al. 1988).

Fish unprepared for saltwater environments could be under physiological stress in estuaries due to osmoregulatory constraints. Smolts are capable of regulating their plasma sodium concentration at or near the normal level (140-170 mmol/L) within 24 hours of transfer to seawater (Clarke and Blackburn 1977). Although salmonid parr can survive many months in seawater, they display slower growth rates and suffer a substantial elevation of some plasma electrolytes which may persist for several days (Clarke and Nagahama 1977). Previous experiments have also shown that when pre-smolts are transferred from freshwater to seawater, there is an initial critical phase characterized by significant increases in Na^+, Cl^- and Mg^{++} concentrations in plasma that may last for several days (Miles and Smith 1968; Weisbart 1968; Clarke and Blackburn 1977, 1987; Bath and Eddy 1979). It is energetically costly to maintain internal homeostasis, particularly within dynamic environments, and this may contribute additional stress. If the effects of any stressors exceed tolerance limits, physiological dysfunctions may result in impaired growth, immune system function or survival (Wedemeyer et al. 1990).

Although several studies have examined the physiological changes associated with smoltification and the changes in saltwater tolerance with age (reviewed by Folmar and Dickhoff 1980), these have been static salinity experiments. In nature, young estuarine salmonids are faced with a dynamic environment. Salinities in typical estuarine habitats may vary by 10-20‰ twice per day, as tidal flux mixes with the river flow. Previous studies also have examined saltwater tolerance and osmoregulation with immediate transfer into saltwater verses a gradual introduction of seawater (Weisbart 1968; Wagner et al. 1969; Clarke et al. 1981). These studies overlook the tidal fluctuations that juvenile Chinook salmon (*Oncorhynchus tshawytscha*) encounter in a prolonged estuarine residency.

The objective of this study was to investigate the physiological effects of fluctuating salinity environments, similar to estuarine tidal marshes, have on juvenile Chinook salmon. We designed an experiment with treatments that were similar to the salinity changes observed in estuarine tidal habitats. We expected to see initial increases in plasma electrolytes within the first 24 hours, followed by adaptation but with reduced growth or increased physiological stress in fish that experienced a fluctuating salinity treatment. Understanding how dynamic habitats, such as tidal marshes, can influence physiological function, development, and growth of juvenile Chinook salmon could provide information on the habitat conditions that estuarine restoration projects should try to re-create.

METHODS

Fish Maintenance and Experimental Design

Spring (stream-type) Chinook salmon fry were obtained from the Oregon Department of Fish and Wildlife's Marion Forks Hatchery on the North Fork of the Santiam River, Oregon. The fish were raised to an age of 12 months at Oregon State University's (OSU) Fish Performance and Genetics Laboratory (FPGL) in Corvallis, Oregon. We held the fish in 2 m circular tanks with ~12°C aerated, pathogen-free well water in a flow-through design. The fish were fed a commercial diet of semi-moist pellets (BioOregon^{TM}) daily. On 10 February 2003, the yearling Chinook salmon were transported for approximately one hour in oxygenated tanks to the Fish Disease Laboratory at Oregon State University's Hatfield Marine Science Center in Newport, OR. The fish were transferred to 1 m circular (water depth ~60 cm) tanks supplied with aerated, pathogen-free, freshwater (12-14°C). Fish remained in freshwater for a 14 day acclimation period. The mean ($\pm$ standard error) weight (g) recorded for fish at the end of the acclimation period was 47.24 $\pm$ 0.87 g.

Treatments consisted of triplicate tanks with freshwater (FW), saltwater (SW) (22-25‰); and fluctuating salinity (SW/FW) (2-25‰). Treatments were based on typical

salinity fluctuations found in estuarine habitats. Salinity was monitored with a YSI 30™ salinity, temperature, and dissolved oxygen meter. The saltwater and fluctuating salinity treatments were produced by introducing pathogen-free, filtered seawater (~13°C, 28-31‰) into the freshwater. Salinities were adjusted to the appropriate level by adjusting flow (liters per minute) of seawater and freshwater. The tanks with fluctuating salinities were equipped with a timer that controlled the seawater and freshwater input for all tanks simultaneously. Seawater input was turned on for the fluctuating tanks at 08:00 with the salinity increasing during the following two hours. The seawater was then shut off at 15:00 and gradually decreased in salinity during the following 5 hours. We only introduced one tidal cycle per day in order to provide a conservative model of fluctuating salinity impacts on juvenile Chinook salmon. It is therefore likely that any effects due to fluctuating salinities could potentially be more extreme in estuarine habitats where tides change every 6-7 hours.

Relative Growth and Condition Factor

Ten fish per tank were individually fin clipped and measured for length and weight on days 1 and 28. Growth is expressed as percentage of mean relative growth = $100^* [(W_2 - W_1) / W_1]$ (Busacker et al. 1990), where W = weight. The coefficient of the condition (K) was used to indicate the nutritional status of the fish. Condition factor was calculated as $K = \text{weight} / (\text{length})^3$ (Busacker et al. 1990).

Osmoregulatory Assessment

On days 1, 14, and 28, six fish were sampled from each treatment tank. Prior to sampling, we withheld food for 24 hours. Withholding food prior to sampling minimizes potential stress associated with feeding (Summerfelt and Smith 1990). Fish were rapidly netted in groups of 2-3 fish from the treatment tank. A lethal dose of tricaine methanesulfate (MS-222 200 mg/L buffered with NaHCO3 500 mg/L) was administered and individuals were measured for fork length (mm) and weight (g). After death, blood was collected as quickly as possible from the dorsal aorta with a heparinized Vacutainer®, and plasma was collected by centrifugation. After centrifugation, at least 170 μL of

plasma was separated and placed on ice, then stored at -80°C. Plasma samples were analyzed with a Nova Biomedical CCR analyzer (Waltham, MA), which was configured to provide concentrations of Na^+, Cl^-, K^+, Mg^{++}, and Ca^{++}.

24 Hour Saltwater Challenge

The 24-hour saltwater challenge test provides a measure of osmoregulatory capacity in seawater. We conducted saltwater challenge tests on each sampling day. Separate subpopulations (six fish per tank) were fin clipped for tank origin and were subjected to full strength salinity (28-31‰). With this test, treatment groups were transferred to tanks with full-strength seawater with the same temperature, dissolved oxygen, and pH levels as the treatment tanks. Challenge tests for the treatment groups were started in 2-hour intervals to allow for sampling time at the completion of the test. After approximately 24 hours of exposure to full-strength seawater, the fish were removed and sampled as described previously. Adaptation to seawater has been defined as the ability to maintain Na^+ levels less than 170 mmol/L within 24 hours following introduction to saltwater (Clarke et al. 1981).

Stress Assessment

Plasma cortisol has been shown to increase due to smoltification and environmental stress (Wendalaar Bonga 1997). Cortisol can promote seawater adaptation by increasing secretory activity in numerous tissues, including the gills, gut, and kidney (Clarke and Hirano 1995). Due to low plasma volumes, we pooled plasma from two individuals within tanks sampled on the same day. Individuals were pooled based on similar weights. Plasma cortisol was measured by radioimmunoassay similar to Redding et al. (1984).

Statistical Analyses

All electrolyte and cortisol data are graphically expressed as mean $\pm$ standard error (SE). We used one-way Analysis of Variance (ANOVA) to determine differences between replicates when the assumptions of normality and homogeneity of variances

were met. If variances were not homogenous, we applied non-parametric tests (Kruskal-Wallis rank sum test). Where differences were not detected ($P > 0.05$) between replicate tanks, the data from the tanks were pooled within a treatment group. Groups were tested for normality and homogeneity of variances and, where applicable, parametric statistical procedures were applied. Tukey's honestly significant differences (HSD) post-hoc test was used to determine which treatments were significantly different (Crawley 2002). If significant differences were detected between the treatment groups with non-parametric tests, we then carried out pairwise Wilcoxon rank tests (Dytham 2003). Any differences were considered significant if the $P \leq 0.05$. All analyses were performed with the S-Plus 6.1™ statistical program.

RESULTS

Relative Growth and Condition Factor

There were no significant differences among the tank replicates within each treatment (ANOVA; P=0.65-0.95); therefore, all data for relative growth and condition factor were pooled into the treatment groups. The greatest difference in the relative growth of the fish was between the SW/FW treatment and the FW treatment; however, the SW treatment was not significantly different from the FW or the SW/FW treatment (ANOVA; $P = 0.055$, followed by Tukey's HSD test) (Figure 2.1). Condition factor was analyzed using the Kruskal-Wallis rank sum test for non-parametric data. There were no significant differences among the groups (Kruskal Wallis; $P = 0.230$) (Table 2.1).

Figure 2.1. Mean percentage of relative growth (+SE) for juvenile spring Chinook salmon in three treatment groups. The growth rate was calculated as the percent change from the initial body weight throughout the 28 day experiment. For the FW and the SW treatments, n = 29. For the SW/FW treatment, n = 30. Letter A indicates which treatments were different ($P = 0.055$).

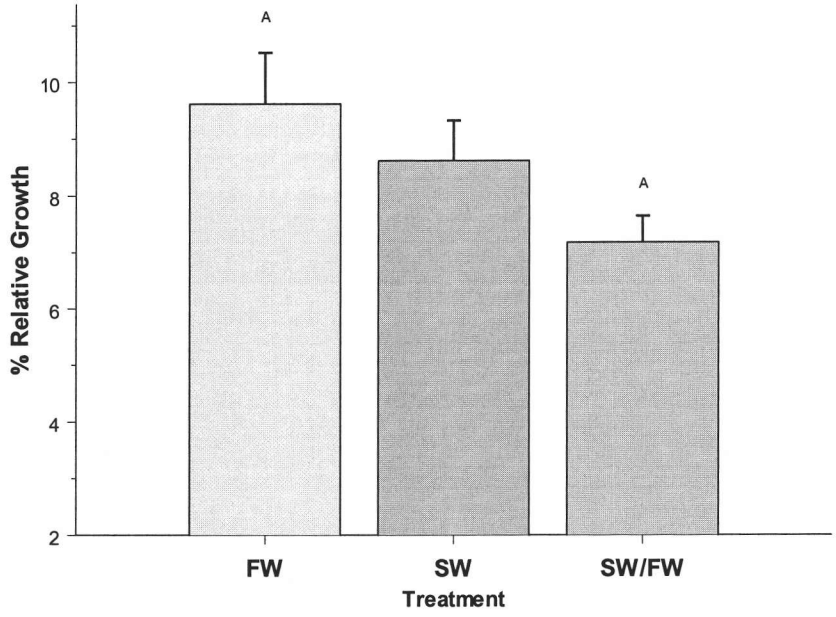


Table 2.1. Statistical analyses and means (SE) for condition factor. For the FW and the SW treatments, n = 29. For the SW/FW treatment, n = 30.

	FW	SW	SW/FW	Statistical Test
Condition Factor				
	0.113	0.113	0.110	Kruskal-Wallace
	(0.0013)	(0.0087)	(0.0011)	($P = 0.230$)

Osmoregulatory Assessment

For this analysis, only the Na^+ and Cl^- concentrations were included. The other plasma electrolytes showed no significant trends or correlations with Na^+ and Cl^-, which are the primary indices of osmoregulatory ability since sodium chloride is the major component of the surrounding seawater. On days 1, 14, and 28, the tank replicates for the Na^+ and Cl^- plasma levels were not significantly different (ANOVA or Kruskal-Wallis; P = 0.164-0.997). All data from the replicate tanks on the different sampling days were pooled into their treatment groups. An among-treatment comparison on day 1 showed that the Na^+ levels of the fish in the SW/FW and the SW treatments were significantly higher than those of the fish in the FW treatment (ANOVA; $P = 0.008$, followed by Tukey's HSD test) (Figure 2.2). After day 1, there were no significant differences among the treatments for the Na^+ levels of the fish sampled on days 14 and 28 (ANOVA; P = 0.283 and $P = 0.767$, respectively). We then looked within the treatment groups for differences among the Na^+ levels of the fish sampled on days 1, 14, and 28. For the FW treatment, the Na^+ levels were significantly lower on days 1 and 14 when compared to day 28 (ANOVA; $P = 0.006$, followed by Tukey's HSD test). For the SW and the SW/FW treatments, the Na^+ levels were not significantly different among days 1, 14, and 28 (ANOVA; $P = 0.067$ and $P = 0.357$ respectively), although the SW treatment results suggest that Na^+ levels were also higher on day 28.

An among-treatment comparison on day 1 showed that the Cl^- levels of the SW/FW treatment fish were much higher than those of the FW and the SW treatments (ANOVA; $P = 0.001$, followed by Tukey's HSD test) (Figure 2.3). On days 14, and 28, the treatment groups were not significantly different (ANOVA; $P = 0.659$ and $P = 0.423$, respectively). Within the treatment groups, there were no significant differences for Cl^- levels of the fish in the FW and the SW treatments on days 1, 14 and 28 (ANOVA; P = 0.982 and $P = 0.214$, respectively). However, the Cl^- levels of the fish in the SW/FW treatment did exhibit significantly higher levels on day 1 when compared to day 14. The SW/FW treatment fish also had significantly lower Cl^- levels on day 14 compared to day 28 (ANOVA; $P = 0.042$, followed by Tukey's HSD test).

Figure 2.2. Mean Na^+ Levels (+SE) for juvenile spring Chinook salmon in treatment groups. For each treatment, n = 18.

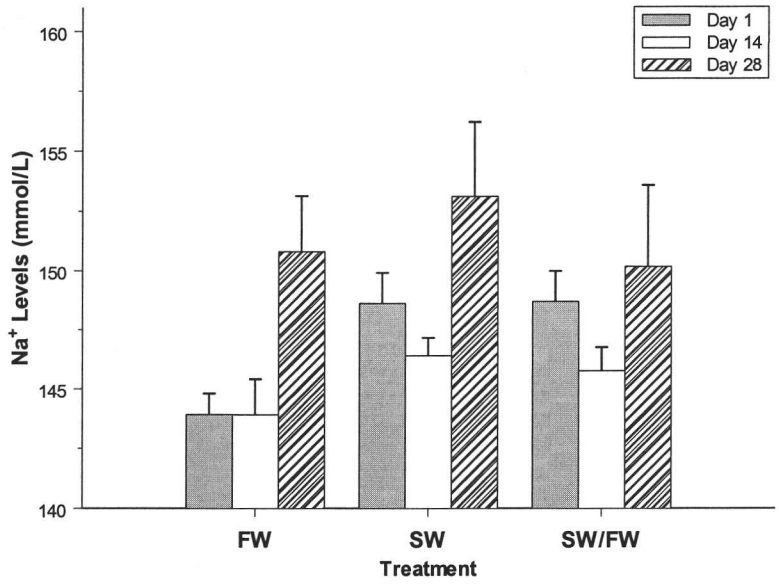
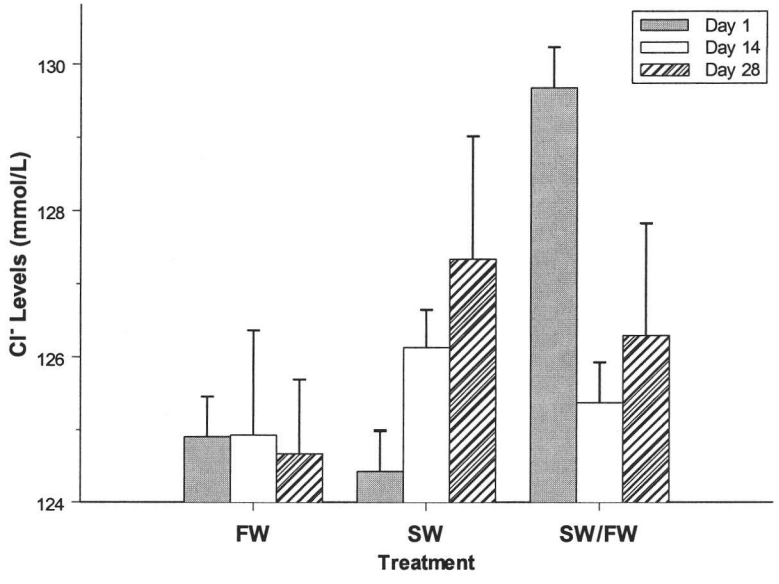


Figure 2.3. Mean Cl^- levels (+SE) for juvenile spring Chinook salmon in treatment groups. For each treatment, n = 18.



24-Hour Saltwater Challenge

After the challenge tests, the Na^+ and Cl^- levels were tested for significant differences among the replicate tanks. No significant differences occurred in Na^+ or Cl^- levels among the replicate tanks after the 24-hour saltwater tests (ANOVA or Kruskal-Wallis; P =0.089-0.941). Therefore, the data were pooled into treatment groups. The fish within a treatment group exhibited significant increases in Na^+ and Cl^- levels after the saltwater challenges (Student's t-test; $P \leq 0.05$), except for the Cl^- levels of the SW/FW treatment fish after the day 1 saltwater challenge (Student's t-test; $P = 0.194$) and the Na^+ levels of the SW treatment fish after the day 28 saltwater challenge (Student's t-test; $P = 0.135$).

After the day 1 saltwater challenge, there were three mortalities. Two of the mortalities were from the FW treatment and one was from the SW treatment. The exact cause of death was unknown and these fish could not be used for any plasma analyses. The remaining fish were sampled after the day 1 challenge. These results showed that the FW treatment fish responded more negatively to the saltwater challenge compared to the other two treatment groups after the day 1 saltwater challenge test only. The SW and the SW/FW treatment had significantly lower Na^+ levels than the FW treatment (ANOVA; P = 0.034, followed by Tukey's HSD test) (Figure 2.4).

The Cl^- levels of the fish sampled after the saltwater challenges showed a different trend. Although all treatment groups had highly variable Cl^- levels, the only statistically significant difference after the saltwater challenges was on day 14 when the SW/FW treatment fish had significantly higher Cl^- levels than the FW treatment (ANOVA; $P = 0.014$, followed by Tukey's HSD test) (Figure 2.5). After the day 28 challenge, the Cl^- levels of the SW/FW treatment fish had decreased and there were no significant differences among the treatment groups.

Figure 2.4. Mean Na^+ Levels (+SE) for juvenile spring Chinook salmon after the 24-hour saltwater challenge tests. For FW treatment, n = 16; SW treatment, n = 17; SW/FW treatment, n = 18.

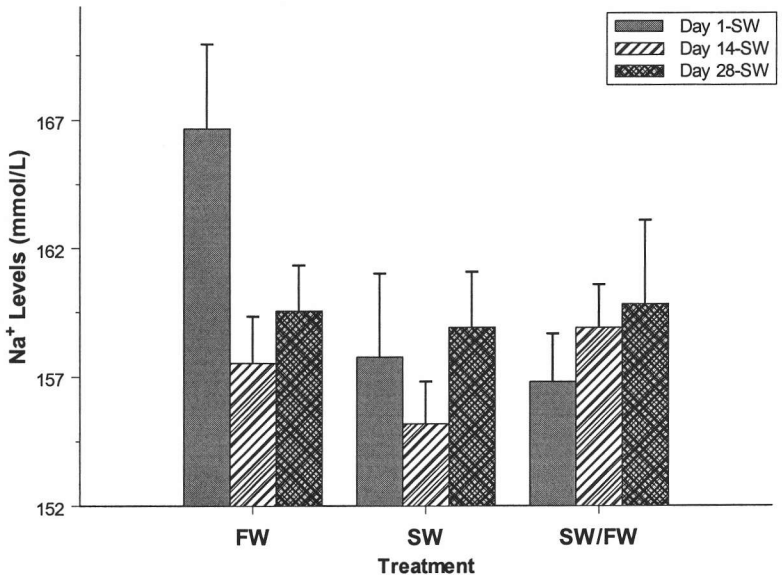
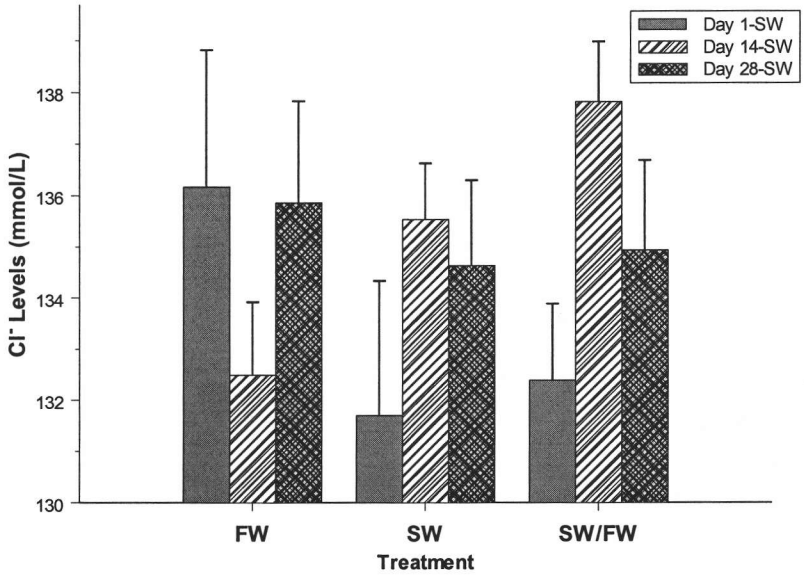


Figure 2.5. Mean Cl^- levels (+SE) for juvenile spring Chinook salmon after the 24-hour saltwater challenge tests. For FW treatment, n = 16; SW treatment, n = 17; SW/FW treatment, n = 18.



Stress Assessment

The plasma cortisol levels did not have homogenous variances; therefore, we used non-parametric statistical tests to analyze the data. There were no significant differences among the replicates (Kruskal-Wallis; $P = 0.067$-0.099); therefore, we pooled the tanks into their treatment groups. Throughout the experiment, the cortisol levels increased in fish sampled directly from the treatments and in those sampled after the 24-hour saltwater challenges. There was not any particular treatment group that appeared to be more variable. After the day 1 saltwater challenge, the cortisol concentrations of the SW treatment fish were significantly lower than those of the FW and SW/FW treatments (Kruskal-Wallis; $P = 0.002$, followed by Wilcoxon rank tests) (Table 2.2). Plasma cortisol levels were typically higher after the 24-hour saltwater challenge tests.

Table 2.2. Mean plasma cortisol levels (SE) for days 1, 14, and 28. SW indicates saltwater challenge tests. Asterisk indicates that the cortisol levels of the SW treatment fish were significantly lower than those of the other treatment fish after the day-1 saltwater challenge (Kruskal-Wallis; $P = 0.002$). Individuals were pooled into groups of two in order to obtain enough plasma for the assay. For each treatment, n = 9, except on Day 1-SW, where the FW treatment, n = 8; SW treatment, n = 8; and SW/FW treatment, n = 9.

Cortisol (ng/mol)						
Treatment	**Day 1**	**Day 1-SW**	**Day 14**	**Day 14-SW**	**Day 28**	**Day 28-SW**
FW	27.19 (6.14)	34.48 (5.98)	40.08 (17.52)	63.34 (6.96)	99.04 (8.77)	84.95 (15.91)
SW	15.99 (3.43)	11.26* (2.88)	44.18 (10.79)	53.90 (4.41)	83.82 (10.24)	84.41 (11.64)
SW/FW	29.23 (8.43)	37.32 (6.82)	21.82 (3.57)	59.31 (15.73)	100.25 (8.76)	70.51 (11.14)

DISCUSSION

Even in a dynamic fluctuating salinity, juvenile Chinook salmon are remarkably tolerant of rapid change from a hyper- to hypo-osmotic environment. In a constant salinity environment, the first 24 hours following introduction can be very crucial to survival and future growth potential of juvenile Chinook. We expected to see an initial increase in the plasma electrolytes following the start of the SW and SW/FW treatments. In our experiment, the fish sampled on day 1 were the only fish exhibiting a clear electrolyte increase to the treatments. Although there were some increases in plasma electrolytes on days 14 and 28, the concentrations for all days and saltwater challenges were within recorded levels for *Onchorhynchus* sp. (i.e. $[Na^+] < 180$ mmol·L^{-1}, $[Cl^-] <$ 160 mmol·L^{-1}; Conte et al. 1966; Clarke and Blackburn 1977; Folmar and Dickhoff 1980; Morgan and Iwama 1991; Wagner et al. 1969), indicating that the fish were regulating their plasma ions competently throughout the experiment. Furthermore, the fluctuating salinity treatments did not provide any significant advantages or disadvantages regarding osmoregulatory capabilities in full strength seawater.

While immediate transfer to saltwater is an extreme test for a fish's osmoregulatory ability, all of the experimental fish were able to survive in the SW and the SW/FW treatments and most of the fish survived the full-strength 24 hour saltwater challenge tests. In natural conditions, juvenile Chinook salmon would undergo a more gradual introduction to full strength salinity during their migration downstream into the estuary and then the ocean. Despite the direct transfer to full strength saltwater during the challenge tests, there were only three mortalities after the day 1 saltwater challenge out of the 162 fish that were tested. However, there is no definite way to link these mortalities to the full strength saltwater challenge test and there were no mortalities after the challenge tests on days 14 and 28. If the fish in the FW treatment were unable to survive 24 hours in full strength saltwater then there likely would have been severely elevated plasma electrolytes or additional mortalities after the day 14 or day 28 challenge tests as well. Furthermore, the size of fish was not correlated with osmoregulatory performance in full-strength saltwater. Although survival alone is not an adequate test of

saltwater tolerance, the experimental fish were able to adapt their osmoregulatory systems in order to survive the full-strength seawater.

Cortisol is considered to be an important hormone promoting adaptation to seawater in teleosts (Johnson 1973; Folmar and Dickhoff 1980). Although few other studies have investigated the relationship among juvenile spring Chinook salmon, cortisol levels and smoltification, our results suggest that juvenile spring Chinook salmon experience an increase in cortisol levels as they age during the smoltification process. Elevated plasma cortisol levels have also been observed in coho (*O. kisutch)* (Specker and Schreck 1982; Barton et al. 1985) and Atlantic salmon (*Salmo salar*) (Langhorne and Simpson 1981; Virtanen and Soivio 1985) during the parr-smolt transformation. Although the cortisol levels did increase five times the initial concentrations, the fish did not exhibit any corresponding secondary stress responses (i.e. plasma electrolyte changes) or behavioral changes. It is possible that the cortisol levels were slightly elevated due to environmental stressors; however, it is unlikely that this was the only cause for the elevations in cortisol. Based on the work of Barton et al. (1985), we expected the fish to be most sensitive to cortisol elevations during the late stages of smoltification. The fish used in our study were readily able to tolerate saltwater physiologically and exhibited a smolt-like appearance. It is possible that the elevated cortisol levels we observed were related to smoltification and could be a pre-adaptation for entry into saltwater.

Numerous studies have documented that salinity resistance and smolting can be size dependent (Hoar 1976; Folmar and Dickhoff 1980). Wagner et al. (1969) also concluded that "salmon which are more rapidly growing possess a regulatory system that is either more functional with respect to a given salt gradient or capable of being initiated more quickly with changes in environmental salinities." However, salinities greater than 20‰ can have significant effects on juvenile Chinook salmon growth due to the metabolic cost of osmoregulation (Shaw et al. 1975; Clarke et al. 1981). Our results confirmed these previous studies that constant salinities between 20-25‰ were not sufficient to significantly affect growth in juvenile spring Chinook salmon, however, this may have been due to their high growth rates or attainment of a critical size threshold by

our experimental fish. Furthermore, in our study the salinities fluctuating between 2-25‰ resulted in a marginally significant reduction in growth without triggering significant cortisol responses. The fluctuating salinities may have negatively affected growth rates by increasing maintenance energetic requirements, thereby, allowing less energy for muscular growth. It is possible that more energy was required to maintain ion and osmoregulatory transport systems, which were constantly switching between hypo- and hyper-osmoregulation and ionregulation due to residence in the dynamic fluctuating salinity environments.

Juvenile Chinook salmon are very plastic in their responses to fluctuating salinities and capable of osmoregulating in full strength saltwater environments. However, the magnitude of this saltwater resistance may be dependent on obtaining a critical size or growth rate prior to entering a marine environment (Conte and Wagner 1965; Folmar and Dickhoff 1980; Clarke and Shelbourn 1985). There also is a rhythmical change in this salinity resistance. It develops immediately before and during migration, is independent of the smolt transformation, and it regresses when salmon or anadromous trout are retained in freshwater (Conte and Wagner 1965; Hoar 1976). Therefore, if this experiment had been conducted at a different time of the year, it may have yielded different results.

In summary, juvenile Chinook salmon are capable of osmoregulating and growing in a wide variety of saltwater situations, including fluctuating salinities. Although freshwater is the natural habitat at this life stage, the FW treatment did not provide any growth or osmoregulatory advantages over the SW treatment. Furthermore, the FW and SW treatments had larger variation in growth rates while the FW/SW treatment had marginally significant reductions in growth with less variation. However, there is still little known about the energetic requirements of juvenile salmonids during their seaward migration through estuaries in fluctuating salinities. This small reduction in growth rate was potentially due to switching between hyper- and hypo-osmoregulation and ionregulation on a daily basis. These differences may be different in natural situations where tidal fluctuations are more frequent, resources may be limited, and energetic expenditure for prey is potentially greater. Depending on the developing saltwater

tolerance, size, and growth rate, it is therefore possible that entry into the dynamic estuarine habitats could affect juvenile salmonid health and survival.

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CHAPTER 3

Diet and Osmoregulation of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) in Freshwater-Saltwater Transition Zones

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ABSTRACT

Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) exhibit diverse life history patterns and have a high behavioral and physiological plasticity to environmental conditions. These characteristics allow them to exploit a wide variety of resources in many types of habitats, including estuarine tidal marshes. We observed habitat use, diet, and abundance of juvenile Chinook salmon in estuarine tidal marshes and experimentally examined physiological costs of a diet reduction on saltwater tolerance in the laboratory. Juvenile Chinook salmon were collected for growth and diet information from three tidal marsh sites located in the Nehalem Bay, Oregon. Juvenile river residents also were collected from the Nehalem River in June. Analysis of stomach contents indicated a seasonal increase in the consumption of allochthonous prey items (i.e. terrestrial prey resources), with a concurrent decrease in crustaceans and aquatic and semi-aquatic insects. Our laboratory results indicated that a reduction in diet significantly affected osmoregulatory capabilities during a 24-hour saltwater challenge test. This suggests that there is a minimum energetic requirement in order to maintain proper ion- and osmoregulation in marine conditions. Estuarine habitat conditions could potentially influence juvenile Chinook salmon health and survival depending on their developing saltwater tolerance, diet quality, size, and growth rate.

INTRODUCTION

Estuarine tidal marshes play an important role in the feeding ecology of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) during their estuarine residency (Shreffler et al. 1992; Miller and Simenstad 1997), potentially affecting their health and survival during the seaward migration. It has been shown that Pacific salmon use estuaries for 1) productive foraging, 2) physiological transition, and 3) refugia from predators and tidal fluctuations (Simenstad et al. 1982; Healey 1982; Iwata and Komatsu 1984). In most cases, the estuary has been treated as one habitat with little distinction among salinity

zones, habitat types, and degrees of habitat accessibility. Although some studies have addressed the general ecological importance of the freshwater and saltwater transition zone (Odum 1971), very few studies have addressed the role this ecotone and surrounding vegetation plays in the estuarine residency of juvenile Chinook salmon.

Among salmonids, Chinook salmon show the greatest variation in juvenile life history patterns and behaviors (Reimers 1973; Fisher and Pearcy 1989; Healey 1991). This variation in behavior includes an assortment of estuarine residency patterns. Chinook salmon show two primary patterns of smoltification and migration: "ocean-type" juveniles smolt and migrate to sea during the first summer after emergence, and "stream-type" juveniles reside in fresh water for 1-2 years before smolting and migrating quickly through the estuary (Healey 1991). For "ocean-type" juveniles this seaward migration behavior is more variable and occurs over an extended period of time during which the estuarine residency may vary from days to months (Healey 1980; Shreffler et al. 1990; Miller and Simenstad 1997). In the estuary, juvenile Chinook salmon complete their transition from a freshwater to a saltwater environment. Abiotic and biotic conditions in the estuarine tidal zones may have strong effects on salmonid year class survival and health (Macdonald et al. 1988; Solazzi et al. 1991). For example, faster growth rates in the estuary and larger sizes upon entry into the ocean have been shown to increase early marine survival of juvenile Chinook salmon (Reimers 1973; Macdonald et al. 1988).

Estuaries are regarded by ecologists as some of the most biologically productive and critical ecosystems for many marine and estuarine juvenile fishes (Odum 1971; Thom 1987). The benefits of estuaries to juvenile salmon inhabitants have been shown to be related to the biological productivity and the availability of suitable prey resources (Healey 1991; Miller and Simenstad 1997; Gray et al. 2002). Some fish will even migrate down into the estuary before they are ready to smolt (Healey 1982). Those juveniles residing in the estuarine habitats often have more food in their stomachs than those in the riverine habitats (Congleton et al. 1981) and they often exhibit the greatest growth rates of their lifetime during estuarine residency (Shreffler et al. 1990). It has been documented that juvenile Chinook salmon will return to estuaries when transported

to sites close to the mouths of the estuaries in order to feed and reside in estuaries for extended periods (Macdonald et al. 1988; Solazzi et al. 1991). Juveniles will also move with the tidal cycles in order to feed on insects associated with the emergent vegetation of marshes and sloughs (Healey 1991; Levy and Northcote 1992; Lott 2004). The marine food entering the estuary via tides along with inputs from rivers potentially provides a large variety and amount of prey available to juvenile salmonids (Kask et al. 1986; Macdonald et al. 1987). It is this abundance of diverse prey items in estuarine habitats that may drive fish to stay longer than required by physiological constraints.

While few studies have focused on the marshes habitats of the freshwater-saltwater transition zone, numerous studies have investigated the functional importance of the estuarine channels and marshes (Macdonald et al. 1987; Healey 1991; Gray et al. 2002) and freshwater wetlands that are bordered by emergent vegetation (Levings et al. 1995) for juvenile salmonids. However, it is in this freshwater-saltwater transition zone that juvenile Chinook fingerlings, fry, and smolts are first encountering an increase in salinity and must switch from hyper- to hypo-osmoregulation (Healey 1991). These juveniles may therefore, have an increased reliance on the surrounding habitats in order to gradually acclimate to a saltwater environment. Understanding the dietary requirements, prey selection, and osmoregulatory abilities of fish using the fluctuating-salinity estuarine habitats could clarify the importance of these habitats during the estuarine residency.

The objectives of our field study were: to determine osmoregulatory capabilities and diet patterns of juvenile Chinook salmon using tidal marshes in transitional, fluctuating salinity zones; to determine if diet differed between marsh sites; and to determine if the number of fish utilizing these marshes differed due to proximity to deep channel refugia. To help understand cost-benefits of osmoregulatory demands versus trophic benefits, we complemented the field study with a laboratory study designed to determine if food quantity would have an effect on future osmoregulatory abilities of juvenile Chinook in full strength saltwater. In the Nehalem Bay along the Oregon coast, we sampled estuarine tidal marshes in order to determine whether Chinook salmon stomach contents reflect the type of prey associated with these habitats. We also studied

the effects of three different diet volumes on saltwater tolerance of juvenile Chinook salmon in a laboratory experiment. It is our belief that despite high physiological plasticity of juvenile Chinook salmon, energy intake can affect maintenance metabolic requirements, including osmoregulatory performance. Understanding how estuarine habitat use and diet correlate with physiological condition could explain more clearly why juvenile Chinook salmon use these habitats and how this habitat could affect their survival.

METHODS

Field Research

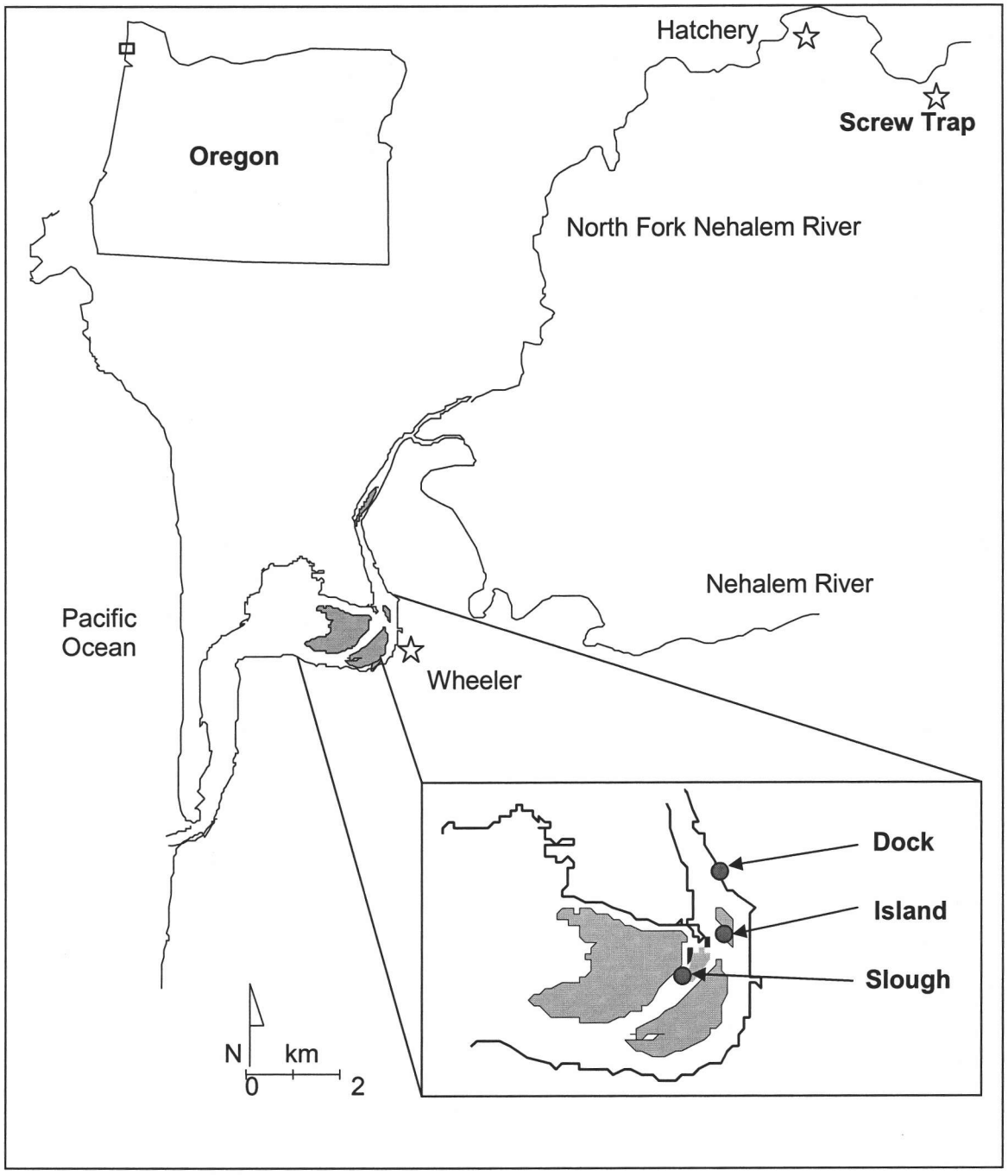
Study Area

The Nehalem River estuary is located in Tillamook County, Oregon along the Pacific Ocean (Figure 3.1). The Nehalem River is one of the longest rivers in Oregon, with a length of approximately 195 km. The North Fork Nehalem River has a steelhead (*O. mykiss*) and coho salmon (*O. kisutch*) hatchery approximately 18 km upriver of the confluence. Both forks of the Nehalem River have naturally occurring wild runs of juvenile fall Chinook salmon. The Nehalem Bay is classified as a shallow draft development estuary with maintained jetties, a main channel that is dredged to a 6.7 m depth or less, and with areas designated for development, conservation, and natural management (IMST 2002). Near the town of Wheeler, a sharp bend in the Nehalem Bay distinguishes upper from lower estuarine habitats. The upper estuary has backwater sloughs, tidal marshes, and various types of habitat structure, such as woody debris and pilings. The lower estuarine habitats contain similar habitat structure but also have mudflats and channels. The network of sloughs and channels generally lack connectivity to upland freshwater sources and dewater at low tides.

Our study focused on the upper estuary tidal habitats in the freshwater-saltwater transition zone. We chose three sites where juvenile Chinook salmon are present, marsh grasses are accessible at high tide, and salinities are similar (within ± 5‰) (Figure 3.1).

The salinities for the sampling sites ranged between 0.1-7.1‰ (low tide) and 11-27.5‰ (high tide) depending on the tidal size and river flow. The three sites varied in the slope gradient, which we measured as the water depth at 9 m from the bank of the marsh grasses. The sampling site called "Slough" had a low slope gradient and was approximately 1 m deep from the bank. "Dock" had a medium slope gradient with a 1.8 m depth while the "Island" had a high slope gradient with a 3 m depth. The variation in the gradients among the sites affected the distance to deep channels and the amount of time the marsh grasses were flooded and available for foraging. For comparisons between estuarine and river resident juvenile fall Chinook salmon, we collected fish at a screw trap operated by the Oregon Department of Fish and Wildlife (ODFW) on the north fork of the Nehalem River.

Figure 3.1. Field site locations in the Nehalem Bay and Nehalem River along the northern Oregon coast. Three sampling sites (Dock, Island, and Slough) were located along the tidal marshes of the Nehalem Bay and one sampling site (Screw Trap) was located in the North Fork Nehalem River.



Fish Collection

We used beach seines (9.14 x 1.83 m with a 4.76 mm mesh size) to catch fish at high tides in the three estuarine sites. Temperature, salinity, and depth were measured with an YSI 30^{TM} during high and low tides at the sampling sites. The three estuarine marsh sites were sampled at two-week intervals, May through September. Because no fish were caught during September, sampling was discontinued. On each sampling day, we collected a maximum of 20 fall Chinook salmon per site. Individual marsh sites were sampled with a maximum of four seine pulls moving in 9.14 m transects down the shoreline. On two sampling days during June, we collected fish from the Oregon Department of Fish and Wildlife's screwtrap in the north fork of the Nehalem River. All fish were euthanized with tricaine methanesulfate (MS-222, 200 mg/L buffered with NaHCO3 500 mg/L), weighed and measured, then sampled for stomach contents and dorsal muscle tissue.

Muscle Water Concentration

Muscle dehydration is an indicator of an insufficient drinking rate or insufficient retention of water by the body. If fish are unable to osmoregulate in a saltwater environment, then the muscle tissue would likely dehydrate due to osmosis (Eddy 1981). To measure muscle water concentration, a small piece of dorsal muscle tissue ($\geq$10 mg) was dissected from all individuals sampled in the estuary and 22 individuals from the river. The samples were placed into pre-weighed and pre-labeled vials, and stored on ice. These samples were dried to a constant weight at 60°C (usually within a 48 hour period) and the percentage of muscle water was calculated as the weight difference between the fresh and dried tissue. The proportional data of muscle water concentration were transformed using arcsine square root, and the means were analyzed using one-way Analysis of Variance (ANOVA).

Diet Analysis

Changes in the food habits of juvenile fall Chinook salmon were determined by analysis of stomach contents from individuals collected at the three estuary sites

throughout the sampling period. We removed, injected and preserved the stomachs in a 10% solution of buffered formalin (v/v) for two weeks. After two weeks the stomachs were transferred to a 70% solution of ethyl alcohol (v/v). Stomach contents were analyzed for the origin of prey items using the Point-Estimate method adapted from Hynes (1970). The volume or area that a prey item occupied in the stomach was assessed by using a reference object of known area (2 x 2 mm square on graph paper). The contents were dissected from the stomach lining under a dissecting microscope, separated into content categories, identified to taxonomic Order if possible, and assessed for the number of squares the categories occupied. The number of squares that the categories occupied provided results for the percentage composition of the diet. The content categories included aquatic crustaceans, aquatic insects, semi-aquatic insects, terrestrial insects, unidentified insect parts, unidentified digested material, and nonfood items. Terrestrial insects included any insect that does not live in water or on the water surface. Semi-aquatic insects were any insect that was associated with the water surface or with intertidal zones or littoral habitats. Aquatic insects included any insects that normally inhabit the water column or were associated with benthic habitats (Borror et al. 1989). These categories were used to determine the importance of aquatic verses terrestrial derived (allochthonous) prey items.

We visually assessed seasonal diet patterns within sampling sites to determine trends. There appeared to be a seasonal shift in the middle of the sampling period with some of the diet categories. No other patterns were discernable. For statistical analyses, only the discernable prey items were included (i.e. crustaceans, aquatic insects, semi-aquatic insects, terrestrial insects, and insect parts). In order to determine if content categories from the individual sampling days could be grouped into "Early" (pre-July 14) and "Late" (post-July 14) periods, we performed parametric and non-parametric statistical tests (one-way ANOVA and Kruskal-Wallis tests) between sampling days and within sites. All data are graphically expressed as mean (+ standard error) percentage of individual diet categories. These stomach content categories were also compared among sites using Schoener's (1970) index, which provides a reasonable measure of dietary overlap while requiring few assumptions (Crowder 1990). The index C_{xy} is:

$$C_{xy} = 1 - 0.5(\Sigma \left| p_{xi} - p_{yi} \right|)$$

where p_{xi} is the proportion of resource i used by species x and p_{yi} is the proportion of resource i used by species y. This index varies form 0 when samples x and y have no food items in common to 1 when they are the same in terms of proportional composition of stomach contents. The proportional data from the diet categories were pooled ($P > 0.05$) and arcsine square transformed in order to compare categories within a site but between early and late season sampling. One-way analysis of variance (ANOVA) with a significance level set at $P \leq 0.05$ was used to compare the transformed proportions of content categories between early and late season sampling.

Laboratory Experiment

Fish Maintenance and Experimental Design

Sub-yearling spring ("stream-type") Chinook salmon fry were obtained from the Oregon Department of Fish and Wildlife's Marion Forks Hatchery on the North Fork of the Santiam River, Oregon. The fish were raised to an age of 7 months (average weight 22.0 $\pm$ 5.4 g) at Oregon State University's (OSU) Fish Performance and Genetics Laboratory (FPGL) in Corvallis, Oregon. We held the fish under a natural photoperiod in approximately 1 m diameter circular tanks with flow-through system using ~12°C well water that was aerated and pathogen-free. Prior to the start of the experiment, the fish were transferred individually into anesthetic (MS-222 50 mg/L buffered with NaHCO3 125 mg/L) and commercially available Stress Coat® to prepare them for tagging. Once anesthetized, each fish was measured and any external abnormalities noted. The fish was then securely held while a pit tag was injected into the ventral body wall anterior to the pelvic girdle. This procedure makes a very small incision (<0.5 cm) that requires no closure. The fish were then returned to their treatment tanks for recovery. Fish were given three days to recover and resume their normal appetites and behaviors prior to the start of the experiment.

The treatments consisted of three feeding rates: three tanks fed 0.75% (LOW) biomass of tank; three tanks fed 3% (HIGH) biomass; and three tanks without food (NONE) during the experiment. Total fish weights per tank were recorded at the start of the experiment and were used to determine total feed amounts during the experiment. The treatments were randomly assigned to the tanks. Fish were fed a diet of Bio-Oregon semi-moist pellets. Fish fed 3% biomass of tank were fed twice a day, while fish fed 0.75% biomass were given their entire ration for the day in the morning. Tanks were cleaned every week in the late afternoon at least four hours after feeding. Prior to sampling, we withheld food for 24 hours in order to minimize potential stress associated with feeding. Eight fish from each tank were sampled for plasma electrolytes on days 12 and 24. A separate eight fish per tank were subjected to 24-hour saltwater challenge tests after days 12 and 24. All fish were individually marked with surgically implanted pit tags. During sampling, we determined fish growth rates in each tank to calculate total fish weights for modifying amounts of food.

Sampling Methods

Fish were rapidly netted from treatment tanks and transferred to holding tanks under similar conditions. A lethal dose of anesthetic (MS-222, 200 mg/L buffered with NaHCO3 500 mg/L) was administered and individuals were measured for fork length (mm) and weight (g). After death, blood was collected as quickly as possible from the dorsal aorta with a heparinized Vacutainer® and 20 gauge needle. After centrifugation, plasma was separated and the samples were stored at -80°C until analysis. Plasma samples were analyzed with a Nova Biomedical CCR analyzer (Waltham, MA), which was configured to provide concentrations of electrolytes (Na^+, Cl^-, K^+, Mg^{++}, Ca^{++}) and bioenergetic (lactate, glucose) constituents. In order to obtain the minimum amount of 150 μL for analysis with the Nova analyzer, individuals were pooled into groups of two.

Fish Condition and Osmoregulatory Assessment

The 24-hour saltwater challenge test provides a measure of osmoregulatory capacity in full strength seawater (Clarke and Blackburn 1987). On days 12 and 24, we

randomly selected eight fish per tank and placed them into three different tanks with full-strength saltwater (28-31‰), prepared in advance using Instant Ocean®, in order to determine the effects of feeding rates on seawater tolerance. Individuals were identified by their pit tags. The challenge tanks were started in 2 hour intervals to allow for sampling time at the completion of the test. Saltwater challenge tanks were aerated with pumps during the 24 hour tests. After approximately 24 hours of exposure to full-strength seawater, the fish were removed, given a lethal does of anesthetic (MS-222 200 mg/L buffered with NaHCO3 500 mg/L), and sampled as described previously. Adaptation to seawater has been defined as the ability to maintain Na^+ levels at less than 170 mmol/L within 24 hours following introduction to saltwater (Clarke et al. 1981). After the 24-hour saltwater challenge tests, we also dissected a small piece of dorsal muscle tissue (>10mg) from individuals for muscle water concentration using the methods described previously.

All data are graphically expressed as mean + standard error (SE). Where differences were not detected (one-way ANOVA; $P > 0.05$) between replicate tanks, we pooled the tanks by treatments. If differences were significant among replicate tanks, these tanks were not pooled for further analysis. One-way ANOVA with Tukey's honestly significant differences (HSD) post-hoc test was used when the assumptions of normality and homogeneity of variances were met. Because variances were not homogenous for all variables measured, we also used the Kruskal-Wallis rank sum test. If significant differences were detected between groups, we carried out pairwise Wilcoxon rank tests. The proportional data of muscle water concentration was transformed using arcsine square root and the means were analyzed using one-way Analysis of Variance (ANOVA).

RESULTS

Field Research

Site salinity and temperature measurements were similar for all estuary sampling sites (Figure 3.2 and 3.3). The Dock site, which is the most upstream, showed somewhat

lower salinities than the other sites after July, when rainfall was minimal. Peak catches of juveniles in the Dock and Island marsh sites were during the month of June. The Slough site had the lowest abundance of juvenile Chinook salmon with catches ranging from 0-11 fish on a sampling day. The lengths (mm) of juvenile fall Chinook salmon increased throughout the sampling season, while numbers of fish caught decreased (Figure 3.4). The mean length of the 50 fish collected from the screw trap on the North Fork Nehalem River in June was 69.3 ± 9.4 mm.

Figure 3.2. Salinity (‰) at high tide for the Nehalem Bay sampling sites during collection.

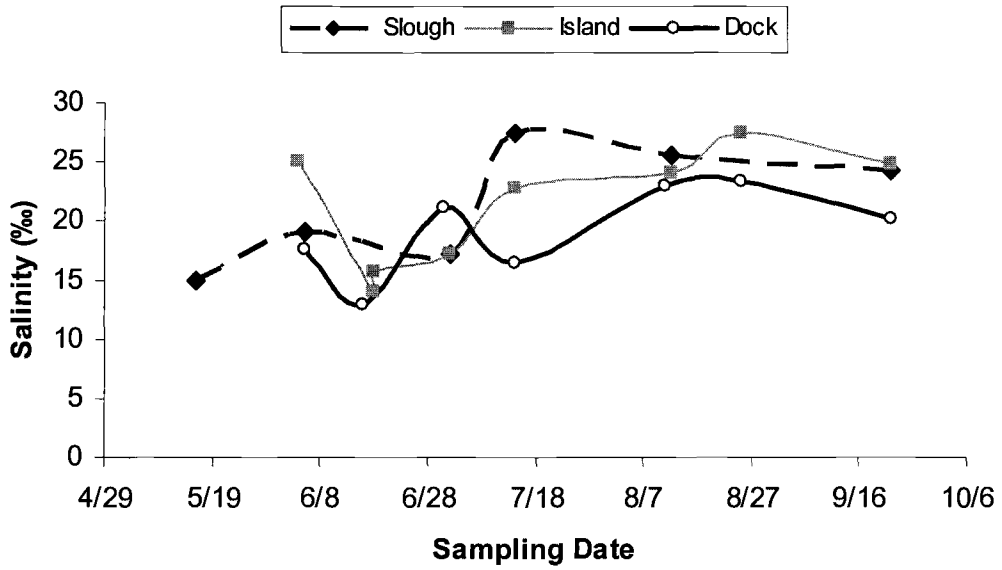


Figure 3.3. Temperature (°C) at high tide for the Nehalem Bay sampling sites during collection.

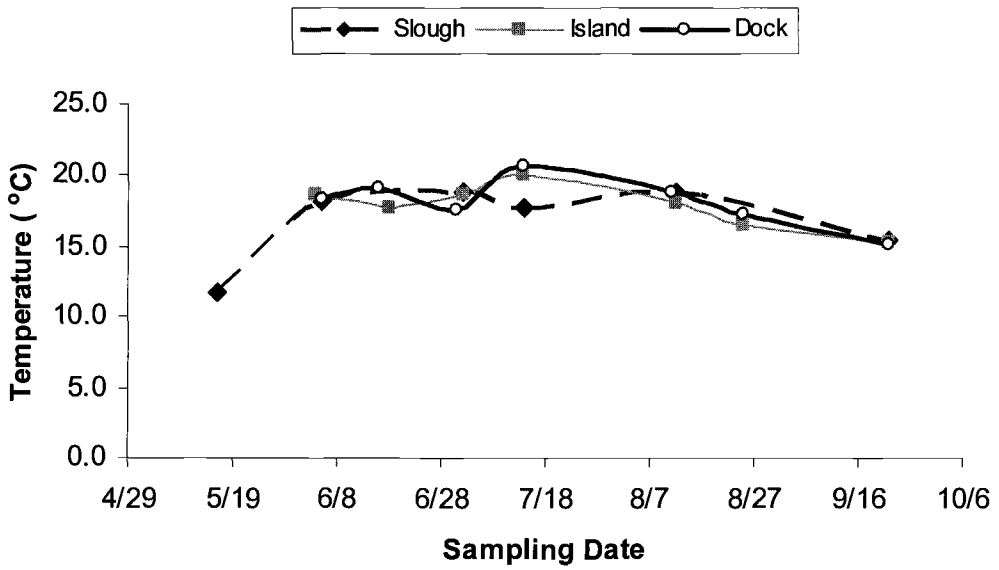
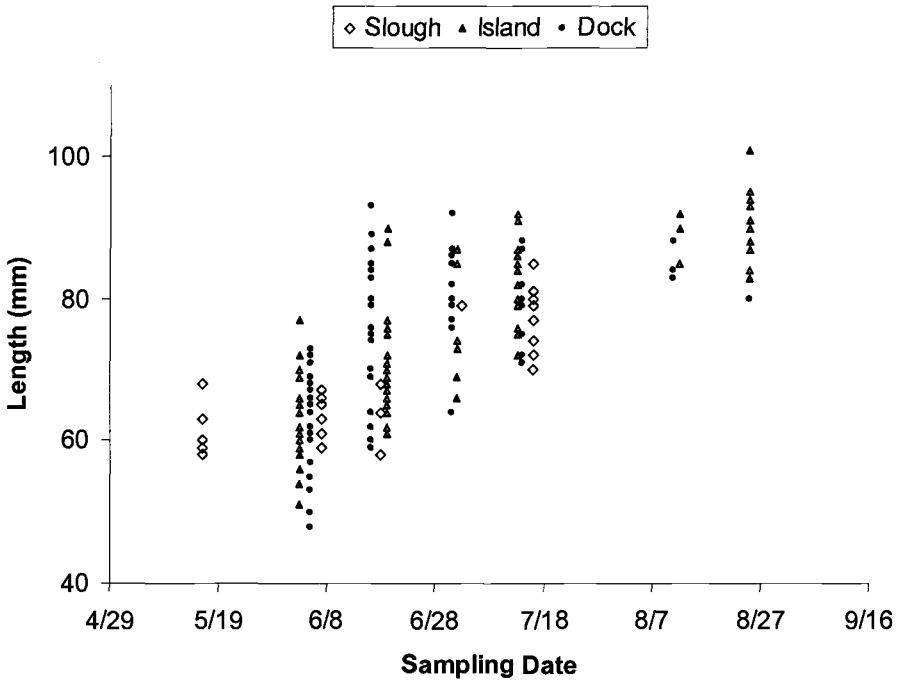


Figure 3.4. Fork length (mm) for juvenile fall Chinook salmon collected from the Nehalem Bay sampling sites throughout the sampling season. All sites were sampled on all dates.

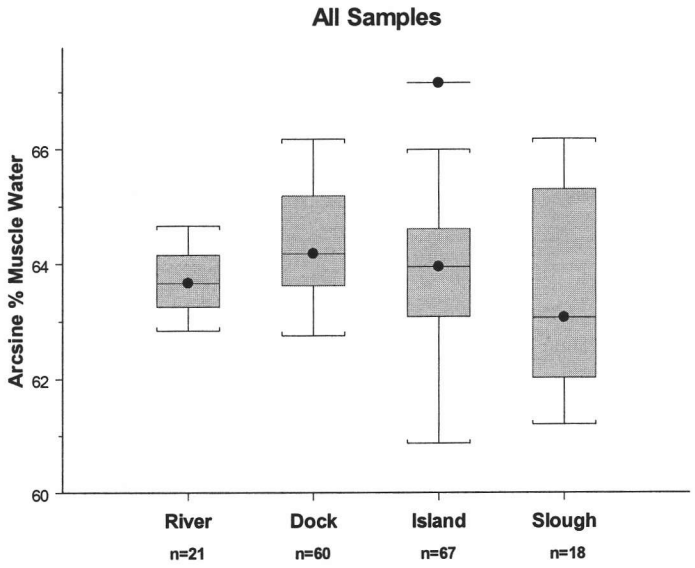


Muscle Water Content

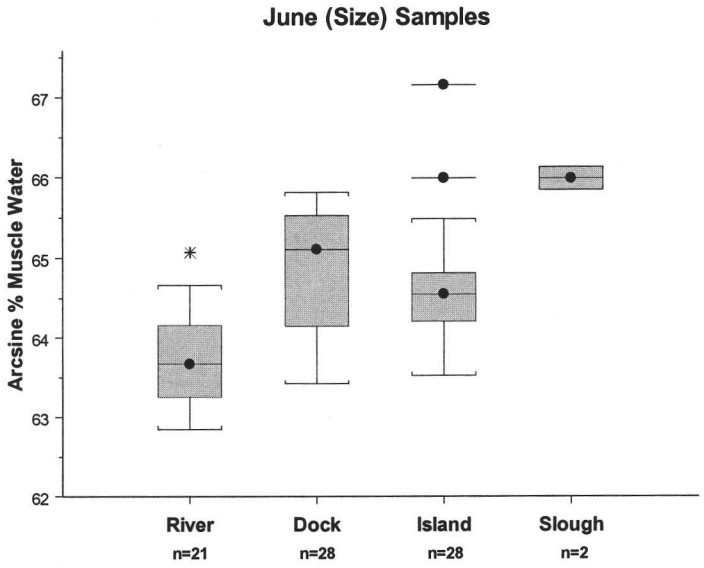
Analysis of arcsine transformed data on muscle water content for all fish collected during the sampling period showed that no significant differences existed between the river and estuary sampling sites ($P = 0.2366$; Figure 3.5a). We further refined this analysis by controlling for time of sampling (June only) and size (length) differences between the estuary and river fish. We used the range of sizes for the river fish (68-91 mm and 3.50-7.32 g) as a guideline for the estuarine fish. The "Slough" sampling site was not included in the statistical analysis due to small sample size (n=2). Although counter-intuitive, this analysis indicated the mean of the river fish had significantly lower muscle water than the means of the "Dock" and "Island" estuary sites (ANOVA, $P = 0.0000$; Figure 3.5b). The mean (SE) percentage of muscle water content of the June fish for the sites was: River = 80.79 (0.13); Dock = 82.06 (0.19); Island = 81.63 (0.17); Slough = 83.00 (0.29).

Figure 3.5. Box Plots of arcsine square transformed % muscle water content for juvenile Chinook salmon. Boxes represent middle 50% of data, the upper and lower whiskers are >~25% and <~25% of the data respectively, the line through the box is the median, and individual points are extreme values. Figure 3.5a shows all sampled collected throughout the sampling period. Figure 3.5b shows only sampled collected during June and only the estuarine fish that were within the same size range as the river fish (68-91 mm). Asterisks indicates the sampling site (River) that is significantly lower (one-way ANOVA; $P = 0.0000$)

a)



b)



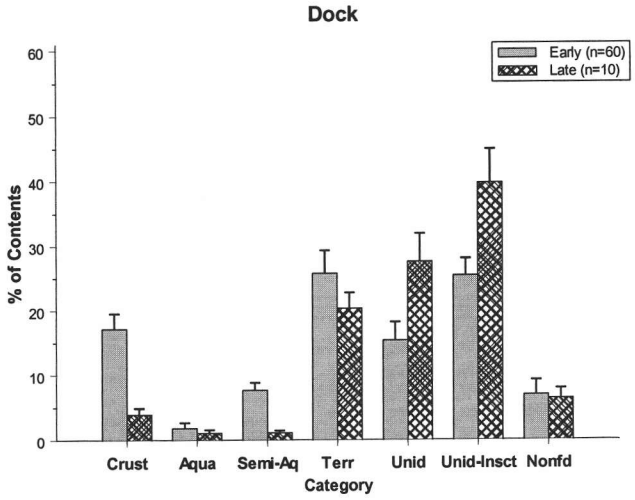
Diet Analysis

Crustaceans and insects dominated juvenile Chinook salmon stomach contents (Figure 3.6). The taxonomic composition of stomach contents for fish collected was similar for the three sites in the estuary. Juvenile Chinook salmon consumed prey from a total of 16 Orders with Amphipoda, Diptera, Homoptera, Hemiptera, and Coleoptera dominating the contents. We also identified insects from the following Orders: Heteroptera, Thysanoptera, Collembola, Araneida, Pscoptera, Hymenoptera, and Lepidoptera. The stomach contents of the fish did not exhibit any signs of specialized feeding.

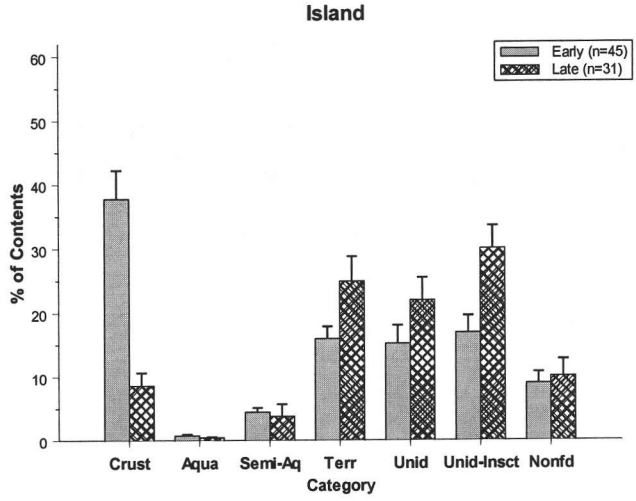
Generally, there were no significant differences among the estuary sites. Schoener's index of overlap showed that all three sites were very similar when compared among sites and within sampling time (i.e. Early or Late) (Table 3.1). Average percentage of terrestrial insects in the stomach contents from fish collected at all marsh sites was significant: 16-25% in "Early" period and 20-49% in "Late" period. However, the diet composition did change seasonally (Table 3.2). The content categories for the individual sampling days within a site were not statistically different (ANOVA and Kruskal-Wallis; $P = 0.06$-0.98); therefore, we pooled the data into two groups, "Early" and "Late". In general, the proportion of terrestrial insects and insect parts in the diet either increased or stayed the same while the proportion of crustaceans, aquatic insects, and semi-aquatic insects decreased or stayed the same throughout the sampling period. Furthermore, it could be assumed that the insect parts category would represent the same proportion of insects from the terrestrial, semi-aquatic, and aquatic insect categories.

Figure 3.6. Percentage of categories in the stomach contents from juvenile fall Chinook salmon sampled in the three estuary sites. The three estuary sites are a) Dock, b) Island, and c) Slough. Percentage of contents is expressed as the mean (+ standard error) of the points (2 x 2 mm square) assigned to each stomach content category. Content categories include: aquatic crustacea (Crust); aquatic insects (Aqua); semi-aquatic insects (Semi-Aq); terrestrial insects (Terr); unidentified digested material (Unid); unidentified insect parts (Unid-Insct); nonfood items (Nonfd). "Early" is sampling prior to July 14 and "Late" is sampling post July 14.

a)


Dock
Early (n=60)
Late (n=10)
% of Contents
0
10
20
30
40
50
60
Crust
Aqua
Semi-Aq
Terr
Unid
Unid-Insct
Nonfd
Category

b)


Island
Early (n=45)
Late (n=31)
% of Contents
0
10
20
30
40
50
60
Crust
Aqua
Semi-Aq
Terr
Unid
Unid-Insct
Nonfd
Category

c)

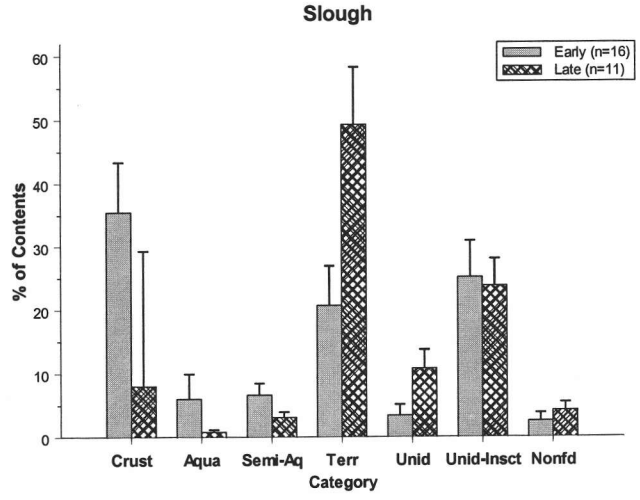

Slough
Early (n=16)
Late (n=11)
% of Contents
0
10
20
30
40
50
60
Crust
Aqua
Semi-Aq
Terr
Unid
Unid-Insct
Nonfd
Category

Table 3.1. Schoener's index of diet overlap for Nehalem Bay sampling sites. The first six comparisons are between sites and within early or late sampling. The last three comparisons are within sites and between early and late sampling. Schoener's index is calculated using the average proportions for diet categories including aquatic crustaceans, aquatic insects, semi-aquatic insects, terrestrial insects, and unidentified insects. The index is $C_{xy} = 1 - 0.5(\Sigma | p_{xi} - p_{yi} |)$, where p_{xi} is the proportion of resource i used by species x and p_{yi} is the proportion of resource i used by species y. The index varies from 0 (x and y have no food categories in common) to 1 (x and y are the same in proportional composition of stomach contents).

	Comparison	Schoener's Index of Overlap
Early	Dock vs. Slough	0.86
Early	Dock vs. Island	0.78
Early	Island vs. Slough	0.89
Late	Dock vs. Slough	0.75
Late	Dock vs. Island	0.89
Late	Island vs. Slough	0.84
Dock	Early vs. Late	0.79
Island	Early vs. Late	0.73
Slough	Early vs. Late	0.66

Table 3.2. Two-sample *t*-test on arcsine square transformed proportional data of contents comparing *between* sampling time (i.e. early vs. late) and *within* sampling site. The total *df* for the sites are: Dock = 70; Island = 72; Slough = 27.

Comparison	**Crustacean**		**Aquatic Insect**		**Semi-Aquatic Insect**		**Terrestrial Insect**		**Insect Parts**	
	t	***P***	***t***	***P***	***t***	***P***	***t***	***P***	***t***	***P***
Dock	2.74	0.0078	0.04	0.9667	2.74	0.0078	0.13	0.8991	-2.24	0.0285
Island	0.47	0.6392	5.60	0.0000	1.49	0.1411	-2.17	0.0335	-3.11	0.0027
Slough	2.99	0.0058	1.31	0.2007	1.16	0.2573	-2.85	0.0082	-0.11	0.9123

Laboratory Experiment

Fish Condition and Osmoregulatory Assessment

We found no significant differences among the tank replicates within each treatment (ANOVA; $P = 0.06\text{-}0.94$); therefore, all data were pooled into the treatment groups. After the saltwater challenge on day 12 of the experiment, plasma electrolyte and lactate levels increased significantly for the treatment groups (Table 3.3). However, glucose did not significantly change for the treatment group that received no food (NONE). After the saltwater challenge test on day 24 of the experiment, the freshwater levels for the Na^+, Cl^-, Ca^+, and Mg^+ plasma electrolytes increased significantly in all the treatment groups. Although the K^+ and the glucose levels of the LOW and HIGH treatments increased, the NONE feed treatment did not significantly change after this challenge. The lactate levels for the LOW and NONE treatments did not significantly change; however, the lactate levels for the HIGH treatment group decreased after the day 24 saltwater challenge.

Table 3.3. Statistical comparisons (one-way ANOVA) *within* three food treatment groups and *between* the sampling day and the subsequent 24-hour saltwater challenge test (SW Challenge). Replicate tanks were pooled by treatments. Means for each day were based on pooling replicate tanks for each treatment group, and the degrees of freedom (*df*) are total *df*'s.

Comparison	NONE			LOW			HIGH		
	Df	*F*	*P*	*df*	*F*	*p*	*df*	*F*	*p*
Day 12/12-SW Challenge									
Na^+	1	260.96	0.0000	1	203.41	0.0000	1	98.99	0.0000
Cl^-	1	114.83	0.0000	1	64.55	0.0000	1	72.10	0.0000
Ca^{++}	1	7.29	0.0128	1	8.46	0.0081	1	31.28	0.0000
Mg^{++}	1	27.58	0.0000	1	19.31	0.0002	1	20.54	0.0002
K^+	1	19.11	0.0002	1	8.58	0.0077	1	4.23	0.0527
Glucose	1	0.93	0.3441	1	70.31	0.0000	1	40.16	0.0000
Lactate	1	7.34	0.0125	1	22.37	0.0001	1	16.81	0.0005
Day 24/24-SW Challenge									
Na^+	1	216.14	0.0000	1	120.64	0.0000	1	24.19	0.0000
Cl^-	1	146.73	0.0000	1	94.93	0.0000	1	43.39	0.0000
Ca^{++}	1	0.07	0.7867	1	9.01	0.0060	1	16.28	0.0004
Mg^{++}	1	18.35	0.0002	1	47.39	0.0000	1	23.49	0.0000
K^+	1	0.12	0.7298	1	6.45	0.0177	1	6.28	0.0190
Glucose	1	0.58	0.4504	1	44.15	0.0000	1	24.65	0.0000
Lactate	1	0.90	0.3513	1	0.48	0.4934	1	6.03	0.0214

After the saltwater challenges on days 12 and 24, the Na^+ levels for the NONE treatment group were significantly higher than those of the HIGH treatment (Table 3.4, Figure 3.7a). However, the Na^+ levels for the LOW treatment were not significantly different from either the NONE or the HIGH treatment groups. The Cl^- levels for the NONE treatment after the day 24 challenge were significantly higher than the Cl^- levels of the HIGH and LOW treatment (Figure 3.7b). After the day 24 saltwater challenge, the Ca^+ levels for the fish in the NONE treatment were significantly lower than the levels of the HIGH and LOW treatment (Figure 3.7c).

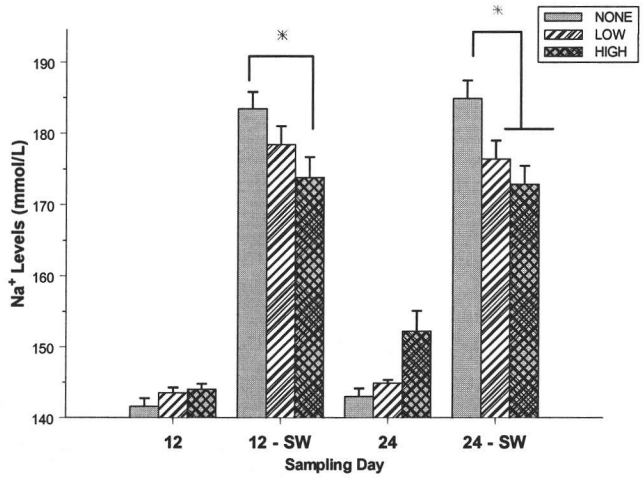
After the saltwater challenges on days 12 and 24, the glucose levels for the HIGH and the LOW treatments were significantly higher than the NONE treatment (ANOVA followed by Tukey's HSD tests) (Table 3.4). Furthermore, the lactate levels for the LOW treatment after the day 12 challenge were significantly higher than the NONE treatment (ANOVA followed by Tukey's HSD test). The muscle water concentrations were not significantly different among the treatment groups after the saltwater challenges on day 12 or 24.

Table 3.4. Statistical comparisons (one-way ANOVA) *among* three food treatment groups and *within* the 24-hour saltwater challenge day. Replicate tanks were pooled by treatments.

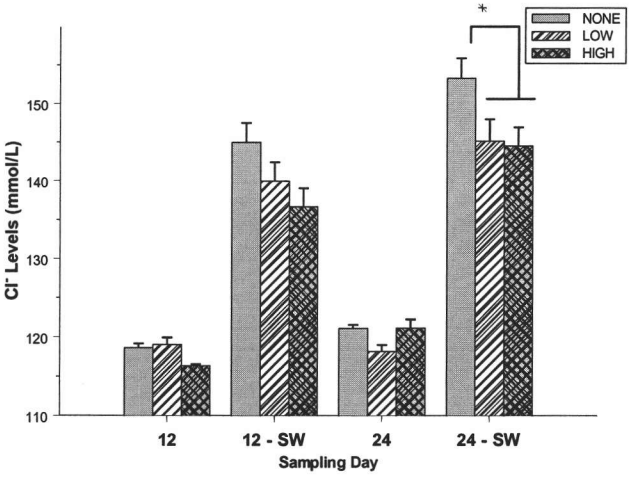
Comparison	**Day 12 – SW Challenge**			**Day 24 – SW Challenge**		
	df	**F**	**p**	***df***	**F**	**p**
Na^+	2	3.457	0.0437	2	5.773	0.0068
Cl^-	2	2.997	0.0641	2	3.566	0.0389
Ca^{++}	2	2.375	0.1091	2	12.974	0.0000
Mg^{++}	2	0.968	0.3908	2	1.741	0.1902
K^+	2	0.846	0.4383	2	0.348	0.7084
Glucose	2	22.024	0.0000	2	47.734	0.0000
Lactate	2	3.705	0.0357	2	1.337	0.2756
Muscle H_2O	2	0.060	0.9419	2	0.899	0.4160

Figure 3.7. Mean Na^+, Cl^-, and Ca^{++} levels (+standard error) for juvenile spring Chinook salmon in treatment groups on days 12 (12), day 12 saltwater challenge (12-SW), day 24 (24), and day 24 saltwater challenge (24-SW). For each treatment on each sampling day, n=8. Figure 3.7a is the Na^+ levels. Figure 3.7b is the Cl^- levels. Figure 3.7c is the Ca^{++} levels. After the saltwater challenge tests on day 12 and 24, there were significant increases over the freshwater levels for the Na^+, Cl^-, and Ca^{++} plasma electrolyte levels in all the treatment groups. Significant statistical differences (ANOVA, $P \leq 0.05$) between treatment groups for comparisons within each saltwater-challenge day are indicated by asterisks.

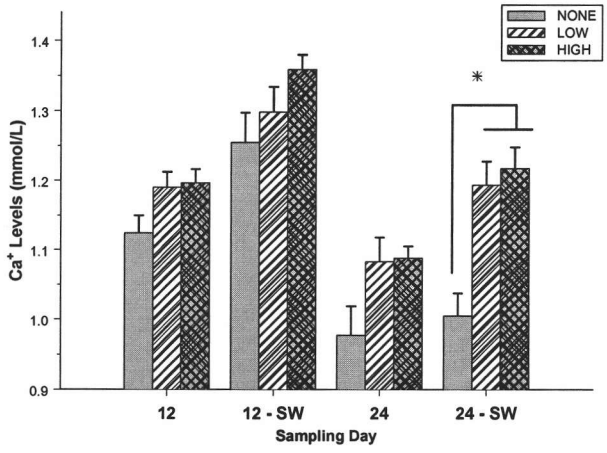
a)


NONE
LOW
HIGH
*
*
190
180
170
160
150
140
Na+ Levels (mmol/L)
12
12 - SW
24
24 - SW
Sampling Day

b)


NONE
LOW
HIGH
*
150
140
130
120
110
Cl- Levels (mmol/L)
12
12 - SW
24
24 - SW
Sampling Day

c)


NONE
LOW
HIGH
*
1.4
1.3
1.2
1.1
1.0
0.9
Ca+ Levels (mmol/L)
12
12 - SW
24
24 - SW
Sampling Day

DISCUSSION

Juvenile Chinook salmon of different sizes utilize a number of estuarine habitats during their lengthy estuarine residence, which has been shown to last up to 60 days in some locations (Levings et al. 1986). Our sampling locations were somewhat different from those of other studies because ours represented conditions that would be the first encountered as fish left riverine environments. In other words, our sample sites were at the very "top" of the estuary close to totally freshwater river habitat. Juvenile Chinook salmon even this high up in the system appear capable of adapting to the dynamic fluctuating salinity conditions found in the tidal marsh habitats of the Nehalem Bay. All of the juvenile fall Chinook we collected along the marsh habitat were robust in appearance, which could indicate good rearing conditions. However, the Dock and Island sites were more heavily utilized by juvenile Chinook than the Slough site, which likely reflects the length of time these sites were available and their proximity to deeper channels. The wide range of fork lengths for the estuarine juveniles which were sampled on later dates probably reflects size differences between recent recruits and fish that had been in the estuary for several weeks. It is also possible that abundance decreased due to larger fish moving into deeper waters or continuing their seaward migration. Juvenile Chinook salmon have been observed in other systems to colonize the estuary by first occupying tidal creeks high in the marsh area and later the outer estuary (Healey 1982). Kjelson et al. (1982) noted that Chinook fry (<70mm) appear to be most abundant in the nearshore upper estuarine habitats, while the larger juvenile Chinook salmon are found further offshore. There also may be a size threshold determining the movement of fish into deeper or higher salinity water (Healey 1980). Wagner et al. (1969) found that juvenile Chinook salmon could tolerate full sea water upon reaching the size of 70 mm.

Some of the feeding habits identified in our study may be unique to aquatic habitats bordered by emergent riparian vegetation; for example some of the juvenile Chinook salmon fed on *Collembola, Araneida sp.,* and *Homoptera sp.*, which live primarily in the soil, litter, and vegetation of riparian habitats. Extensive invertebrate surveys in other estuaries have not reported these taxa in samples of benthos, epibenthos

or drift from the intertidal zone (Higgs et al. 1995). Numerous other studies have also confirmed that a significant portion of juvenile fall Chinook fry and sub-yearlings utilize tidal sloughs for foraging habitats and consume allocthonous organisms that would be associated with the surface film or surrounding riparian vegetation (e.g. adult Diptera, adult Hymenoptera, other adult insects, and spiders) (Simenstad et al. 1982; Levings et al. 1995). Levy and Northcote (1982) found that juvenile Chinook salmon will repeatedly move in and out of the marshes and sloughs with the tidal cycles and were among the last fish to vacate the tidal channels at low tide.

Our data indicate that juvenile fall Chinook salmon were opportunistically consuming similar prey items at all three estuarine marsh sites. However, the composition of the diet changed over time, including a proportional increase in terrestrial insects and a decrease in aquatic crustaceans and insects. This suggests a seasonal shift in the proportion of prey categories consumed during the later sampling period, which could have been due to a change in targeted species or availability of prey species. As juvenile salmonids grow and move seaward, other studies have documented a decreased dependence on the surface layer for physiological adaptation and for food supply, which has been reflected in a dietary shift to benthic and marine organisms (Wolf et al. 1983; Macdonald et al. 1987). We did not see this trend in our study most likely due to our limited scope of sampling only nearshore marsh habitats. Overall, juvenile Chinook salmon consume a diverse prey spectrum, which reflects extended estuarine residence, diversity of available prey resources, and the emergent vegetation of the marsh habitats utilized.

It has been suggested that estuarine habitats are important areas for the osmoregulatory adjustment of juvenile salmonids (Macdonald et al. 1988; Iwata and Komatsu 1984). We used muscle water content as an indication of osmoregulatory capabilities in the fluctuating salinities encountered in the tidal marsh habitats. If the osmoregulatory ability differed for fish collected at the different estuarine sites, we would have expected fish with greater seawater adaptation or higher saltwater tolerance to have higher muscle moisture. For example, recent migrants from the river may have higher percentages of muscle moisture in saltwater due to an insufficient acclimation period. In

salmonids, this regulative period is approximately one day (Conte et al. 1966; Clarke et al. 1995), which could necessitate a gradual acclimation to avoid disruption of the ionic and osmotic balance. The first analysis of all samples indicated that the river and estuarine fish were osmoregulating within the same capabilities. However, the analysis of the mean muscle water content after controlling for time and size indicated that the fish sampled in the river during June had less muscle moisture than those of the estuarine sites. This contradicts our expected results. Even if the river resident fish had been under stress they would have likely experienced an increase in muscle water content. During periods of stress, body fluid concentrations may become altered. However, the body fluids of freshwater fish are more concentrated than the surrounding medium and water would enter osmotically while salts or electrolytes would diffuse outwards (Eddy 1981). Therefore, it is more probable that the differences in the muscle water content for the fish sampled during June were due to sampling errors involving small sampling sizes.

During their estuarine residency, juvenile Chinook salmon have access to a diverse prey base and have been shown to exhibit some of the highest growth rates experienced during their lifetime (Kjelson et al. 1982; Simenstad et al. 1982). Higher growth rates offer significant advantages for migrating juvenile salmonids as it has been shown that large salmon are more likely to be physiologically prepared for movement to the marine environment than smaller salmon (McInerney 1964). The major metabolic costs to juvenile salmonids during their seaward migration are due to migration swimming and maintenance metabolism. Wissmar and Simenstad (1988) discovered that during the lowest prey levels at the beginning and the end of the migration, total metabolic costs either approached or exceeded the energy intake. Their results suggested that during this stage of migration the amount of energy left for fish growth appears to be dependent on higher levels of prey resources available in the estuary.

The results from our laboratory experiment confirm the importance of optimum feeding conditions for migrating juvenile Chinook salmon. Our data suggest that during a freshwater or estuarine residency, energy intake can directly affect future osmoregulatory abilities as well as growth and size. Throughout the experiment, the fish on restricted diets were most likely to exhibit weak hypo-osmoregulatory abilities in full-

strength seawater. It is possible that the due to photoperiod or size, the experimental fish were already at an osmoregulatory disadvantage. However, this alone does not account for the decrease in saltwater tolerance of the pre-smolt juvenile Chinook salmon with low feed quantity. Considering the magnitude of the physiological changes required during smoltification, it is likely that juvenile Chinook salmon benefit from an abundance of diverse prey resources present in the freshwater-saltwater transition zones of estuaries.

During their estuarine residency, Chinook salmon utilize various tidal habitats for feeding and facilitating physiological adaptation to seawater. The importance a habitat, however, cannot always be measured by the amount of time it is occupied by fish (Larimore and Garrels 1985). Although juvenile Chinook salmon may occupy tidal marshes and sloughs for a relatively small amount of time, terrestrial and marsh vegetation are among the major primary producers upon which the estuarine detrital food chain depends (Healey 1980; Wolf et al. 1983; Miller and Simenstad 1997). Our research confirms that importance of productive estuarine transition zones to juvenile Chinook salmon as feeding grounds for a diverse spectrum of prey including allochthonous items. Changes reducing the biological productivity or habitat quality could negatively impact the diet quality, growth, osmoregulatory performance, and health of juvenile Chinook salmon. Furthermore, Magnusson and Hilborn (2003) found that juvenile fall Chinook salmon residing in severely altered estuarine habitats had lower survival rates than those residing in natural, pristine habitats. Therefore, it is critical for estuarine restoration projects to identify the habitat conditions that could affect the health and survival of juvenile Chinook salmon during their seaward migration.

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CHAPTER 4

GENERAL CONCLUSIONS

In this thesis, I demonstrated that juvenile Chinook salmon exhibit highly adaptive behavioral responses in order to exploit resources during their estuarine residency. Juvenile Chinook salmon are very plastic in their capabilities to deal with a wide variety of saltwater conditions. In Chapter 2, the data showed that the experimental fish did exhibit a clear electrolyte increase to the treatments involving saltwater. Although plasma electrolytes increased on days 14 and 28, the concentrations for all days and saltwater challenges were within recorded levels for *Oncorhynchus* sp. (i.e. $[Na^+] < 180$ mmol·L^{-1}, $[Cl^-] < 160$ mmol·L^{-1}; Conte et al. 1966; Wagner et al. 1969; Clarke and Blackburn 1977; Folmar et al. 1980; Morgan and Iwama 1991), indicating that the fish were regulating their plasma ions competently throughout the experiment.

Juvenile Chinook salmon were responsive to fluctuating salinities and capable of osmoregulating in full-strength saltwater. However, the magnitude of this saltwater resistance may be dependent on obtaining a critical size or growth rate prior to entering a marine environment (Conte and Wagner 1965; Folmar et al. 1980; Clarke and Shelbourn 1985). Wagner et al. (1969) concluded that "salmon which are more rapidly growing possess a regulatory system that is either more functional with respect to a given salt gradient or capable of being initiated more quickly with changes in environmental salinities." However, salinities greater than 20‰ can have significant effects on juvenile Chinook salmon growth due to the metabolic cost of osmoregulation (Shaw et al. 1975; Clarke et al. 1981). These results confirmed these previous studies that constant salinities between 20-25‰ were not sufficient to significantly affect growth in juvenile spring Chinook salmon. However, when salinities fluctuated between 2-25‰ there was a small but marginally significant reduction in growth without triggering significant stress responses. This negative impact on growth rates may have been due to increased maintenance energetic requirements. Constantly switching between hypo- and hyper-osmoregulation and ionregulation during fluctuating salinities may have required more energy to maintain ion and osmoregulatory transport systems. These differences may

become more or less pronounced in natural situations where tidal fluctuations are more frequent, resources may be limited, and energetic expenditure for prey is potentially greater.

Juvenile Chinook salmon of different sizes utilize a number of estuarine habitats during their lengthy estuarine residence. The results from Chapter 2 suggest that juvenile Chinook salmon are capable of adapting to the dynamic fluctuating salinities typical of many estuarine habitats. However, the cost of residing in a fluctuating salinity may be additional energetic requirements. From the field research, I did not see any negative impacts from residing in the fluctuating salinity marshes. All of the juvenile fall Chinook salmon we collected along the marshes were robust in appearance and the majority had full stomachs, which could indicate good rearing conditions. The wide range of fork lengths seen in the later sampling probably reflects differences in sizes between recent recruits and fish that had been in the estuary for several weeks. It is also possible that the catch size decreased due to larger fish moving into deeper waters or continuing their seaward migration.

Some of the prey items identified in Chinook salmon stomachs in Chapter 3 may be unique to aquatic habitats bordered by emergent riparian vegetation. Numerous other studies have also confirmed that a significant portion of juvenile Chinook salmon utilize tidal sloughs for foraging habitats and consume organisms that would be expected to be associated with the surface film or surrounding riparian vegetation (e.g. adult Diptera, adult Hymenoptera, other adult insects, and spiders) (Simenstad et al. 1982; Levings et al. 1995; Healey 1995). The data from this study indicate that juvenile fall Chinook salmon were opportunistically consuming similar prey items at the marsh sites, despite differences in slopes and habitat availability during high tide cycles. However, there were significant changes in the proportional diet composition over time. The proportion of terrestrial insects increased later in the sampling, while the crustaceans and aquatic insects either decreased or stayed the same. This seasonal shift in the prey consumed could have been due to a change in targeted species or availability of prey species.

The results from the laboratory experiment and field research confirm the importance of good rearing grounds for migrating juvenile Chinook salmon. There likely

is a metabolic cost for ion- and osmoregulating in a hyper-osmotic environment; this is suggested by the differential growth of fish under the various salinity regimes. The experimental data from Chapter 3 suggests that during a freshwater or estuarine residency, energy intake can directly affect future osmoregulatory abilities as well as growth and size. Throughout this experiment, the fish on restricted diets were most likely to exhibit weak hypo-osmoregulatory abilities in full-strength seawater.

During their estuarine residency, Chinook salmon utilize various estuarine habitat types for feeding and zones of increasing salinity which can facilitate physiological adaptation to seawater. The importance of these estuarine habitats, however, cannot always be measured by the amount of time it is occupied by fish (Larimore and Garrels 1985). Although tidal marshes and sloughs may only be occupied by juveniles for short periods of time, these habitats help support the terrestrial and marsh vegetation upon which the estuarine detrital food chain depends (Healey 1980; Wolf et al. 1983; Miller and Simenstad 1997). Depending on their developing saltwater tolerance, size, growth rate, and diet quality, juvenile Chinook salmon encountering dynamic estuarine habitats may be more dependent on productive rearing grounds in the freshwater-saltwater transition zone. Although juvenile Chinook salmon are capable of adapting to a wide-range of environmental conditions, changes which reduce biological productivity of critical estuarine marsh habitats could affect the growth and health of juvenile salmonids during their seaward migration.

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APPENDICES

APPENDIX 1

Osmoregulation and Growth of Juvenile Spring Chinook Salmon in Fluctuating Salinities – Additional Plasma Electrolytes

INTRODUCTION

During the process of adaptation to marine life, or smoltification, a number of behavioral, morphological, and physiological changes occur that affect numerous organs, hormones, and processes associated with metabolism and osmoregulation (Hoar 1976; Hoar 1988). Although several studies have examined the physiological changes associated with smoltification and the changes in saltwater tolerance with age (Folmar and Dickhoff 1980), in nature young estuarine salmonids are faced with a dynamic environment. Salinities in typical estuarine habitats may vary by 20‰ twice per day depending on the tidal fluctuations and river flow. It can be energetically costly to maintain internal homeostasis within these dynamic conditions and this may contribute additional stress. The objective of this study was to investigate the physiological effects that fluctuating salinity environments, such as estuarine tidal marshes, could have on juvenile Chinook salmon (*Oncorhynchus tshawytscha*). We designed an experiment with treatments that were similar to the salinity changes observed in estuarine tidal habitats. We expected to see initial increases in plasma electrolytes within the first 24 hours. In addition, juvenile Chinook unprepared for the saltwater and fluctuating salinity treatments could have a poor osmoregulatory performance.

METHODS

Fish and Experimental Design

Spring Chinook salmon fry were obtained from the Marion Forks Hatchery on the North Fork of the Santiam River, Oregon and were raised at Oregon State University's (OSU) Fish Performance and Genetics Laboratory (FPGL) in Corvallis, Oregon. Spring Chinook salmon were held under a natural photoperiod in approximately 2 meter diameter circular tanks with flow through, aerated, pathogen free, 12 °C well water and fed a diet of Bio-Oregon semi-moist pellets ad libidum twice per day. During February, 2003,yearling spring Chinook (12 months old) were transported for approximately one

hour in oxygenated tanks to the Fish Disease Laboratory (FDL) at Oregon State University's Hatfield Marine Science Center in Newport, OR, where 54 fish each were transferred to 1 m diameter (water depth ~60 cm) tanks supplied with aerated, pathogen-free, freshwater (temperature 12-14°C).

Treatments consisted of tanks 1, 2, and 3 with freshwater, tanks 4, 5, and 6 saltwater (salinity ≈23‰); and tanks 7, 8, and 9 with fluctuating salinity (2 - 25‰). Salinity was monitored with an YSI 30™ salinity, temperature, and dissolved oxygen meter. Fish remained in freshwater for a 14 day acclimation period. The saltwater and fluctuating salinity treatment tanks then had pathogen free, filtered seawater introduced (13-14°C, 28-31‰) into the freshwater. Salinity levels were adjusted by altering flow (liters per minute) of seawater and freshwater. The tanks with fluctuating salinities were equipped with a timer that controlled the seawater and freshwater input for all tanks simultaneously. Seawater input was turned on for the fluctuating tanks at 08:00 with the salinity increasing to 23‰ in the following two hours. The seawater was then shut off at 15:00 and gradually decreased in salinity during the following 5 hours.

The fish were fed ad libidum twice a day for the duration of the experiment. Prior to sampling, we withheld food for 24 hours prior to minimize potential stress associated with feeding. On days 1, 14, and 28, six fish from each tank were sampled for plasma electrolytes. A separate subpopulation of six fish per tank were marked for tank origin and subjected to 24 hour saltwater challenge tests. We measured plasma electrolytes at the completion of each test. The mean (± standard error) weight recorded for fish sampled on day 1 was 47.24 (± 0.87) g.

Sampling Methods

Fish were rapidly netted in groups of 2-3 fish from the treatment tank. A lethal dose of tricaine methanesulfate (MS-222 200 mg/L buffered with NaHCO3 500 mg/L) was administered and individuals were measured for fork length (mm) and weight (g). After death, blood was collected from the dorsal aorta with a heparinized Vacutainer® and 20 gauge needle and centrifuged for 10 minutes at 4000 rpm. After centrifugation, plasma was separated and stored in a -80 °C freezer. Plasma samples were analyzed with

a Nova Biomedical CCR analyzer (Waltham, MA), which provided concentrations of electrolyte (Na^+, Cl^-, K^+, Mg^{++}, Ca^{++}).

24 Hour Saltwater Challenge Tests

We conducted saltwater challenge tests on each sampling day. Six fish per tank were subjected to full strength salinity (29-31‰). With this test, treatment groups were immediately placed into full-strength seawater with the same temperature, dissolved oxygen, pH levels as the treatment tanks. After approximately 24 hours of exposure to full-strength seawater, the fish were removed, given a lethal does of anesthetic (MS-222), and sampled as described previously.

Table 1.1: Mean (Standard Error) of K^+, Ca^{++}, and Mg^{++} levels from Fluctuating Salinity experiment. Tanks 1, 2, and 3 are freshwater treatments. Tanks 4, 5, and 6 are constant saltwater treatments. Tanks 7, 8, and 9 are fluctuating salinity treatments. SW indicates that these fish were sampled after a 24 hour saltwater challenge test.

	Tank	1	2	3	4	5	6	7	8	9
Day 1	K	4.50 (0.95)	5.92 (0.28)	6.23 (0.49)	6.74 (0.58)	4.86 (0.50)	6.43 (0.43)	4.11 (0.60)	6.11 (0.43)	4.82 (0.39)
	Ca	0.85 (0.04)	0.86 (0.03)	0.90 (0.03)	0.97 (0.02)	0.98 (0.03)	1.03 (0.02)	0.91 (0.02)	0.90 (0.03)	0.91 (0.03)
	Mg	0.45 (0.04)	0.43 (0.02)	0.47 (0.03)	0.49 (0.05)	0.49 (0.02)	0.51 (0.01)	0.50 (0.03)	0.50 (0.03)	0.53 (0.02)
Day 1 SW	K	5.01 (0.56)	6.81 (0.79)	5.01 (0.45)	3.14 (0.62)	4.99 (0.51)	4.09 (0.51)	5.36 (0.34)	5.65 (0.76)	5.51 (0.24)
	Ca	1.08 (0.02)	1.15 (0.08)	1.07 (0.03)	1.08 (0.01)	1.09 (0.02)	1.11 (0.04)	1.12 (0.02)	1.09 (0.03)	1.17 (0.04)
	Mg	0.49 (0.01)	0.69 (0.23)	0.51 (0.04)	0.63 (0.15)	0.57 (0.05)	0.48 (0.01)	0.57 (0.04)	0.58 (0.01)	0.53 (0.03)
Day 14	K	5.46 (0.52)	4.92 (0.42)	5.19 (0.22)	6.17 (0.35)	6.37 (0.35)	4.71 (0.30)	6.07 (0.38)	5.40 (0.27)	4.42 (0.34)
	Ca	1.05 (0.06)	1.03 (0.03)	1.05 (0.03)	1.04 (0.03)	1.09 (0.03)	1.07 (0.02)	1.00 (0.03)	1.01 (0.04)	0.98 (0.02)
	Mg	0.48 (0.04)	0.43 (0.01)	0.44 (0.01)	0.46 (0.01)	0.49 (0.01)	0.53 (0.03)	0.47 (0.02)	0.47 (0.01)	0.44 (0.03)
Day 14 SW	K	4.08 (0.24)	3.59 (0.12)	4.58 (0.64)	3.75 (0.63)	3.38 (0.34)	3.94 (0.53)	4.11 (0.40)	4.69 (0.33)	4.12 (0.46)
	Ca	1.21 (0.04)	1.24 (0.03)	1.15 (0.03)	1.11 (0.02)	1.21 (0.05)	1.20 (0.05)	1.29 (0.04)	1.27 (0.03)	1.21 (0.02)
	Mg	0.59 (0.03)	0.57 (0.02)	0.57 (0.03)	0.56 (0.01)	0.58 (0.02)	0.59 (0.02)	0.59 (0.03)	0.58 (0.01)	0.57 (0.02)
Day 28	K	3.69 (0.32)	3.42 (0.25)	3.48 (0.18)	3.88 (0.33)	3.09 (0.30)	4.18 (0.23)	3.46 (0.28)	3.22 (0.27)	4.20 (0.29)
	Ca	1.12 (0.05)	0.99 (0.09)	1.08 (0.05)	1.11 (0.03)	1.13 (0.07)	1.16 (0.05)	1.13 (0.06)	1.04 (0.04)	0.96 (0.05)
	Mg	0.48 (0.02)	0.47 (0.01)	0.47 (0.01)	0.49 (0.18)	0.54 (0.02)	0.58 (0.04)	1.04 (0.04)	0.44 (0.01)	0.43 (0.01)
Day 28 SW	K	4.09 (0.42)	4.00 (0.27)	4.35 (0.34)	3.98 (0.23)	4.04 (0.44)	3.43 (0.40)	3.28 (0.36)	3.51 (0.37)	3.17 (0.43)
	Ca	1.27 (0.07)	1.12 (0.03)	1.18 (0.06)	1.09 (0.05)	1.08 (0.03)	1.24 (0.09)	1.12 (0.04)	1.08 (0.04)	1.16 (0.04)
	Mg	0.63 (0.06)	0.59 (0.03)	0.66 (0.03)	0.53 (0.02)	0.57 (0.03)	0.73 (0.04)	0.62 (0.03)	0.55 (0.03)	0.63 (0.05)

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APPENDIX 2

Stomach Contents from Juvenile Fall Chinook Salmon Collected in Estuarine Tidal Marshes

INTRODUCTION

Estuarine tidal marshes play an important role in the diet and growth of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) during their estuarine residency (Shreffler et al. 1992; Miller and Simenstad 1997). The benefits of estuaries to juvenile salmon have been shown to be related to the biological productivity and the availability of suitable prey resources. The marine food entering the estuary via tides along with inputs from rivers potentially provides a large variety and amount of prey resources available to juvenile salmonids (Kask et al. 1986; Macdonald et al. 1987). This diversity of prey items in estuarine habitats may drive fish to stay longer than required by physiological constraints.

Typically, few studies have focused on fish habitat use in estuarine tidal habitats bordered by emergent vegetation with terrestrial or riparian affinities. Studying the diets of fish using the tidal estuarine habitats could clarify if the emergent vegetation is providing access to allochthonous prey. The objectives of our field study were to determine diet patterns of juvenile Chinook salmon using the tidal marshes in fluctuating salinity zones and if the tidal marshes may be influencing the diet of these juveniles. In the Nehalem Bay along the Oregon coast, we sampled estuarine tidal marshes in order to determine the diet of juvenile Chinook salmon and if the prey items consumed potentially could reflect the habitat occupied.

METHODS

Fish Collection

We used beach seines (9.14 x 1.83 m with a 4.76 mm mesh size) to catch fish at high tides in the three estuarine sites. Temperature, salinity, and depth were measured with an YSI 30^{TM} during high and low tides at the sampling sites. The three estuarine marsh sites were sampled at two-week intervals, May through September. Because no fish were caught during September, sampling was discontinued. On each sampling day,

we collected a maximum of 20 fall Chinook salmon per site. Individual marsh sites were sampled with a maximum of four seine pulls moving in 9.14 m transects down the shoreline. All fish were euthanized with tricaine methanesulfate (MS-222, 200 mg/L buffered with NaHCO3 500 mg/L), weighed and measured, and then sampled for stomach contents.

Diet Analysis

Changes in the food habits of juvenile fall Chinook salmon were determined by analysis of stomach contents from individuals collected at the three estuary sites in the Nehalem Bay throughout the sampling period. We removed, injected and preserved the stomachs in a 10% solution of buffered formalin (v/v) for two weeks. After two weeks the stomachs were transferred to a 70% solution of ethyl alcohol (v/v). Stomach contents were analyzed for the origin of prey items using the Point-Estimate method adapted from Hynes (1970) (see Chapter 2). The contents were dissected from the stomach lining under a dissecting microscope, separated into content categories, identified to taxonomic Order if possible, and assessed for the number of squares the categories occupied. The content categories included nonfood items, digested material, insect parts, terrestrial insects, semi-aquatic insects, aquatic insects, and aquatic crustaceans. Terrestrial insects included any insect that does not live in water or on the water surface. Semi-aquatic insects were any insect that was associated with the water surface or with intertidal zones or littoral habitats. Aquatic insects included any insects that were normally inhabit the water column or were associated benthic habitats (Borror et al. 1989).

RESULTS

Table 2.1. Stomach content categories and identified Taxa, Order, and life stages for prey items.

Content Category	Taxa	Order / Life Stage
Aquatic Crustacean		
	Amphipoda	
	Amphipoda	Corophium
	Bivalve	
	Cirripedia	
	Cyripidae	
	Decapoda	
	Isopoda	
Aquatic Insect		
	Coleoptera	larvae
	Diptera	chironomidae larvae
	Diptera	chironomidae pupae
	Diptera	larvae
	Diptera	pupae
Semi-Aquatic Insect		
	Coleoptera	
	Coleoptera	Histeridae
	Coleoptera	larvae
	Coleoptera	Staphylindae
	Diptera	
	Diptera	midge
	Hemiptera	
	Heteroptera	nymph
	Pseudoscorpiones	Chelonethida
	Trichoptera	emerging pupae
Terrestrial Insect		
	Araneida	
	Coleoptera	
	Coleoptera	Carabidae
	Coleoptera	Chrysomelidae
	Coleoptera	Cucujidae
	Coleoptera	Curculionidae
	Coleoptera	Elateridae
	Hemiptera	
	Hemiptera	lace bug
	Hemiptera	Miridae
	Hemiptera	Pentatomidae
	Hemiptera	Tingidae
	Heteroptera	nymph

Table 2.1. Continued.

Terrestrial Insects continued	Homoptera	
	Homoptera	nymph
	Homoptera	Sternorrhyncha
	Hymenoptera	
	Hymenoptera	ants
	Lepidoptera larvae	
	Pscopetera	
	Pseudoscorpiones	
	Thysanoptera	Aeolothripidae
Nonfood		
	algae / plant / seeds	
	Feather / mesh / string	
	mite	
	parasite	eggs / Nematode / Trematode
	sand	
	fish scales	

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APPENDIX 3

RNA:DNA Ratio Method for Juvenile Chinook Salmon Muscle Tissue

INTRODUCTION

Analysis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) concentrations has become an established method for estimating size, condition and growth of fish (Bulow 1970; Buckley et al. 1999; Smith and Buckley 2003). The foundation for this work is the theory that the amount of DNA is relatively constant per cell, while the amount of RNA per cell varies due to protein synthesis and growth. This ratio of RNA:DNA (R/D) in whole fish and various tissues has been used for over 30 years as an indicator of short-term and current growth rates. It has been established that R/D ratios are not comparable between different species, life histories, and sizes as growth and body composition will vary with these factors (Bulow 1987). Furthermore, the origin of the specimens must be considered as the R/D can also be affected by the environmental temperature which subsequently affects the rates of metabolism and growth.

In addition to the inherent complications with R/D, a lack of communication and establishment of a standard protocol has made the use of this method very tenuous and comparison of data results highly difficult. There is a great deal of variation among laboratories in methods, standards, and quality control checks. Some of the earliest techniques were based on UV absorption by purified nucleic acids (Wilder and Stanley 1983). Newer techniques now include fluorometric methods utilizing various fluorochromes which bind to the RNA and DNA (Buckley et al.1999). Several protocols are based on the fluorescence of the dye ethidium bromide (EB) after it is bound to the nucleic acids. The fluorescence of the nucleic acid-EB complex is then measured before and after digestion of RNA and DNA by RNase and DNase, respectively (Canino and Caldarone 1995). To complicate matters further, there are variations in the R/D as a result of the type and origin of the tissue analyzed. Some studies have found differences in the R/D of liver, kidney, muscle, and scale-associated tissue from the same fish (Smith and Buckley 2003). Numerous studies have found sub-sampling muscle tissue to be an effective method; nevertheless, other studies have also shown that sub-sampling muscle tissue is ineffective and provides misleading results (Bulow 1987). Our objective was to

develop a high-through put method that could analyze sub-samples of dorsal musculature tissue for R/D ratios from juvenile spring Chinook (*Oncorhynchus tshawytscha*).

METHODS

Fish Maintenance and Experiment Design

The experiment was conducted at the FPGL in Corvallis, Oregon. Zero-age spring Chinook (7 months old) were held under a natural photoperiod in approximately 1 m diameter circular tanks with flow through, aerated, pathogen free, 12°C well water. The treatments consisted of three different feeding rates: three control tanks fed 0.75% body weight ("Low"); three tanks fed 3% body weight ("High"); and three tanks were fasted ("None") during the experiment. Total fish weights per tank were recorded at the start of the experiment and were used to determine total feed amounts during the experiment. The treatments were randomly assigned to the tanks. Fish were fed a diet of Bio-Oregon semi-moist pellets. Fish fed 3% body weight were fed twice a day, while fish fed 0.75% body weigh were given their entire ration for the day in the morning. Tanks were cleaned every two weeks in the late afternoon at least four hours after feeding. Prior to sampling, we withheld food for 24 hours in order to minimize potential stress associated with feeding. Eight fish from each tank were sampled for plasma electrolytes on day 12. All fish were individually marked with surgically implanted pit tags in order to monitor growth rates throughout the experiment. As fish were sampled, we determined growth rates for the tanks and used this to calculate total fish weights per tank in order to modify amounts of food.

Pit Tag Implantation

Fish were transferred individually into anesthetic (MS-222 50 mg/L buffered with NaHCO3 125 mg/L) and commercially available Stress Coat® to prepare them for tagging. Once anesthetized, the fish was measured and any external abnormalities noted. The fish was then securely held while a pit tag injector was used to insert the pit tag into

the ventral body wall anterior to the pelvic girdle. The fish were then transferred to their respective treatment tanks for recovery. Fish were given three days to recover and resume their normal appetites and behaviors prior to the start of the experiment.

Fish Sampling

Fish were rapidly netted from treatment tanks and transferred to holding tanks under similar conditions. A lethal dose of tricaine methanesulfate (MS-222 200 mg/L buffered with NaHCO3 500 mg/L) was administered and individuals were measured for fork length (mm) and weight (g).

Sample Collection

The whole fish or dorsal musculature tissue samples (>30mg) for R/D ratio analysis were individually wrapped in foil and flash frozen in liquid nitrogen. Samples were transferred from liquid nitrogen into -80°C freezer until further analysis.

Sample Preparation

Total RNA and DNA were extracted from muscle tissue with a 1% N-lauroylsarcosine, Tris-EDTA-HCl buffer solution. We followed the methods as outlined by Caladrone et al. (2001) with a few modifications to allow for easier processing of muscle tissue. A control homogenate of juvenile spring Chinook muscle tissue was analyzed along with the standards and unknowns for every plate that was assayed. The control homogenate was stored at -80°C in 50 μl aliquots of muscle tissue and DEPC-treated water in 1.5 ml Rnase-Dnase free microcentrifuge tubes. When thawed for use, 50 μl of 2% N-lauoroylsarcosine-proteinase K-Tris-EDTA-HCl buffer was added to the control homogenate and further homogenized. Subsequently, the controls were treated the same as all other samples.

For the unknown samples, 20-30 mg of dorsal muscle tissue was homogenized with a Kontes® hand-held pellet pestle in 100 μl of 1% N-lauroylsarcosine-proteinase K-Tris-EDTA-HCl buffer for 60 seconds. Following homogenization, all samples were incubated at 37°C for 30 minutes.

Sample Dilution

After incubation, samples were vortexed and then diluted with 0.9 ml Tris-EDTA-HCl buffer. This brings the final concentration of the solution to 0.1% N-lauroylsarcosine, Tris-EDTA-HCl. The samples were vortexed and then centrifuged for 20 minutes at 13,800 g.

Microplate Loading and Processing

A working solution of EB (10 μg/ml) was prepared by adding 80 μl of a 1 mg/ml EB stock solution to 80 ml of Tris-EDTA-HCl (pH 7.5) buffer. Prior to adding any solutions, an empty Greiner black with clear, flat bottom well-plate was read using a fluorescence microplate reader used with permission by the Nucleic Acids and Protein Facilities and Services Core. The 96 well-plate was read on the 365 nm excitation and 590 nm emission range, which is appropriate for ethidium bromide. Following the centrifugation after dilution, the supernatant was removed and 75 μl of the nucleic acids suspended in 0.1% sarcosyl Tris-EDTA buffer were pipetted into the 96 well-plate along with RNA (18S and 26S rRNA from calf liver 40 μg/ml) and DNA (Salmon sperm DNA, 20 μg/ml) standards. All the wells then had an additional 75 μl of the EB working solution (10 μg/ml) added. Following the second well-plate reading, the RNA was eliminated with Ribonuclease and then read again for the DNA fluorescence. Finally, the DNA is eliminated with RNase-free DNase and the plate is read once more for the residual background fluorescence. These data were then used to calculated concentrations of RNA and DNA from the standards and expressed as ratios of RNA to DNA.

Method Validation

The coefficient of variation (CV) among control homogenates was calculated by dividing the standard deviation among replicates by the average among the control replicates. Each batch of control homogenates was thoroughly tested to ensure that replication is within a satisfactory range. We also tested the extraction efficiency of the

method by spiking the control homogenates with a known concentration of the DNA and RNA standards. Quality control checks such as these should be done to validate the technique for every species, as this assay is very dependent on environmental conditions and life-stage of species being studied.

RESULTS

For this method, the coefficient of variation between control homogenates was approximately 4-9% variation. However, when we tested replication for R/D ratios extracted from different 20-30 mg samples of muscle tissue from the same fish, the coefficient of variation was approximately 15%. Average extraction efficiency after spiking the control homogenates with RNA and DNA was $99 \pm 2\%$.

The R/D ratios for the "High" feed treatment were significantly higher than the ratios for the "Low" and the "None" feed treatments (one-way ANOVA, $P < 0.0001$, followed by Tukey's HSD test) (Figure 3.1). However, the growth rates for the "High" and the "Low" feed treatment were significantly higher than the "None" treatment, while the "High" and the "Low" treatments were not significantly different from each other (one-way ANOVA, $P = 0.0015$, followed by Tukey's HSD test) (Figure 3.2). Although, the R/D ratio was significantly related to the % growth per day, there were numerous outliers (GLM; $P = 0.0484$, $R^2 = 0.0551$; Figure 3.3).

Figure 3.1. Box and Whisker Plots of RNA:DNA ratios for tissue from fish sampled from High, Low, and None feed treatments. Boxes represent middle 50% of data, the upper and lower whiskers are >~25% and <~25% of the data respectively, the line through the box is the median.

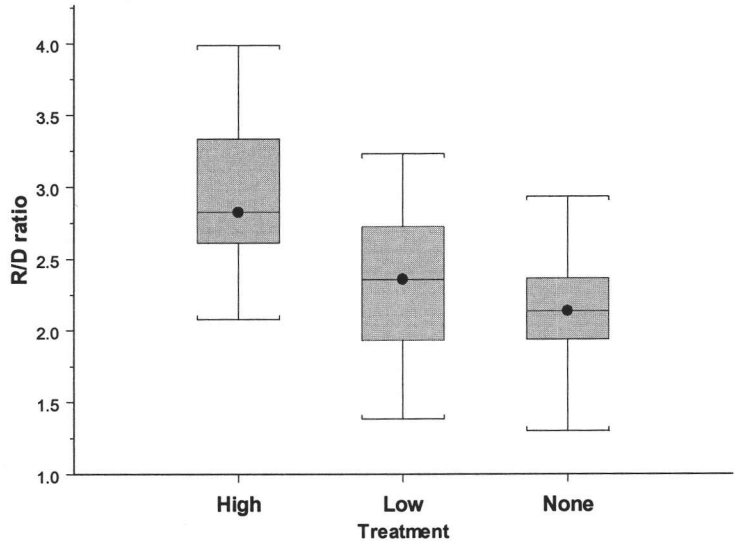


Figure 3.2. Box and Whisker Plots of % Growth per day for fish sampled from High, Low, and None feed treatments. Boxes represent middle 50% of data, the upper and lower whiskers are >~25% and <~25% of the data respectively, the line through the box is the median, and individual points are outliers.

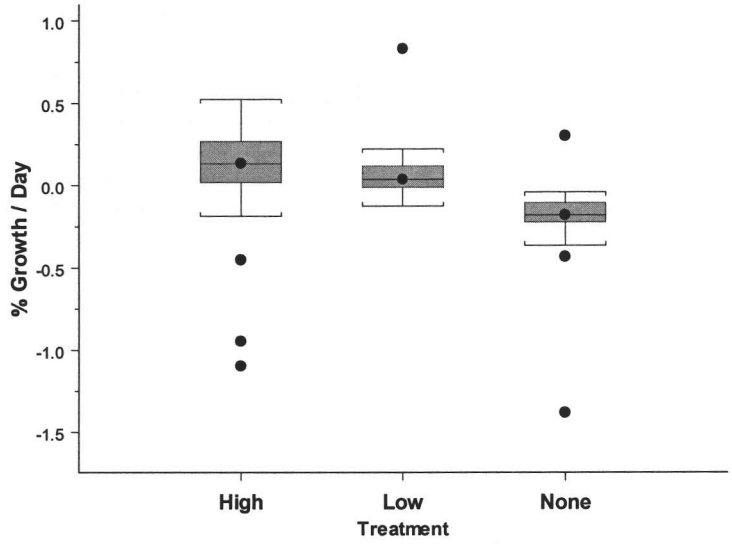
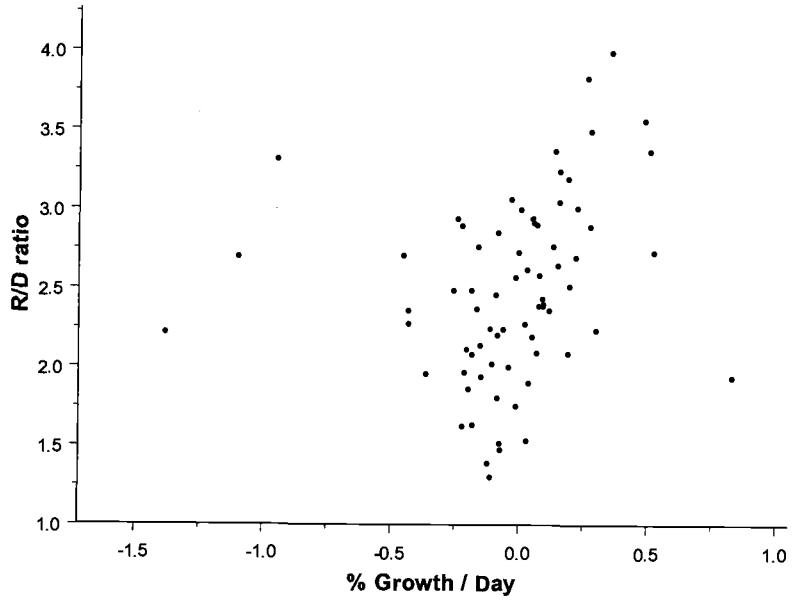


Figure 3.3. Scatter plots of R/D ratios verses growth per day. Points represent individual fish sampled after day 12.



DISCUSSION

The R/D ratio seems to be indicative of the recent growth for the entire population. However, our data shows that R/D ratio is not an accurate indication of an individual's short-term or recent growth. Other problems arise in using R/D ratios when sub-sampling tissue is necessary due to the fish's size. For the purposes of this method, sub-sampling muscle tissue for analysis of R/D ratios as an indication of growth and condition may not be the most representative method. This method should be applied carefully and sample size should be considered, particularly since the tissue requirements may require lethal sampling. Caladrone et al. (2001) noted that different sub-samples from the same fish can have different R/D values; therefore the ratio from a sub-sample cannot be directly compared to the ratio of the whole fish. It appears from our data that sub-sampling muscle tissue potentially has some additional problems with replication that may introduce additional variation. This additional variation may reduce accurate detection of differences in growth and feeding condition. Prior to utilizing R/D ratios as

a measure of growth, it should be tested and validated that this method is appropriate for the fish size and life stage that is of interest.

A quantitative analysis of nucleic acids can provide a relatively simple and immediate measure of recent growth rates and physiological condition. Many laboratory studies have documented this method's accuracy for estimating growth with larval fish (Buckley 1982; Buckley 1984; Clemmensen 1993), however, other life stages tend to be more difficult to analyze. During the juvenile period, concentrations of both RNA and DNA decrease while concentrations of protein, lipid and ash increase with development (Love 1970). Furthermore, weight specific growth begins to decrease. This method becomes even more complicated when comparing juveniles of different sizes and from different environments.

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