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## Fish Out of Salt Water: Smoltification in Subyearling Chinook Salmon from the Laurentian Great Lakes

Steve Sharron, *The University of Western Ontario*

Supervisor: Dr Yolanda Morbey, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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**FISH OUT OF SALT WATER: SMOLTIFICATION IN SUBYEARLING  
CHINOOK SALMON FROM THE LAURENTIAN GREAT LAKES**

(Thesis format: Monograph)

by

Steve Sharron

Graduate Program in Biology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## ABSTRACT

The timing of smoltification in juvenile anadromous salmonids is important to ensure individuals match their preparedness with their migration timing and the optimal conditions in the environment. I performed the first study of smoltification in adfluvial juvenile Chinook salmon naturalized in the Laurentian Great Lakes. In a hatchery study, I found that juveniles from one of these populations have similar patterns of smoltification timing to individuals from anadromous populations. Their  $\text{Na}^+/\text{K}^+$  ATPase activity, a common indicator of smolt status, peaked at 7.7  $\mu\text{moles ADP/mg protein/hour}$  on July 1 in freshwater. During the peak period, individual body size was not a good predictor of ATPase activity ( $R^2 = 0.05$ ,  $P = 0.168$ ). This is evidence that body size is not as important to an individual's decision to smolt and out-migrate as seasonal timing. My study also provides a valuable data set for future studies investigating rapid adaptation in Pacific salmon introduced into adfluvial environments.

### **Keywords**

Smoltification, Pacific salmon, rapid adaptation, Chinook salmon, *Oncorhynchus tshawytscha*, adfluvial, ontogenetic niche shifts, Laurentian Great Lakes, introduced species

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Science truly is a collaborative effort.

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# INTRODUCTION

## ***Timing of Ontogenetic Niche Shifts***

Prescient timing of major life history events can have important fitness benefits for many organisms. Ontogenetic niche shifts, where individuals change their resource use at some point in their life, generally require temporal matching of their individual state (e.g. size) to environmental conditions (e.g. food availability) (Werner and Gilliam 1984). For example, many lacustrine fish species including bluegill sunfish (*Lepomis macrochirus*) shift their foraging habitat and tactics as their body size increases and seasonal food availability varies (Werner and Hall 1988). The timing of ontogenetic niche shifts ultimately balances the trade-off between survival and growth. For example, maximum fitness occurs when choosing the environment that minimizes the ratio of mortality ( $\mu$ ) over growth ( $g$ ) (Werner and Gilliam 1984). Organisms should attempt to gain as much body mass or condition while paying the lowest mortality cost due to predation or other hazards. Oftentimes, the environment with the greatest food availability also carries with it the largest density of predators or other dangers (Lima and Dill 1990).

There could also be a time constraint for the ontogenetic shift, whereby individuals should be prepared for and make the niche shift before conditions become unsuitable. This time constraint means that there is a time window where the fitness advantages for shifting niches is the greatest. For example, mayfly (Order Ephemeroptera) emergence timing is predicted to occur before a critical time period when reproductive success of late emerging individuals is zero (Rowe and Ludwig 1991). Past this critical time, their offspring are unable to compete for the diminishing food sources and have decreased fitness. This cost outweighs the advantage of higher fecundity in late reproducing individuals (Rowe and Ludwig 1991).

Each individual makes a decision about when to make the ontogenetic shift based on their morphological or physiological state and environmental conditions. Their ability to read

cues from their environment and use them to make the correct decisions about the timing of ontogenetic niche shifts is therefore extremely important. In the era of global climate change, where temperatures are rising and many species are being exposed to novel environments, there may be a mismatch between the environmental cues and the environmental conditions. Global mean annual temperatures are expected to rise over the coming decades anywhere between 1-7 °C and the temperate regions of the world will experience the largest effects (IPCC 2014). Adaptation of phenology, the timing of life history events, to changing temperatures is one of the most documented forms of rapid or contemporary adaptation (Bradshaw and Holzapfel 2001, Walther et al. 2002). Contemporary adaptation of many traits, including phenological traits such as diapause or migration timing, is also seen in displaced species that are either unwanted invaders or species purposefully introduced into new environments (Moran and Alexander 2014).

There are many organisms that have been used to study the effect of environmental change on the timing of ontogenetic niche shifts. For example, many butterfly species (Order Lepidoptera) must time their emergence to coincide with optimum environmental conditions such as ambient temperature. Roy and Sparks (2000) predict that a 1 °C increase in temperature could lead to an advance of emergence by 2-10 days in many British species of butterflies. Mistiming of the arrival to breeding grounds in the migratory pied flycatcher (*Ficedula hypoleuca*) due to climate change leads to population declines of about 90% in the Netherlands (Both et al. 2006). This is thought to be due to the lack of food availability for nestlings when they hatch. Adaptation of ontogenetic timing to altered seasonal environments is therefore paramount for organisms in a changing world.

### ***Rapid Adaptation and Salmonid Fishes***

Local adaptation refers to individuals from one environment having higher fitness within their own environment relative to individuals from another environment. For example, brown trout (*Salmo trutta*) in Newfoundland have higher survival in their home streams versus foreign streams (Westley et al. 2013). This is an important concept in ecological studies because it means that populations have adapted to the conditions presented to

them. This could be via genetic change driven by natural selection or via phenotypic plasticity. Phenotypic plasticity is when an individual can express different traits under different environmental conditions. Testing for the presence of true local adaptation usually requires measuring the relative fitness of populations in a reciprocal transplant or common garden study (Kawecki and Ebert 2004, Merilä and Hendry 2014).

Genetic adaptations are thought to be common in wild populations. In a review of the research on wild populations across taxa, Hendry (2013) found that there is a considerable amount of standing additive genetic variance within populations for natural selection to act upon. Local adaptation is an important phenomenon for divergence of salmonid populations due to large and small scale differences in river traits or conditions (Fraser et al. 2011). Some of these disparate traits include migration distance to and from spawning grounds, water temperature, day length across latitudinal gradients, river flow, and productivity.

For a group of coldwater species such as salmonids, a warming climate could also bring new environmental challenges. Some of these challenges include increased water temperatures, increased disease virulence, and altered river flow patterns (Crozier et al. 2008). Temperature itself can directly affect a myriad of traits including emergence timing, juvenile and adult migration timing, and developmental rates (Crozier et al. 2008).

Evolution and adaptation are known to occur on ecological time-scales, meaning they can be quantified along with the environmental change imposing natural selection (Carroll et al. 2007). Rapid adaptation caused by strong selection in novel or anthropogenically altered environments has been documented numerous times in salmonid fishes (Family Salmonidae). They are thus considered a model species for many types of evolutionary studies, including rapid adaptation of life history traits in response to novel environments. Hendry and Stearns (2003) outline the many advantages of salmonids for evolutionary studies including: 1) high fecundity; 2) variation in life history strategies among populations; 3) partial reproductive isolation because of their natal homing abilities; and 4) the considerable amount of knowledge about heritability and phenotypic plasticity.

Evidence of rapid adaptation has been observed in many salmonid populations. For example, reproductive isolation was observed, via genetic markers and phenotypic differences, in 13 generations among introduced sockeye salmon (*Oncorhynchus nerka*) populations that have undergone adaptive divergence by utilizing different spawning habitat in Lake Washington (Hendry et al. 2000, Hendry 2001). Another well-studied example of rapid adaptation in salmonids is the established populations of Chinook salmon (*Oncorhynchus tshawytscha*) introduced to New Zealand's South Island in the early 1900s. Researchers found evidence of population divergence with differences in many traits in response to new selection pressures (e.g., temperature, photoperiod, and migratory distances) between two established populations with disparate riverine environments. They tested this using controlled common garden hatchery studies. The traits known to diverge between the two populations include juvenile growth rates (Unwin et al. 2000), reproductive output, and timing of the spawning migration (Quinn et al. 2001).

### ***Downstream Migration of Anadromous Juveniles***

Juveniles from anadromous species and populations of salmonines (Family Salmonidae including the Genera *Oncorhynchus*, *Salmo*, and *Salvelinus*) make an ontogenetic niche shift from the freshwater environments of their natal streams or rivers to the brackish or saltwater environments of their later feeding and growth stages. They do this by migrating downstream from fresh water to salt water. These migrations can be anywhere from a few metres in populations of intertidal spawning pink salmon (*O. gorbuscha*) to over 3,000 km in Chinook salmon and chum salmon (*O. keta*) from the Yukon River (Waples et al. 2001).

Anadromous fishes migrate from the sea, where most of their feeding and growth occurs, to freshwater environments for reproduction. While not completely understood, anadromy is considered an adaptation to both increased growth in the nutrient rich saltwater environment and the relatively safe reproductive environment in freshwater streams (Gross 1987, McDowall 1997). In populations of mixed anadromous and resident (remaining in fresh water for their lives) individuals, the anadromous individuals are

larger. Adfluvial populations, on the other hand, migrate from their natal freshwater streams to larger bodies of fresh water such as lakes where they gain from the increase in resource availability. The increased size of migratory salmon confers fitness advantages in the form of increased survival and fecundity (Hutchings 1993, Quinn 2005).

The transition between fresh water to salt water causes osmotic stress to the fish and carries with it some costs to the individual. This is especially true for juveniles entering sea water for the first time. Swimming ability has been shown to be impaired in the days following saltwater transfer. Coho salmon (*O. kisutch*), for example, showed a 15% reduction in critical swimming velocity in a two hour swim test following transfer (Brauner et al. 1994). This is most likely due to reduced osmoregulation negatively impacting aerobic metabolism. Atlantic salmon (*S. salar*) were shown to significantly reduce their feeding for at least one week following saltwater transfer (Damsgård and Arnesen 1998). Osmotic stress can also impair anti-predator response and increase mortality risk in the saltwater environment. Järvi (1990) found that the combination of osmotic stress and increased predation pressure in the salt water can have negative effects on Atlantic salmon released from hatcheries. Smolts that were not acclimated to salt water and exposed to predation risk showed a 90% mortality compared to 43% for individuals acclimated to salt water under the same predation risk (Järvi 1989).

### ***Smoltification in Juvenile Salmonids***

Smoltification is the complex suite of transformations individual juvenile salmonids undergo in preparation for the downstream migration to, and survival in, the marine environment. These changes are preparatory in that most of the transformations begin in fresh water before the individuals reach the marine environment where the new phenotypes are adaptive. While it remains a subject of contemporary research, much work has been done over decades to create a general model of smoltification (Folmar and Dickhoff 1980, Hoar 1988, McCormick et al. 1998, Björnsson and Bradley 2007). These transformations include physiological, behavioural, and morphological changes that are adaptive for some part of this niche shift. There is also olfactory imprinting on their natal

streams during smoltification that allows the adults to return to the same area for spawning (McCormick et al. 1998).

Increasing photoperiod is the main exogenous cue that orchestrates and regulates smoltification by stimulating multiple hormonal pathways. There are also other environmental cues that influence smoltification and initiate downstream migration such as water temperature, stream flow, and lunar phase (Björnsson and Bradley 2007). The onset of smoltification is triggered by a suite of hormonal cues. Briefly, it is stimulated by increases in thyroid hormones (Iwata 1995) and glucocorticoids, such as cortisol (Shrimpton et al. 1994b). Perhaps most importantly, smoltification is accompanied by an increase in hormones associated with growth such as insulin-like growth factor I (IGF-1) and growth hormone (GH) (Groot et al. 1995, Björnsson 1997). There is also inhibition of smolt development and GH from prolactin (Björnsson et al. 2011). The results of smoltification are a reorganization of certain parts of the brain as well as the various organs involved in osmoregulation (Ebbesson et al. 2003). Smolts are then able to tolerate and thrive in marine environments.

Behavioural changes during smoltification are important to individual fish as they shift environments. In freshwater streams, many juvenile salmonid species feed mostly on invertebrates that they intercept as they are carried downstream by the currents (Quinn 2005). To achieve this, the fish orient themselves upstream along the banks of the rivers and swim to maintain themselves in the current. They are waiting for invertebrates that have either been dislodged from benthic sediments or fallen into the water from the terrestrial environment (Quinn 2005). Part of the observed behavioural changes during smoltification includes a propensity for orientation downstream instead of upstream in a feeding position (Iwata 1995).

Juveniles in fresh water are usually solitary or in direct competition with each other for the drifting food. Since food intake is very closely tied to growth rates, individuals that are the best competitors and foragers have growth advantages over the others. As individuals smolt, their behaviour shifts from this solitary tendency to a less agonistic, more gregarious behaviour. Increased schooling behaviour is also observed as the

environmental conditions in the river change (i.e., increased flows). Schooling behaviour is believed to be an adaptation to increased predation risk during their dangerous downstream migration that can be like running the gauntlet (Hoar 1988). This gauntlet includes many waiting predators such as piscivorous birds and predatory fishes.

Migrating as a group can swamp these predators and increase an individual's chances for escape. There is also an increase in salinity preference, or time spent in salt water instead of fresh water, during smoltification as a juvenile salmon's physiology becomes more adapted to saline conditions than freshwater conditions (Whitman 1987, McCormick et al. 1998).

Morphologically, juvenile salmonids undergo significant changes to allow them increased crypsis in deeper waters and improved swimming ability for downstream migration and predator avoidance. The most useful morphological change is the elongation of the caudal peduncle, translating into a longer body. A longer body shape confers greater swimming efficiency in fishes that swim using the posterior part of their body like salmonids (Healey 2001). This lowered aspect ratio (length/width) provides less drag and more thrust per kick of the tail (Blake 1983). Combined with a reduction in total body lipids during smoltification, there is a noticeable reduction in the condition factor of smolts compared to presmolts (Groot et al. 1995). Body condition, traditionally measured as Fulton's condition factor  $K$ , is the ratio of body mass to body length. It is still unclear if the reduction in condition is an adaptation to increase swimming performance or is a non-adaptive outcome of the stress caused by smoltification (McCormick et al. 1998).

The second major morphological change during smoltification of juvenile salmonids is in their body colouration. The riverine environment generally has shallow water and rocky substrate. Dark colouration and parr marks (vertical black markings along the body characteristic of many juvenile salmonids) are known to provide crypsis and are a primary defense against predators in streams and rivers, such as piscivorous fishes and birds (Donnelly and Dill 1984). However, this dark colouration would not be advantageous in the deep coastal and pelagic waters to which juveniles migrate. Here, a lighter colour with reflectance (i.e., countershading) is more adaptive for predator avoidance (McCormick et al. 1998). During smoltification, juvenile salmon alter their

body colour from a dark colour with distinct parr marks to a more silvered colour and lose their parr marks to increase their countershading in deeper waters. This silvering is the result of deposited guanine and hypoxanthine in their skin (Denton and Saunders 1972).

Physiological changes during smoltification are perhaps the most important for juvenile salmonids because they need to become adapted to sea water. Seawater adaptability is the ability to maintain homeostasis in the newly encountered hyper-osmotic environment of the sea. Salmonids generally have a higher metabolic rate in saline waters, indicating a higher cost to life in salt water (Morgan and Iwama 1991, Tseng and Hwang 2008). By decreasing their osmotic stress through physiological changes, individuals can lessen these costs. The major tissues involved in osmoregulation include the kidney and urinary bladder, the intestine, and the gills. Urine flow is decreased when the fish are in salt water, but it is still unclear whether there is a reduction in urine flow during smoltification in fresh water (Clarke and Hirano 1995). In the intestine, a major site of water absorption in vertebrates, there is a marked increase in absorption when introduced to seawater (McCormick and Saunders 1987). During smoltification, there are changes in the epithelial components of the intestine such as an increase in aquaporin water channels that allow water to pass into the body. Tipmark et al. (2008) found that there was a significant increase during smoltification in intestinal mRNA expression of one of the three aquaporin isoforms important in seawater osmoregulation. While smoltification causes a change in many body tissues, much work has implicated gills as the major site of change for increasing saltwater preparedness.

In fish gills, there are specialized cells, ionocytes, with increased concentrations of proteins that move ions in and out of the cells. The major proteins involved in branchial (gill) ionoregulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in the gills are: cystic fibrosis transmembrane conductance regulator (CFTR), a  $\text{Cl}^-$  channel;  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter1 (NKCC1) which facilitates the movement of these three ions across the cell membranes; and  $\text{Na}^+/\text{K}^+$  ATPase (NKA) which actively pumps  $\text{Na}^+$  against the osmotic gradient out of the cells (Perry 1997, Hwang et al. 2011). The altered functionality of branchial NKA enzymes during smoltification has been extensively studied and is considered the most important

index of physiological changes during smoltification (McCormick et al. 2009, Christensen et al. 2012, Handeland et al. 2014). Increased seawater capability is positively correlated with increased NKA enzyme activity and there is a marked increase in branchial NKA activity during the smoltification process (McCormick and Saunders 1987). There is also a shift in isoform expression of the catalytic subunit (alpha,  $\alpha$ ) of NKA in ionocytes, from freshwater adapted isoforms to saltwater adapted isoforms (Richards et al. 2003). The alpha subunit has at least two isoforms in salmonids,  $\alpha_{1a}$  and  $\alpha_{1b}$  and they have different affinities for sodium ions. Many studies have observed a shift from the  $\alpha_{1a}$  isoform, when NKA activity is lower, to the  $\alpha_{1b}$  isoform which is the saltwater adapted isoform and correlates with higher NKA enzyme activity (e.g., Richards et al. 2003, McCormick et al. 2013).

While smoltification itself takes months to complete, the noticeable change in NKA enzyme activity over the natural smoltification period in fresh water is fairly rapid and can be seen over a few weeks (McCormick et al. 2009, Björnsson et al. 2011). The period during which physiological smoltification indices are at their peak is referred to as the “smolt window” (McCormick et al. 1998). This is the period when the individual is best prepared for the saltwater environment and it should be timed with downstream migration and seawater entry. This does not, however, mean that individuals cannot adapt to seawater if introduced outside of this smolt window. When introduced to salt water as presmolts, many individuals are able to quickly up-regulate and express these osmoregulatory adaptations. For example, rainbow trout (*Oncorhynchus mykiss*) showed a significant increase in NKA  $\alpha_{1b}$  expression and concomitant decrease in NKA  $\alpha_{1a}$  expression after one day of exposure to sea water (Richards et al. 2003).

### ***Timing of Smoltification – Environmental Factors and Body Size***

Based on hatchery studies, changing photoperiod is known to be the main environmental cue that initiates and orchestrates the timing of smoltification in juvenile salmonids. Photoperiod is known to be an important environmental cue that connects an individual’s biological cycle to the Earth’s natural rhythm in many species (Bradshaw and Holzapfel 2007). This is especially important for ontogenetic changes that occur in preparation for

future environmental conditions. Researchers have known for years that photoperiod has this effect and this has aided the salmonid aquaculture industry immensely (Saunders and Henderson 1970). Juvenile Atlantic salmon can be induced to smolt at any time of the year by manipulating the photoperiod they experience in a hatchery. This induction can be caused by artificially shortening the photophase to a winter length of approximately 9 -12 hours daylight for weeks and then following this with an increase in photophase to a summer length of approximately 14 -16 daylight hours (Berge et al. 1995, Duston and Saunders 1995). Smoltification can also be induced in a hatchery setting with an abrupt switch from ambient winter photophase to artificial, long summer daylight hours (McCormick et al. 2000). Photoperiodic information is received and processed in the retina and pineal organ of the fish and the signals are transmitted to the necessary organs via hormones such as melatonin and GH (Ebbesson et al. 2003). There is evidence that light intensity can affect smolt quality as well. The growth of Atlantic salmon was positively correlated to light intensity and the highest smolt quality, as measured by body silvering and body shape, was found at intermediate light intensities (Handeland *et al.*, 2013a).

Salmonids are ectotherms that have evolved in and are adapted to temperate coldwater environments (Quinn 2005). As our understanding of smoltification timing improves, the interaction between photoperiod, temperature, and individual growth is being elucidated through hatchery studies (Björnsson and Bradley 2007). Handeland et al. (2013b) found that there was a significant interactive effect between water temperature and photoperiod on smoltification timing in hatchery raised Atlantic salmon. Individuals raised in colder water temperatures (8 °C versus 13 °C) and simulated natural photoperiod had a higher peak NKA enzyme activity than fish raised at low temperature and continuous light. In a hatchery setting, McCormick et al. (McCormick et al. 2000) found that in lower, ambient winter water temperatures (approximately 2 °C), increasing the photoperiod to a long day length did not result in a normal time course of smoltification compared to fish in 10 °C water and the same increase in photoperiod. The fish raised at ambient temperatures experienced later NKA enzyme activity increases, lower levels of activity, and a shorter duration of the elevated levels. This suggests an optimal water temperature, and probably growth rate, for the photoperiod cues to result in normal expression of smoltification

traits and progression of timing. They also found that growth hormone increased under the higher temperature conditions when the photoperiod was lengthened. The GH increase was less in a shorter photophase treatment. GH levels were also highly correlated with NKA enzyme activity. This further speaks to an interaction between photoperiod as the *Zeitgeber* and other environmental factors that modify smoltification.

Warm water temperatures can cause physiological stress and inhibit growth and smoltification in salmonids. There is an optimal temperature for maximizing growth before blood oxygen levels decrease and the metabolic scope for these fish decreases (Brett et al. 1969, Richter and Kolmes 2005). In other words, there is a parabolic relationship between temperature and growth rates. This optimum can influence when the ideal out-migration timing will be. When food intake was rationed, the optimal growth temperature was shown to decline in Chinook salmon from 19 °C to 15 °C (Clarke et al. 1981). It has also been shown in hatchery studies with Atlantic salmon that high temperature and growth conditions can actually inhibit an increase in NKA enzyme activity and smoltification (Handeland et al. 2000). Juvenile Chinook salmon from the Sacramento River in California were found to express impaired smoltification indices, such as NKA enzyme activity, when exposed to higher temperatures than normal (21-24°C) in a laboratory setting (Marine and Cech 2004).

Growth during a specific time period of development are important in determining if and when individuals smolt. GH and IGF-1 levels, along with increased growth in spring, are correlated to high NKA activity levels and other smoltification traits in Chinook salmon (Björnsson 1997, McCormick et al. 2000). In masu salmon (*O. masou*) from a hatchery study, growth rates between March and April predicted smoltification status at the beginning of June (Shimoda 2002). By increasing feeding and growth in the summer, Beckman et al. (2003) were able to induce fall smolting in subyearlings from a population of spring Chinook salmon that do not normally smolt as subyearlings. This was in contrast to juveniles that were of the same size at age but whose maximum growth rates occurred in the fall.

Juvenile growth rates have played a role in the divergent evolution of two populations of introduced Chinook salmon in New Zealand. Fish that have adapted to colder waters (7-8 °C during incubation) had slower growth rates in a controlled growth experiment compared to individuals from warm water streams (11-13 °C during incubation) (Unwin et al. 2000). These growth differences are related to the disparate life history strategies between populations. Slower growth observed in the cold water streams are consistent with the stream-type life history strategy found in some populations in the native range of Chinook salmon. The stream-type life history strategy is characterized by a downstream migration as yearlings rather than subyearlings as in the ocean-type life history. Faster growth in ocean-type individuals are thought to be an adaptation to the time constraints for individuals to out-migrate as subyearlings. This is consistent with theory of flexible growth rates under varied time constraints when optimal body size is necessary for an ontogenetic shift (Abrams et al. 1996).

Decades after Elson (1957) first proposed the idea of a minimum winter body length of 10 cm as a threshold for predicting Atlantic salmon migration in the spring, researchers still agree that individual body size is important for the timing of smoltification in salmonid fishes. Reaching a large enough body size before downstream migration can decrease the ratio of mortality risk versus growth ( $\mu/g$ ) because larger fish generally have fewer predators (piscivorous fish are gape-limited) and can eat a wider range of foods to increase growth. However, there is much diversity in threshold sizes and age at smoltification among salmonid species and populations within species (Quinn 2005). Smoltification in Atlantic salmon has been described in life history models as a negative development decision that individuals make only if they have not reached the proper state conditions for maturation (Hutchings and Myers 1994, Thorpe and Metcalfe 1998, Mangel and Satterthwaite 2008). Models for the development decisions in rainbow trout are very similar to Atlantic salmon models (Satterthwaite et al. 2009, Beakes et al. 2010). These models consider that there is a “decision window” where an individual’s state during this time period is correlated with their fitness probabilities, dependent on their decision whether to smolt and migrate downstream or mature and remain in freshwater habitats at this time.

Both Atlantic salmon and rainbow trout have the ability to forgo smolting and downstream migration in a given year. Other populations exhibit a life history strategy where a majority of juveniles migrate at a given age. The models explaining individual variation in timing of downstream migration in these species speak of a threshold size that can be reached via different growth trajectories (Dodson et al. 2013). Their large body size can be reached through increased growth in that season or simply via advanced age. Some populations of salmonids, such as ocean-type Chinook salmon, generally migrate as subyearlings (Waples et al. 2004). Juvenile salmon that migrate downstream as subyearlings reach a large body size through faster growth over the short period of development experienced before migration (i.e. months). Size thresholds may be less important if smolting is obligatory, as in migrating subyearlings. In one study of stream-type Chinook salmon, fish from both large and small size classes (>85 mm and <75 mm fork length in July) did not differ in the timing of the peak NKA enzyme activity, although they did differ in the intensity of that peak (Beckman and Dickhoff 1998).

### ***Optimal Timing of Smoltification & Out-migration in Wild Populations***

While smoltification prepares the individual for the marine environment, the ontogenetic shift actually takes place when they move downstream towards the sea. In wild populations, the river conditions in that first year can have significant effects on the optimal timing of the downstream migration. There are many factors influencing the suitability of streams for juvenile salmonids. Some of these include water temperature, water flow rate, and food availability. Water temperatures are generally influenced by solar irradiation, depth, canopy cover, and the prevalence of groundwater inputs (Caissie 2006). Rivers and streams are subject to higher daily temperature variation than lakes or oceans due to their vertical mixing and shallow depths. These warm temperatures are often mitigated by cold ground water entering the rivers. Water flow is a function of runoff from precipitation or snow melt in the surrounding area and geomorphological characteristics of both the river bed and the entire watershed (Vannote et al. 1980).

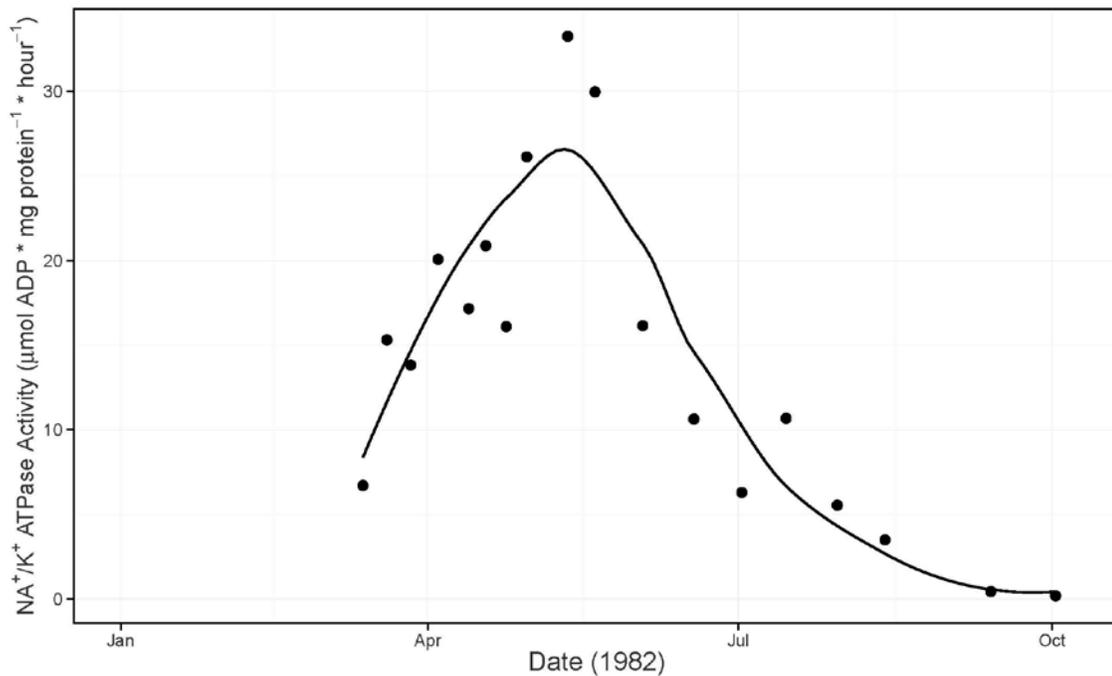
There may be strong pressure to out-migrate before water temperatures increase in the spring and summer. In a river where the forest canopy was lost to logging, Holtby et al.

(1989) found that there was a shift towards earlier age at smoltification and out-migration. They observed a shift from 50% of wild coho salmon (*O. kisutch*) smolting as subyearlings and yearlings before the logging, to 85% of individuals smolting as subyearlings after the logging. This shift towards earlier migration may be due to increased growth in warmer waters allowing them to reach threshold body sizes as subyearlings. It could also be because of pressure to leave the stream environment when temperatures reach thermal stress limits. Out-migration timing in Atlantic salmon is known to be correlated with river temperature. For example, water temperature increases and mean temperature in the spring are major predictors of migration timing in the Isma River, Norway (Jonsson and Ruud-Hansen 1985). Researchers have found that the timing of downstream migration in juvenile Atlantic salmon across the North Atlantic Basin has advanced over 50 years along with average air, river, and ocean temperatures (Otero et al. 2014). On average, downstream migration of juveniles has advanced by 2.5 days per decade.

The factors that affect out-migration timing in wild populations are complex and can be different in disparate systems. For example, four populations of coho salmon on the Pacific Coast of North America showed differences in the factors that explained out-migration timing (Spence and Dick 2013). They found that all populations relied on photoperiod, temperature, and the interaction between the two. However, other factors such as water flow and lunar phase differed between sites, as did their interaction with the other variables. Stream flow can also act as a release factor for downstream migration of juvenile salmonids (McCormick et al. 1998). As the season progresses, stream flows in temperate climates generally decline because there is less snow melt and precipitation. This can lead to warmer temperatures due to the shallower water as well as a decrease in the rate at which food is available in the downstream current.

There is evidence that early out-migration may have fitness advantages for subyearlings. In both Chinook salmon and rainbow trout from one tributary of the Columbia River, there was a 4-50 fold increase in survival to adults for individuals migrating downstream earlier in the spring (mid-June) than those migrating one month later (mid-July) (Tiffan et al. 2000). The date of out-migration had more of an effect on survival than water

temperature in their models. They also found that NKA enzyme activity was lower in mid and late migrants. This pattern has been seen in other studies as well (Giorgi et al. 1997, Scheuerell et al. 2009). This corroborates the evidence in hatchery studies of juvenile anadromous salmon raised in fresh water showing a distinct rise, peak, and fall of NKA enzyme activity in the spring before smoltification. This pattern is seen in Chinook salmon from the University of Washington hatchery, which were derived from the same broodstock as Great Lakes Chinook salmon (Figure 1).



**Figure 1.** Plot of mean Sodium Potassium ATPase enzyme activity of juvenile Chinook salmon from the University of Washington salmon hatchery over the spring and summer, 1982. LOESS curve is included to illustrate the general pattern (Adapted from Whitman 1987).

## ***Introduced Chinook Salmon Populations***

Salmonid fishes have been introduced into many novel temperate environments around the world because of their value as a prized sport fish and their capacity to adapt to new conditions. Many introduced salmonid populations are now considered naturalized. A naturalized population is one that sustains itself without assisted reproduction and has become incorporated in the local community (Copp et al. 2005). Chinook salmon are one of the most common species used for non-native transplantations because they have the largest scope for phenotypic plasticity and adaptation of the Pacific salmon (Waples et al. 2004). Naturalized Chinook salmon populations can be found today in South America (Soto et al. 2007), Southern New Zealand (Unwin et al. 2000), and the Laurentian Great Lakes (Kerr 2006).

There is a long history of stocking non-native fishes in the Great Lakes. Pacific salmon (*Oncorhynchus* spp.) have been introduced into the Great Lakes numerous times since the late nineteenth century. At one time or another, four of the five salmon species (i.e., pink; Chinook; coho; and sockeye) and rainbow trout were present in the lakes due to the focused stocking of juveniles (Kerr 2006). Supplementation efforts of rainbow trout, Chinook salmon and coho salmon continue today in both the United States and Canada. The Great Lakes salmon recreational fishery is now a large part of the economy surrounding the lakes (Melstrom and Lupi 2013). There is ongoing debate about the merits of continuing these supplementation programs, but they have mostly been accepted as part of the ecology in the Great Lakes (Bunnell et al. 2014). Chinook salmon, for example, are currently found in all of the lakes except Lake Erie, where the water temperatures are thought to be too warm for their survival.

In the 1960s, there began intensive efforts to establish Chinook salmon populations in Lakes Michigan and Huron from state governments in the U.S. and Canada. They began their stocking programs ostensibly to control the increasing population of non-native alewife (*Alosa pseudoharengus*) and as a target for the international sport fishery (Weeder 2005, Johnson et al. 2007, Johnson 2010). The source stock for these introductions was the Green River hatchery on the Green River in Washington and they

were introduced between 1966 and 1968 (Weeder 2005). In the decades since these stocking events and after approximately ten generations, there appears to be a genetic structure among Lake Huron populations (Suk et al. 2012). This evidence suggests that there is genetic isolation in these naturalized populations and the potential for local adaptation. The genetic structure found at this point seems to be due to random founder effects (Suk et al. 2012). This leaves the evolutionary ecologist with many questions as to what traits could be under selection, if any, in these populations. To date, there is only one study that has examined this. Purcell et al. (2014) found evidence of genetic divergence in resistance to bacterial kidney disease (BKD) amongst Lake Michigan Chinook salmon populations and divergence from their ancestral population.

Pacific salmon were introduced into a novel environment with many differences in environmental conditions including photoperiod, temperature, salinity, and changes in photoperiod due to latitudinal differences. For the most successful Great Lakes Chinook salmon populations of Southern Georgian Bay, there is a slight difference in photoperiod from their ancestral green river in Washington. On the summer solstice (e.g., 21 June 2014), Southern Georgian Bay experienced 15:33 hours of daylight compared to 15:42 hours at the outlet to the Green River. Temperatures are also very important for the physiology and phenology of these salmon. During the spawning season, temperatures in the Sydenham River, Ontario were found to be warmer (12.1 °C in 2010 and 14.2 °C in 2011, Gerson et al. 2014) compared to the spawning grounds of the founding population (10.4 °C from 1972-2000, Quinn et al. 2002). The most obvious environmental difference is their growth in fresh water of the Great Lakes versus the salt water of the Pacific Ocean. While temperature and photoperiod are cues affecting the timing of smoltification, salinity is the environmental factor for which smoltification is adapted.

### ***Smoltification in Adfluvial Environments***

There are examples of salmonid populations in freshwater environments retaining at least some of their ability to undergo smoltification. Many times, adfluvial populations are derived from anadromous populations that have become land-locked due to anthropogenic causes such as dam installation. For example, rainbow trout in a reservoir

created by an impassable dam in southwestern Idaho still undergo the transformations of smoltification after 49 years (Holecek 2012). Much of the population showed increased skin silvering and a doubling of NKA enzyme activity over the spring. Other populations have been geographically isolated by natural phenomena such as glacial retreat. Kokanee salmon, which are an adfluvial form of sockeye salmon, were shown to retain aspects of smoltification, including seawater adaptability (Foote et al. 1994). Because there has been no gene flow between most kokanee and sockeye salmon populations for approximately 10,000 years, this indicates that aspects of smoltification may be important for more than just saltwater preparedness, or that there is a linkage between smoltification traits that shields some traits from selection pressure. However, in a population of landlocked Arctic char (*Salvelinus alpinus*), fish did not significantly up-regulate the saltwater adapted  $\alpha_{1b}$  subunit of NKA when exposed to sea water and had lower NKA enzyme activity than fish from an anadromous population (Bystriansky et al. 2007b). However, species of the *Salvelinus* genus have a lowered scope for seawater tolerance compared to those from the *Salmo* or *Oncorhynchus* genera (Bystriansky et al. 2006). There are a few studies showing the existence of adfluvial Chinook salmon populations outside of the Great Lakes (e.g. above a dam on the Willamette River, Oregon, Romer and Monzyk 2014), but no studies have examined smoltification in these populations.

### ***Motivations for my Study***

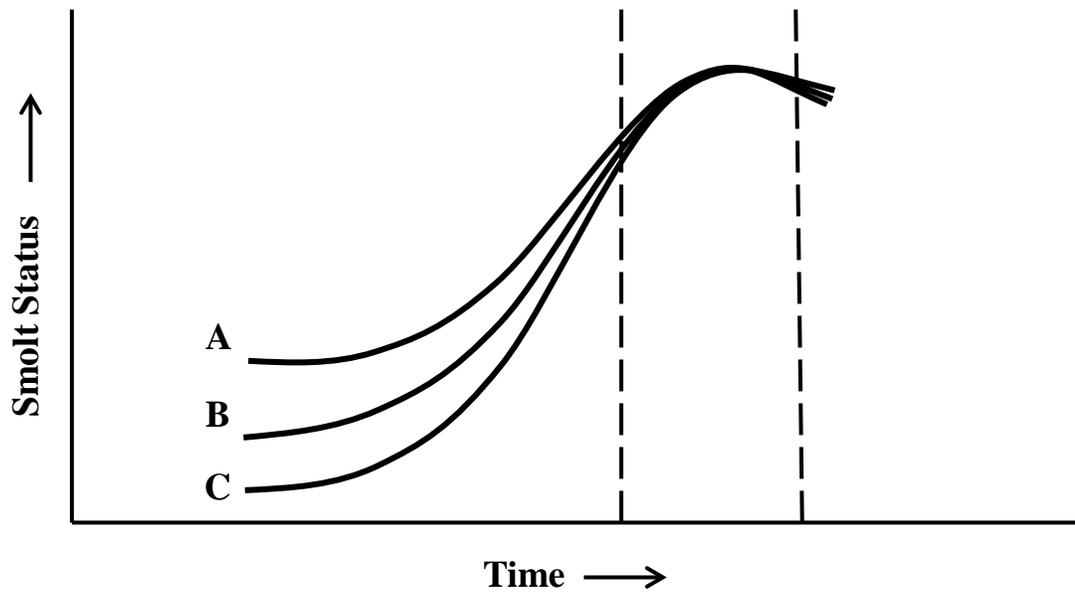
My original study design was to use a common garden experiment to compare smoltification timing in a Great Lakes Chinook salmon population to smoltification timing in a population derived from the same ancestral population. This was not possible due to a decision by the Canadian Food Inspection Agency (CFIA) to deny my request to import gametes from Washington State at the last minute. The reason for the denial was the positive test for infectious haematopoietic necrosis virus (IHN) in the parent fish. My altered study was designed to provide important baseline data on the smoltification timing of juvenile Chinook salmon in the Great Lakes to be used for further study into adaptation of smoltification timing and traits.

I had two primary objectives as possibly the first researcher to study smoltification in these populations. The first was to describe the pattern of smoltification for a population of juvenile Chinook salmon that no longer migrate into a saline environment. There are currently no studies that confirm that both wild and hatchery-raised juveniles from an adfluvial population of Pacific salmon undergo smoltification in the same timing pattern as anadromous juveniles. To this end, I combined a field study, where I sampled fish from a naturalized population, with a controlled hatchery study to monitor the seasonal timing of morphological and physiological changes.

In the hatchery study, I raised juveniles from gametes under controlled environmental conditions to track the progression of smoltification throughout the spring and summer seasons when they would smolt in the wild. I used a battery of smoltification indices to determine their smolt status. My hypothesis was that Great Lakes juveniles would undergo smoltification following the same general pattern as populations in their native range (Figure 1), but that there would be a difference in growth and timing. Because of colder over-wintering temperatures combining with higher summer water temperatures than their ancestral rivers, individuals were expected to grow faster and smolt at smaller sizes. This would lead to selection to be prepared to out-migrate before river conditions deteriorated, even though development would be suppressed during winter. This follows from theory of flexible growth rates under time constraints (Abrams et al. 1996). I predicted that juvenile Great Lakes Chinook salmon would undergo a similar trajectory of NKA enzyme activity to Figure 1 and I also predicted a concomitant increase in body silvering and body elongation over the smoltification period.

My second objective was to examine the relationship of smolt status and body size of individual fish to examine the effects of body size and photoperiod on the timing of smoltification over the spring season. My hypothesis was that smoltification traits would be coupled to a threshold body size as shown in other studies, but because of the earlier time constraints of warmer rivers, smoltification would be faster than in ancestral populations. It would therefore be advantageous for individuals to smolt during the smolt window, regardless of their individual size. I predicted that during the important smoltification periods, all individuals will reach similar smoltification status, regardless

of their body size (Figure 2). There are no studies to date that have investigated the relationship between size and photoperiod in an ocean-type Chinook population. These two factors are the most important for smoltification timing, yet their relative importance during important smoltification periods in subyearling out-migrants is understudied.



**Figure 2.** Model showing the convergence of smolt status (e.g. NKA enzyme activity) of three size classes of subyearling Chinook salmon during the smoltification window. Each curve represents a separate size class with A representing the largest individuals in the population, B representing intermediate sized individuals, and C representing the smallest individuals. The smoltification window is the time between the dashed lines. At this point, all juveniles reach the same smolt status independent of body size.

## METHODS

### PART 1. FIELD SURVEY

#### *Field Sampling – Pine River, Spring 2013*

The first part of my study was to perform a field survey of juvenile Chinook salmon from a naturalized population in Southern Georgian Bay. The goal of this survey was to capture subyearling smolts or out-migrating juveniles to obtain a set of physiological and morphometric data for comparison with the data that I obtained in the hatchery study and with other studies in the literature. Field sampling took place on the Pine River in Southern Ontario, a tributary of the Nottawasaga River (Figure 3). There were three sites on the river that I had access to and permissions to sample from. I used two main fishing tactics practical for fishing in the shallow water conditions in the Pine River: dip-netting and seining. Dip-netting was favourable at the beginning of the season when the juveniles were feeding at the edge of the shore and seining was more effective when the juveniles were large enough to occupy the deeper areas of the river with higher flow rates and less overhead cover. This ontogenetic shift in habitat preference is well-documented (e.g., Quinn, 2004) and allowed me to target the largest size classes of juveniles as the season progressed to ensure I was capturing those individuals in the pre-smolt or smolt stage.



**Figure 3.** Map of Southern Georgian Bay and surrounding areas, including the two rivers sampled in this study. The black star marks the location of the Pine River where juvenile field sampling for Chinook salmon took place. The white star marks the Sydenham River, the site of gamete collection for the hatchery study. (Adapted from National Geographic basemap in ArcGIS).

## ***Sampling Sites on the Pine River***

Site 1 was accessed via Adjala-Tosorontio concession 3 in Everett, ON (UTM: 17T 0582160 4893260). This site is characterized by the low grade from the banks to the river, where the highest bank height was approximately 30 cm. This site became the predominant sampling site due to its easy access and abundance of target fish.

Site 2 was accessed from Tiogo Blvd adjacent to Adjala-Tosorontio County Road 13 (UTM: 17T 0583248 4896969). This site is characterized by high sandy cliffs on both sides of the river with sporadic areas of lowered banks for easy access to the river. There are many riffle stretches with appropriate gravel sizes for spawning by Chinook salmon.

Site 3 was accessed from the Town of Angus soccer fields (UTM = 17T 0589352 4908678). This site is characterized by sandy river banks intermediate in size compared to the other sites. The substrate is predominantly sand with very small stretches of small gravel and no evidence of ideal salmonid spawning habitat. The water flow is higher in this stretch and there is more large woody debris creating a lot of potential habitat that could be used by juvenile salmonids. Despite this observation, there were only a small number of juvenile Chinook salmon caught at this site.

## ***Fishing Tactics and Gears Used***

I performed all of the seining using a 10 m long beach seine with 6 mm mesh and a 1 m<sup>3</sup> bag at the cod-end to encircle and corral fish into it. The net was deployed by two people (Figure 4). Both netters started at the same spot along the shore before one netter waded out into the water and downstream to create a capture zone. The second netter walked along the shore holding onto the net for approximately 5 m. At this point, the two netters met again to gather in the netting to maneuver the fish into the bag. This technique proved to be the more effective of the two techniques for capturing larger pre-smolts and smolts. After the first week of using both capture methods, starting May 8, we only used the beach seine to capture fish because the fish were found predominantly in the deeper portions of the river.

Dip-netting refers to the method whereby fish were captured with a 6 mm mesh minnow net from the river bank or behind the fish in the river. The netter simply scooped the target fish quickly and efficiently before they could escape. This method leads to a greater failure rate when capturing larger fish due to their increased swimming ability. This method is also only functional in accessible near-shore habitats. Due to these inherent size and habitat biases, this method was not used throughout the entire season.



**Figure 4.** Photo showing the seining method for juvenile Chinook salmon. Pictured is myself and field technician, Yelin Xu.

## ***Fish Handling***

All procedures for the capture and handling of fish were approved by the University of Western Ontario's Animal Use Subcommittee (AUP 2007-043-05) and were optimized to ensure minimal impact on each individual. This included anesthetizing the fish during measurement to ensure that the sampling was done in the most efficient manner and to minimize the amount of necessary physical restraint. The dosage of anesthetic was obtained from previous research and was monitored during the entire handling process to ensure that the fish were not being unduly harmed. In total, there were 19 fish mortalities during the handling of 1812 total fish (~1% mortality).

Upon capture, I transferred all fish to plastic five gallon buckets and carried them to the sampling area. Fish were anaesthetized using buffered MS-222 solution (100 g/l in water buffered in 100 g/l sodium bicarbonate; Cho and Heath 2000) for approximately 30 to 60 seconds before length, mass, and species were recorded. Length was measured as fork length (FL) in millimetres. FL is a common method for measuring salmonids; it is the distance between the most anterior point on the rostrum and the end point in the middle of the caudal fin between lobes. Wet mass was measured to the nearest 0.001 g using an Acculab Vicon 120 g balance. I also calculated Fulton's condition factor K, which is the relationship between mass and body length in a fish and is calculated using Equation 1 (Ricker 1975).

### **Equation 1:**

$$K = (10^5) * (\text{Mass (g)}) * (\text{Fork Length (mm)})^{-3}$$

I set aside fish that were identified as smolting individuals in a separate aerated bucket after measurements and photos for lethal sampling. I determined smolting individuals by their large size (> 68 mm FL, > 3 g mass; Negus 2003), streamlined body and head shape, and silvered sides with parr marks that were difficult to see in natural light. They were euthanized in 300 mg/l MS-222 dissolved in river water. In addition to size measurements and photos, I took blood plasma (separated on site in a microcentrifuge)

and gill filament samples from all of these fish and stored them in a dry shipper of liquid nitrogen before transporting them back to the University of Western Ontario.

I transferred all other fish that were not euthanized to a continuously aerated five gallon bucket of river water for recovery. The fish took up to a maximum of two minutes to recover to a state where they were swimming upright and their gill opercula were opening and closing at a steady rate. They remained in the recovery bucket for a minimum of 20 minutes before release back into the same area of the river where they were captured. This refractory period was enough to ensure no lingering effects of the anesthetic on their survival or swimming ability in the river.

### ***Sodium Potassium ATPase Enzyme Activity***

To assess smoltification status of captured individuals, I performed an enzyme activity assay for branchial sodium potassium ATPase (NKA) on the 38 “smolting” fish. While hypo-osmoregulatory ability is known to be the work of many organs and enzymes, gill NKA enzyme activity is known to be one of the most useful indicators of the smoltification status of juvenile salmonids (McCormick 1993, Beckman et al. 1999, Handeland et al. 2014). The protocol that I used for the NKA enzyme activity assays was modified from a protocol developed by McCormick (1993) and is used by many contemporary salmonid researchers studying smoltification (e.g., Flores and Shrimpton 2012, Spangenberg et al. 2014). Generally, this assay quantifies the ability of each ATPase enzyme to transport sodium ions out of the ionocytes and across the gill membrane. It explicitly measures the amount of nicotinamide adenine dinucleotide (NADH) that is oxidized in a reaction mixture. Each mole of  $\text{NAD}^+$  produced represents one mole of adenosine triphosphate (ATP) that is hydrolyzed. ATP is the currency for NKA enzyme function.

For each fish that I sampled, I removed all of the gill arches (there are four on each side of the head) and dissected out the dorsal-most filaments of the anterior-most arch (Figure 5). The gill tissue was then placed in a microcentrifuge tube with 100  $\mu\text{l}$  of SEI buffer (Sucrose (250 mM),  $\text{Na}_2\text{EDTA}$  (10 mM), Imidazole (50 mM),  $\text{pH} = 7.3$ ). Samples were then placed into a freezer at  $-80\text{ }^\circ\text{C}$  until the assay date. They were frozen for no longer

than two months for hatchery samples and ten months for field samples. In the field, all samples were immediately placed into a dry shipper with liquid nitrogen until they could be transported to the University of Western Ontario and placed into a freezer at  $-80^{\circ}\text{C}$ .

Samples were placed in a spectrophotometer in groups of eight samples on 64 well microplates. To each sample in SEI buffer, I added another 50  $\mu\text{l}$  of SEID (SEI buffer with 30 mg/l sodium deoxycholate) and ground up the filaments in the microcentrifuge tube using a pellet pestle to remove any solid, non-filamentous tissue. The sample was then spun in a centrifuge at 5,000 g for 30 seconds and the supernatant was used in the assay. I added 10  $\mu\text{l}$  of sample to each well in quadruplicate. To two of these wells, I added 200  $\mu\text{l}$  of the assay mixture (recipe below) with ouabain (0.7 mM), an inhibitor of NKA, and 200  $\mu\text{l}$  of assay mixture without ouabain was added to the other two wells. This resulted in duplicate wells of the uninhibited NKA sample and the inhibited sample.

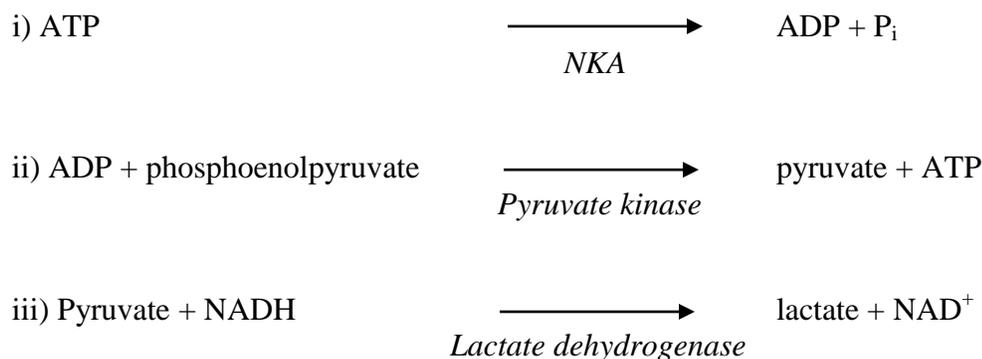
#### **Assay Mixture Recipe:**

- Imidazole buffer (Imidazole (50 mM),  $\text{dH}_2\text{O}$ , pH = 7.5)
- Salt solution (Imidazole (50 mM), NaCl (189 mM),  $\text{MgCl}_2$  (10.5 mM), KCl (42 mM),  $\text{dH}_2\text{O}$ , pH = 7.5)
- Nicotinamide Adenosine Dinucleotide (NADH, 0.22 mM)
- Adenosine Triphosphate (ATP, 0.7 mM)
- Phosphoenolpyruvate (PEP, 2.8 mM)
- Pyruvate Kinase (PK, 5.0 U/ml)
- Lactate Dehydrogenase (LDH, 4.0 U/ml)

All biochemicals were obtained from Sigma Aldrich Corporation. I detected absorbance using a kinetic reading on a plate spectrophotometer (PowerWave X340, BIO-TEK instruments Inc.) at 340 nm wavelengths and  $25^{\circ}\text{C}$ . This wavelength detects absorbance by NADH and the multiple readings track the disappearance of NADH over 11 minutes (60 readings at 11 second intervals). As per Equation 2, each mole of  $\text{NAD}^+$  produced is equivalent to one mole of ATP hydrolyzed. The units for the activity calculations are  $\text{mOD}/10 \mu\text{l}/\text{minute}$ . mOD is milli-units of optical density recorded by the

spectrophotometer. The difference between the measurement without ouabain inhibitor and measurement with ouabain inhibitor gives the final measurement for calculation.

**Equation 2:**



For calculations of the enzyme activity, an NADH standard curve was prepared using four concentrations (0, 10, 20, 40 nmoles NAD/ 10  $\mu$ l imidazole buffer) in quadruplicate and recorded as an endpoint reading on the spectrophotometer. The slope of this standard curve was calculated as 21.7 mOD/nmoles NADH. In addition to the NADH standard curve, I calculated an ADP standard curve for each batch of assay mixture to ensure that the batches remained consistent. Each batch was stored for no longer than four days. I calculated the ADP curve using four concentrations (0, 5, 10, 20 nmoles ADP/10  $\mu$ l) and 200  $\mu$ l of assay mixture per well. The batches were considered fit for use if the slope of the curve was between 17-19 mOD/nmoles ADP.

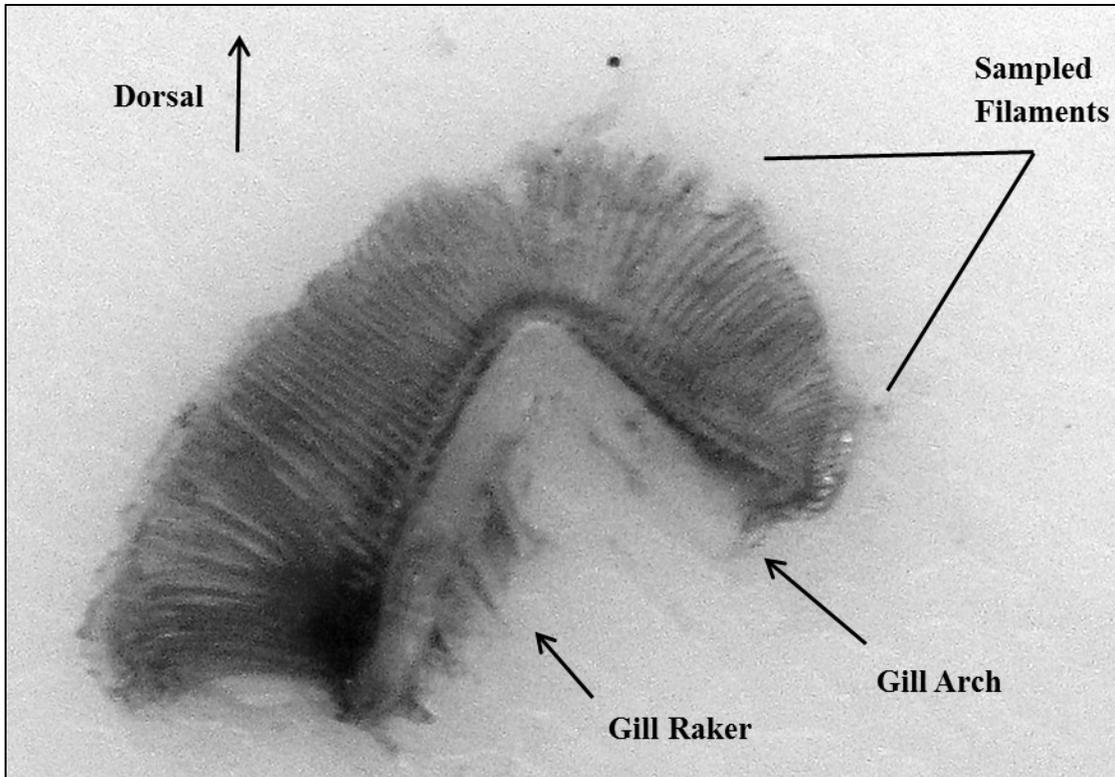
To standardize the activity measurements for individual enzymes, I also obtained a measurement of the total protein in each gill filament sample ( $\mu$ g/ $\mu$ l). I did this using a standard Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology, Rockford IL). I placed 10  $\mu$ l of each sample in another plate of wells in triplicate and 200  $\mu$ l of the BCA mixture was added. After one hour, an endpoint reading at 540 nm wavelength was obtained. The BCA reaction mixture binds to proteins and the sample changes colour depending on the amount of protein in the sample. The sample concentrations were

calculated from the standard curve of four concentrations (0, 5, 10, 20  $\mu\text{g}$  protein/ 10  $\mu\text{l}$ ) of protein added to each plate.

The final calculations to obtain NKA enzyme activity were then performed using Equation 3.

**Equation 3:**

$$\begin{array}{ll}
 \text{Standard curve slope} & = 21.7 \text{ mOD/nmole ADP} \\
 \\
 X = \text{ATPase (without ouabain)} & = X \text{ mOD}/10\mu\text{l}/\text{minute} \\
 \\
 Y = \text{ATPase (with ouabain)} & = Y \text{ mOD}/10\mu\text{l}/\text{minute} \\
 \\
 Z = \text{Na}^+, \text{K}^+ \text{-ATPase} = X - Y & = Z \text{ mOD}/10\mu\text{l}/\text{minute} \\
 \\
 A = \text{protein reading} & = A \mu\text{g}/10\mu\text{l} \\
 \\
 \frac{Z \text{ mOD}/10\mu\text{l}/\text{minute}}{21.7 \text{ mOD/nmole ADP}} & = B \text{ nmoles ADP}/10\mu\text{l}/\text{minute} \\
 \\
 \frac{B \text{ nmoles ADP}/10\mu\text{l}/\text{minute}}{A \mu\text{g protein}/10\mu\text{l}/\text{minute}} & = C \mu\text{moles ADP}/\text{mg} \\
 \\
 (C \mu\text{moles ADP}/\text{mg})(60 \text{ min}) & = \underline{\underline{D \mu\text{moles ADP}/\text{mg protein}/\text{hour}}}
 \end{array}$$



**Figure 5.** Diagram of a single juvenile Chinook salmon gill arch showing the major features and the portion of the arch where filaments were removed for NKA enzyme activity assays.

## ***Plasma Sodium Concentrations***

During smoltification, salmonids alter their ability to regulate salt ions in their tissues and their blood. Consequently, measuring plasma sodium concentrations can be used as an index of their smoltification status. It is especially effective at determining an individual's ability to remove excess salts from their body during a saltwater challenge. I used a flame atomic absorption spectrometer (AAS, Thermo Scientific iCE 3000 AA) to measure plasma sodium concentrations in all of the sampled juvenile Chinook salmon from the field survey and my hatchery study. Briefly, AAS measures the total amount of light absorption by specific atoms in a gaseous sample.

During fish sampling, blood was extracted immediately after euthanasia. I excised the caudal fin and caudal peduncle immediately posterior to the adipose fin, exposing the caudal blood vessels. I then placed a heparinized micro-hematocrit capillary tube adjacent to the vessels until it filled with blood. I then transferred the blood from the tube to a 0.5 µl microcentrifuge tube and the sample was spun in a centrifuge at 5000 g for 3.5 minutes. The plasma was then decanted and stored in microcentrifuge tubes at -80 °C before being used in the assays. Samples were transferred to -80 °C within one hour for hatchery samples and were not stored for longer than two months. Field samples were transferred to a dry shipper of liquid nitrogen within ten minutes and stored for no longer than ten months at -80 °C.

All plasma samples were diluted with deionized water before the plasma sodium concentration assays. I diluted 3 µl of plasma in 10 ml of deionized water on the day of the assays. A standard curve was obtained each day of plasma concentration assays using standards of 0, 0.5, 0.75, 1.0, and 1.5 ppm. A raw concentration of the diluted sample was calculated from this standard curve. This raw concentration was multiplied by the dilution coefficient and divided by the atomic mass of sodium (Equation 4). The resulting number is the plasma sodium concentration in millimoles sodium per litre plasma (mmol).

**Equation 4:**

$$\frac{\text{(Raw concentration calculated from standard curve (mg/l)) (4001/1 dilution)}}{22.99 \text{ g/mol}}$$

***Statistical Analysis***

All statistics for both parts of my study were calculated using R statistical computing software version 3.0.1 (R Core Team 2012). Alpha levels were set at 0.05 for all analyses. All figures include the mean  $\pm$  standard error except for growth statistics, where standard deviations are used and mentioned in the figure captions.

To investigate the relationship between body size and smoltification in juveniles captured in the Pine River, I performed a linear regression of fork length on NKA enzyme activity and plasma sodium concentration.

**PART 2. HATCHERY STUDY**

To assess the time course of smoltification and its sensitivity to body size, I performed a hatchery study from October 2013 until September 2014 using juvenile Chinook salmon from the Sydenham River in Owen Sound, Ontario. The Sydenham River, like the Nottawasaga River, drains into Southern Georgian Bay and has supported Chinook salmon populations since the 1960s (Suk et al. 2012). The difference between the two rivers, however, is that the Chinook salmon population in the Sydenham River is supplemented by the release of hatchery raised smolts each year, while the Nottawasaga does not have a hatchery supplementation program. This river was chosen for gamete collection due to its large returning population size and the fact that there is already infrastructure in place to rapidly capture adults migrating up the river to the spawning grounds.

The fish were hatched from fertilized eggs and raised under natural photoperiod, constant temperature, and feeding regimes. I assessed their smoltification status over the spring and summer seasons by measuring body size, NKA enzyme activity, plasma sodium concentrations, body shape, and body colouration.

### ***Gamete Collection – Sydenham River, Fall 2013***

A total of 21 adults (10 males, 11 females) were captured and used for fertilizations over three days in the Sydenham River (October 11, 12, and 16 2013). I used two predominant methods to capture the fish in the Sydenham River: dip-netting and capture at the dam fishway. Dip-netting was done using 60 cm diameter nets (3 cm mesh) and two or three fishers working together. We targeted specific individuals on the spawning grounds or near the edge of the river. Once an individual was targeted, one fisher would stand downstream approximately three meters while another approached the fish from upstream. This approach would cause the individual to turn and swim downstream, hopefully into the path and net of the downstream fisher. This method proved to be successful and allowed us to be selective of the individuals we were targeting. The major drawback of this method is the fact that I was targeting individuals that were spawning below the Mill Dam. The fish that were in the best condition seemed to be those waiting in the deep pool below the dam, rather than those in spawning positions downstream of this pool. That meant that dip-netting generally resulted in the capture of poor condition adults. Poor condition in this case refers to males with deteriorating fins and underbellies, indicating they have been on the spawning ground for a long time. The amount of milt and motility of spermatozoa are known to decrease over time (Kazakov 1981, Büyükhatoğlu and Holtz 1984). For females, poor condition could indicate either a fish that has fully released all of her eggs, or one that has only a small portion of eggs remaining. Egg quality is also known to decrease over time (Craik and Harvey 1984).

To ensure that I was capturing high quality adults, I used two other methods that took advantage of the migratory behaviour of the fish and the fact that the dam focuses water flow in a small area of the river. By opening up a side water channel next to the dam, we created a heavy downstream flow, via an impassable waterfall, that attracted migrating fish into it. Leaving the channel open for approximately two hours in the morning would attract up to six fish into the channel. I then closed off the downstream portion of the channel and we used our dip-nets to capture individuals in the channel. Aside from a few individual females that were still not yet ripe, all of these fish were of high quality because they were still migrating upstream and had not spawned yet.

I also used the fishway on the Mill Dam to capture migrating fish. The fish ladder was built in 1967 to allow migratory fish to ascend the side of the dam to reach the spawning grounds upstream. This fish ladder has a large basket at the top that can be lowered into the water flow. Any individuals attempting to jump upstream end up in the basket and cannot escape due to the six inch overhanging lip. We then operated the basket lift to capture all individuals in the basket. Fish were anesthetized using MS-222 (150 mg/l river water).

Milt was collected from mature males by gently squeezing their stomachs and proceeding posteriorly to the vent. When they are mature, the milt will flow easily from their vent with little pressure. Approximately 10 ml of milt was collected into 118 ml Whirlpak sample bags and kept on ice until fertilization. Eggs were collected from mature females using a similar method, but usually with a little more pressure. Approximately 500 eggs were collected into a dry plastic Ziploc container and stored on ice. For all gamete collections, special care was taken to ensure the gametes were not exposed to river water. This can lead to a deactivation of the sperm in the milt as well as a closing of the micropyle and hardening of the vitelline membrane in eggs, rendering them incapable of fertilization. All gametes were transported to the University of Western University fish research hatchery within six hours for fertilization.

### ***Hatchery Setup***

I performed all of the fish fertilizations using procedures developed in our lab and following Ontario Ministry of Natural Resources disinfection guidelines (OMNR 2009). The gametes were kept on ice until moments before fertilization. Approximately 2 ml of milt was added to a petri dish of 40 eggs from each female and the petri dish was swirled for about 30 to ensure milt contacted all eggs. The eggs were then transferred into egg cups and rinsed with 5 ml of dechlorinated water at 13°C. This is to remove any excess milt. They were then transferred into plastic containers with a solution of iodophore and dechlorinated water (5 mg/l of Ovadine) to stand for between 30–60 minutes. After this time, they were then transferred to another container of dechlorinated water to further harden for 60 minutes. All eggs were kept in small egg cups made of 2” ABS pipe with

mesh bottoms and drilled holes to allow water flow around the eggs. The egg cups were then transferred to standard Heath tray stacks for incubation and covered to create a dark environment (Figure 6).

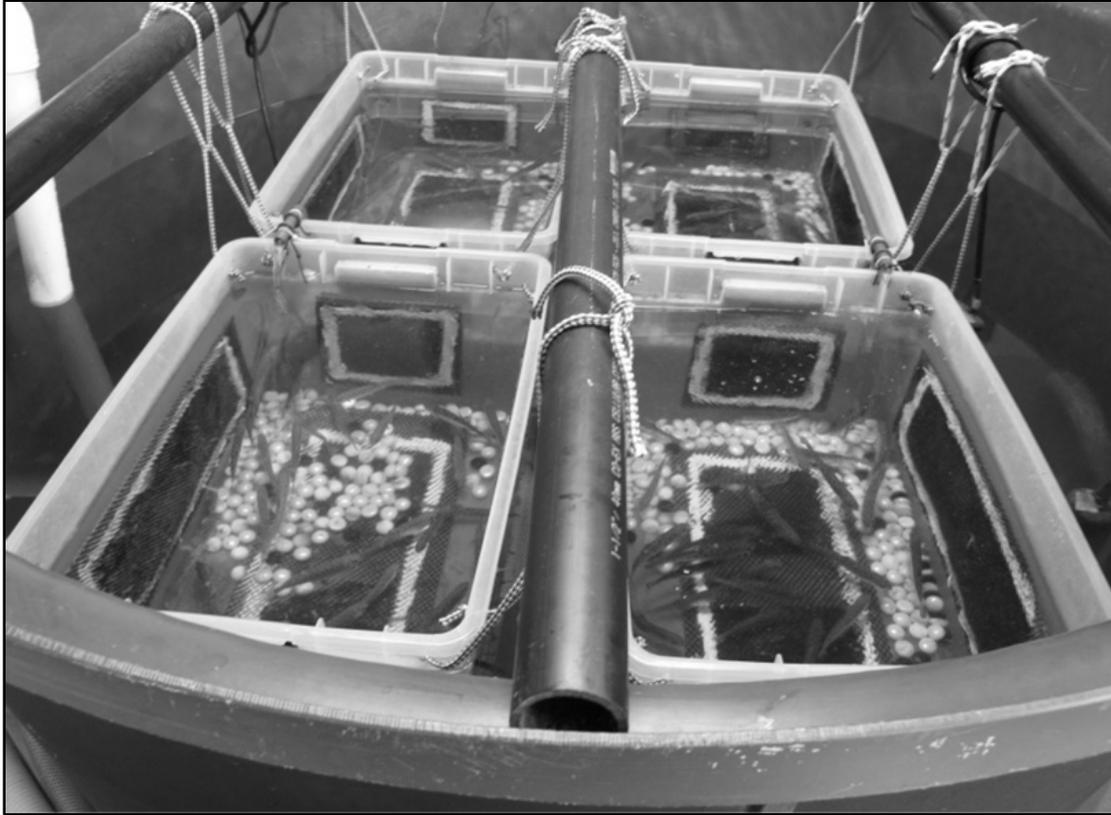
The research hatchery contains three independent, closed-circulation systems fed from the same water source. Water from the City of London water supply is de-chlorinated using an activated charcoal filtration system. The water is then cooled to the desired temperature of each system before being added to the tanks. Each independent system contains two ultraviolet light filters for the destruction of microorganisms and a mechanical filter with foam filters to remove any large particulate matter such as excess fish feed. Each system also contains a biofilter containing bacteria that are able to metabolize excess ammonia to nitrite and then to nitrate. Nitrate is harmless to fish in the environment. The dissolved oxygen levels were maintained at high enough levels to minimize environmental stress ( $>7$  mg/l, Davis 1975). Temperature and dissolved oxygen were both recorded by sensors inside the tanks and total ammonia levels were measured using an ammonia reader at least twice weekly. Anytime ammonia levels exceeded 0.1 mg/l, biofilter media was added to the biofilter and levels were monitored daily until remediation. Remediation usually took place in 1-2 days.

Eggs were kept in the incubation trays and were only disturbed approximately twice a week to remove unfertilized eggs or eggs that did not survive for unknown reasons. Upon hatching (between November 19–27), alevins were kept in the Heath trays until their yolk sacs were mostly absorbed. Fry were then transferred to fry cups inside a larger tank of in the research hatchery. I began to feed the fry on December 16-18 when they were transferred to fry cups. They were fed ground commercial trout feed (Corey Nutrition Co.) and 6% of total fry cup biomass once a day. This was deemed to be enough food as evidenced by the amount of uneaten food accumulated on the bottom of the cups. Cleaning of the cups took place weekly to remove this excess food and tanks were cleaned on a daily basis. On February 9, fish were chosen for the study based on their size and transferred to larger containers to allow them to grow uninhibited at lower densities. The largest 720 fry were transferred from the fry cups to four large containers inside the larger fish tanks (180 fish per container, Figure 7). The fish were separated into

two size categories to ensure a range of body sizes in my sampling group. Two containers had large fish (>42 mm) and two containers had medium fish (>35 and <40 mm FL). They remained in these containers until the end of the study.



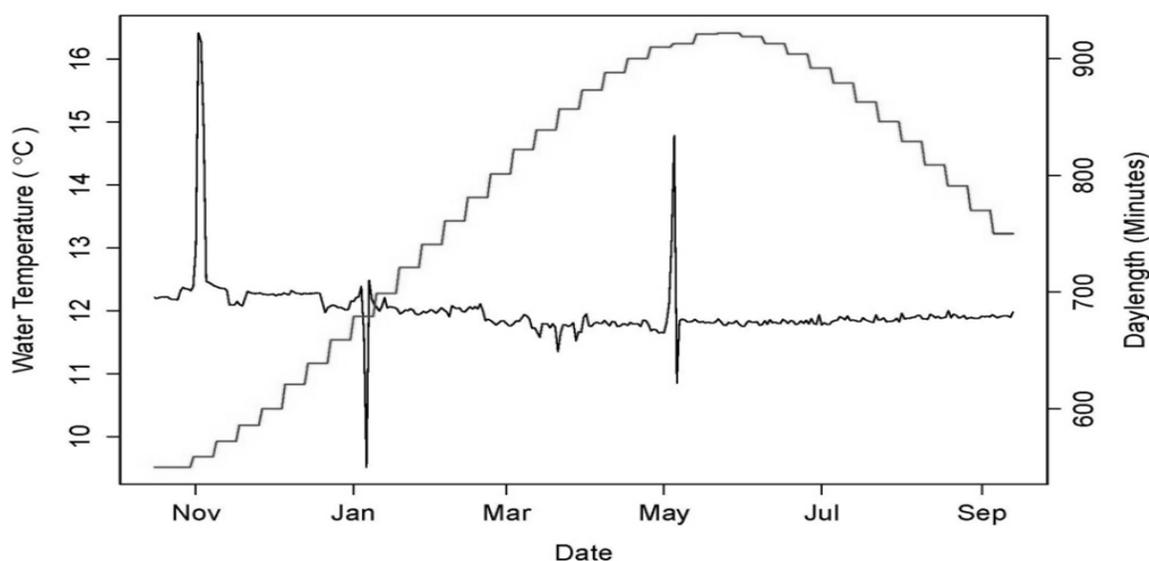
**Figure 6.** Photo of egg cups in Heath incubation trays with lids on them to prevent escape. Each cup contained 40 eggs and was uniquely labeled.



**Figure 7.** Photo of the four containers inside the large tank in the Western University fish research hatchery. Each container housed 180 juvenile Chinook salmon before lethal sampling began.

## Environmental Conditions

I continued to feed the fish 6% of container biomass daily throughout the remainder of the study. The temperature in the tank remained constant at 12 °C throughout the rest of the study (Figure 8). Day length was changed weekly to coincide with the ambient photoperiod in London, ON (42.9837° N, 81.2497° W). Beginning on December 19, the timer on the fluorescent lights in the hatchery was set every Monday morning to turn on at sunrise and turn off at sunset.



**Figure 8.** Plot showing water temperature and day length in the research hatchery throughout the study. The relatively constant lines represent the water temperature and the step-wise increasing parabola represents the day length. The three spikes of temperature were due to human error and lasted no longer than two days.

## ***Saltwater Challenge***

Hypoosmoregulatory ability in salt water is a key component of smoltification in juvenile salmonids. To measure the ability of individuals to eliminate excess salt from their tissues and respond to saline water conditions, I used a haphazard selection method and exposed five juveniles from each container ( $N = 20$ ) to a 24-hour saltwater challenge. This procedure is a simple one and has been performed numerous times in smoltification studies. I modified a protocol by Blackburne and Clarke (1987) for my study. I mixed 360 g of *Instant Ocean salt mix* (Aquarium Systems Inc., Mentor, OH) into a five gallon bucket with 15 litres of hatchery system water (24 ppt). I determined 24 ppt to be an appropriate concentration because it is high enough to detect differences in individual salt flux rates and low enough to minimize undue stress or mortality on the smaller fish that I used in my study (Flores et al. 2012; Shrimpton, personal communication). The buckets were hung inside one of the blue tanks with water temperature in the bucket and outside at 11°C. Each bucket contained four cups made of 4" PVC pipe and plastic screening to compartmentalize the fish from separate grouping containers (Figure 9). Each bucket was aerated using basic aquarium air pumps and small air diffusion stones, as per the animal use protocol (Appendix A). I monitored the fish in the saltwater challenge at hourly intervals for the first 6-8 hours and then every 4-8 hours after that. I removed and euthanized all fish that were found moribund or showed any other signs that they had lost equilibrium. After 24 hours, I removed all fish from the salt water, euthanized them and left them in beakers of freshwater until they could be sampled. They were all euthanized at the same time to ensure that all fish were exposed to salt water for an equal amount of time. All sampling was done within two hours of euthanasia. I performed the saltwater challenge on new fish every two weeks from March 26 to September 3.

Survival was very high in salt water (99.1%,  $n = 218$ ). The only deaths were two fish from the first sampling period (March 26) and these fish were from smallest size class at that date (<50 mm fork length). After this sampling date, no other fish died during the saltwater challenge. All fish from salt water and 20 fish from fresh water were euthanized in an MS-222 bath (300 mg/l in dechlorinated water). Gill filament samples and blood

plasma samples were taken. All assays were done using the same protocols as for the Pine River sampled fish.



**Figure 9.** Photo of apparatus for the 24-hour saltwater challenge. Each Bucket was immersed in 11 °C aerated water (24 ppt). Each of four cups had five fish from each container in the tanks.

## ***Sodium Potassium ATPase Enzyme Activity***

There were two sample groups from this experiment, fish sampled straight out of the freshwater holding tanks (fresh water) and fish sampled after a 24 hour saltwater challenge. These two groups are analyzed separately because there is expected to be a difference in branchial NKA enzyme activity of a fish stressed in salt water and one that has undergone minimal handling stress. The first analysis that I performed was a logistic regression to determine the relationship between fish size on NKA enzyme activity for each sampling group. I then performed an analysis of covariance (ANCOVA) to test for differences in NKA enzyme activity among the four sampling periods when controlling for body size. This also allowed me to test for an interaction between fork length and sampling period in predicting NKA enzyme activity.

I used the timing of NKA enzyme activity in freshwater fish along with the plasma sodium concentrations to divide the sampling dates into four sampling groups. The peak enzyme activity occurred over two sampling dates, June 6 and July 1. There was also a rise in NKA enzyme activity and plasma sodium concentration between May 6 and May 20. I labeled these sampling periods p3 and p2, respectively. I then labeled the pre-smolt period before May 6, p1 and the period after July 1 when the NKA enzyme activity decreased, p4. I used these sampling period groupings throughout the analysis of smoltification timing in my study.

To examine more closely the timing of smoltification as it related to seasonal progression and body size in juvenile Chinook salmon, I examined individual variation at the four different sampling periods over the study. I performed a linear regression analysis to test the effect of body size on NKA enzyme activity for each sampling period.

## ***Body Shape Analysis***

Morphological changes during smoltification are well documented and can be used as an indicator of smolt status. I analyzed the body shape of each individual sampled throughout the experiment to determine if smoltification in my study was accompanied by a general body elongation as seen in other studies (Beeman et al. 1994, Beckman et al.

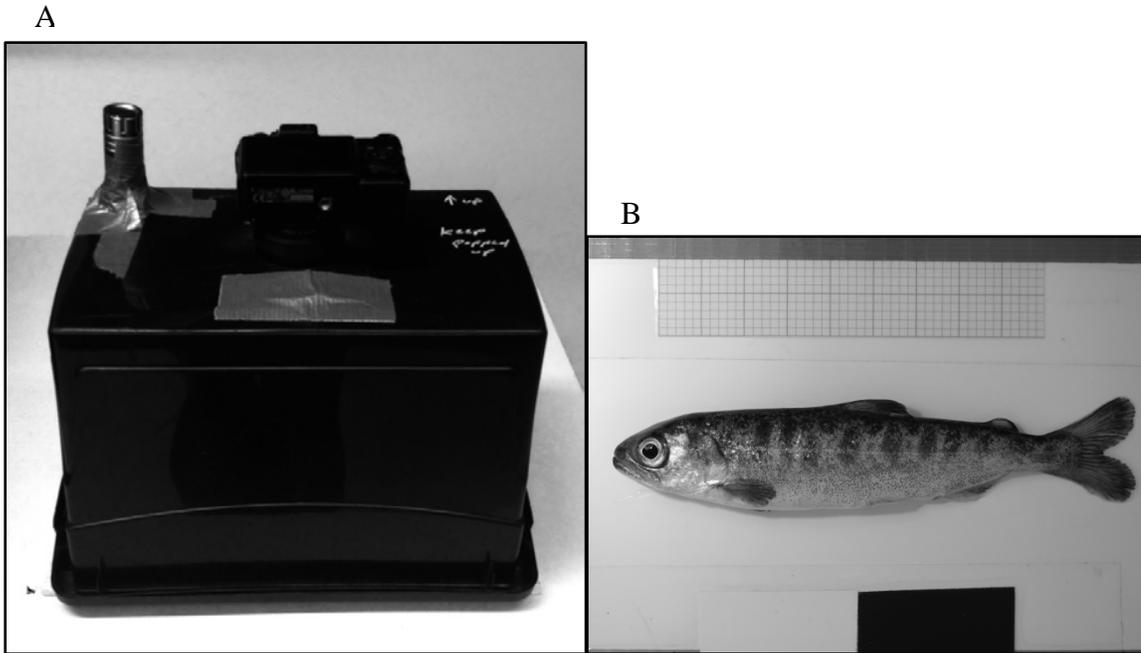
1999). Body shape analysis was performed using thin-plate spline relative warp (TPSW) analysis software from State University of New York Stony Brook (Rohlf 1994). This analysis is similar to a principal component analysis (PCA) and uses the relative distances of each landmark from each other to calculate a body shape.

I captured photos of each sampled fish in this study. I placed each sampled fish inside of a light box in the same orientation to take a photograph for the body shape and body colour analysis. The light box was made of black plastic and had an opening in the top for the camera lens to attach and reach inside the box (Figure 10). An LED light was inserted into the top left of the box which corresponded to the antero-dorsal portion of each photographed fish. This was to standardize all photos that were taken, independent of ambient conditions. This is important because sampling time varied throughout the season and in the field, the amount of sunlight can vary drastically throughout the day and affect the colouration of fish. All photos were taken using a Canon Powershot G10 camera and settings remained the same throughout the sampling period (i.e., no flash, macro setting, automatic focus, 20 cm focus, automatic ISO). A two second timer was used to ensure no movement of the camera occurred during the photography. All fish were positioned in the centre of the frame to ensure that no reflectance from the LED light was seen in the portion of the body posterior to the dorsal fin where colour measurements would take place.

I used a suite of 14 landmarks to model an individual's body morphology (Figure 11). This was adapted from a protocol by (Beaman et al. 1994). To standardize measurements for the difference in size between individual fish, a reference configuration is calculated by the program using a generalized least squares method. All landmarks across individuals are then scaled to the calculated centroid of the reference configuration. The landmarks were then included in the relative warp model to determine which principle warp component (PC) axis explains most of the variation in body shape. A second PC axis is then fitted orthogonal to the first, followed by the fitting of a third component orthogonal to the second, etc. The number of principle warp components that I deemed relevant to the study were chosen by comparing their eigenvalues in a scree plot. I accepted the components for further analysis that were greater than one and that were in

the part of the plot before the slope changed drastically between eigenvalues (Tabachnick and Fidell 2012).

I next performed linear regression analysis for the correlation of body size and NKA activity with body shape principle warp component scores to determine if there is a relationship between body shape and other indices of smoltification. I also performed a one way ANCOVA using sampling period as a factor and fork length as a covariate to determine if there is a significant difference in body shape between sampling periods, independent of body size.



**Figure 10.** A) Photo box used for all photos of sampled fish to ensure that the only light in the box was from the LED light in the top left corner. The camera is inserted in the top middle. B) Photo of a juvenile Chinook salmon from the hatchery study taken inside the photobox.



**Figure 11.** Landmarks used for thin plate spline warp analysis of body shape on juvenile Chinook salmon. 14 landmarks and 601 fish were used in the analysis.

## ***Body Colour Analysis***

In order to assess body colour changes during smoltification, I performed a skin colour analysis using the same standardized photographs as for the body shape analysis. There are many ways that researchers have used to assay body reflectance or silvering. These range from a simple subjective classification system (Whitman 1987, Negus 2003), to the design and implementation of a light reflectance meter (Duston 1995), to biochemical analysis of purines in the fish skin (Staley and Ewing 1992). I chose to compare the relative dark colour in the parr marks to the light colour between the parr marks in a photo set to grey scale. This method allowed me to capture the decrease in dark pigmentation of the parr marks as well as the increase in guanine in the skin that causes silvering. Fish with a more silvered skin and reduced parr marks will show a lower differential darkness value than fish that remain darkened with parr marks.

The data for the colour analysis was extracted from the photos using the image analysis software, ImageJ version 1.49d (Rasband 2012). Before every analysis, each photo was converted to 16-bit grey scale to further standardize photos. For every fish, rectangular areas were identified inside parr marks and between marks (Figure 12). The average optical density of the pixels in each of these rectangles was calculated by ImageJ using a black/white intensity scale and saved in a spreadsheet. Pure black is given an intensity score of 0 and pure white is given a score of 255. I then calculated a relative intensity differential between the skin between the parr marks and the skin with the parr mark. No fish in my study had lost their parr marks completely and therefore all fish were measured in the same manner.

Using these average pixel intensity measures, I combined the first two measures (areas 1-2 in Figure 12, the space between the first two full parr marks posterior to the leading edge of the dorsal fin, directly above and below the lateral line) and the next two measures (areas 3-4 in Figure 12, the second full parr mark past the dorsal fin, above and below the lateral line). I then calculated their difference. The higher of these relative intensity scores indicates a greater difference between parr marks and less skin silvering.

To test for the correlation between fish body size and skin colouration, I performed a linear regression analysis of the relative intensity scores for each individual on their fork length. To test for the difference in body colouration over the entire study, I first performed a one way ANCOVA using the four sampling periods and fork length as a covariate. If there was a significant interaction between fork length and sampling period, I performed a post hoc pairwise test for interactions to determine which slopes differed from each other. As smoltification occurs, body silvering is expected to increase and this should be indicated by decreasing colour differential scores.



**Figure 12.** Photo of juvenile Chinook salmon in gray-scale and showing the four rectangular areas selected for colour analysis. The two areas on the left are between par marks and the areas on the right are over a parr mark.

## ***Using All Measured Variables to Assess Smoltification Status***

As part of my study, I wanted to determine if the suite of measurements of smoltification that I used in this hatchery study were successful in capturing the progression of smoltification in juvenile Chinook salmon. To this end, I performed a principle component analysis (PCA) using the measured indices from my study (fork length, mass, condition factor, NKA enzyme activity, body morphology, and body colour). In general, a PCA combines multiple, correlated variables to extract the maximum amount of variance in a data set of individuals by combining them into components (Tabachnick and Fidell 2012). The components are combinations of the variables that maximize the variance in the scores that each individual receives for that component. The second and subsequent components are always orthogonal to the preceding components. This allows for individuals to be plotted in n-dimensional space along these principle component axes.

Using the R package, FactoMineR, I performed a PCA on all of the juvenile Chinook salmon from fresh water which I had data for each variable. In total, I used 178 individuals from the freshwater group and six variables (fork length, mass, condition factor, NKA activity, body shape (PC1 from the TPSW analysis), and body colour) in my PCA analysis. All of the variables were scaled to have a mean of 0 and a standard deviation of 1. This scaling was done because all of the variables used in the analyses were of different units of measurements. I chose the relevant principle components to use in my analysis using the same method as for the principal warp scores of body shape. The eigenvalues were greater than 1.0 and in a section of the scree plot before the slope drastically changes (Tabachnick and Fidell 2012). The individuals were then grouped according to their sampling period (p1, p2, p3, p4) to assess the differences between these four groups as smoltification progresses. I performed an ANOVA to determine if there were significant differences in PC1 scores between sampling periods. Tukey's post hoc pairwise comparison test was performed to find out which differed from each other.

## ***Plasma Sodium Concentration***

The plasma sodium assays were done in the same way as for the Pine River juveniles. For the first few sample dates when the fish were in the smallest size category, it was difficult to extract enough plasma in the saltwater fish to run the analysis. The sample sizes are therefore less than 20 for these sampling dates.

The concentration of plasma sodium is not expected to follow the same pattern as the other smoltification indices (i.e. the logistic regression models). Past studies have shown that concentration decrease during the peak smoltification periods, as the individual mechanisms of ion expulsion are highest. For this reason, plasma sodium was not used in the PCA of smoltification indices over the study. The assay is useful for assessing the timing of smoltification and specifically the smoltification window when saltwater tolerance is the highest.

## ***Statistical Analysis***

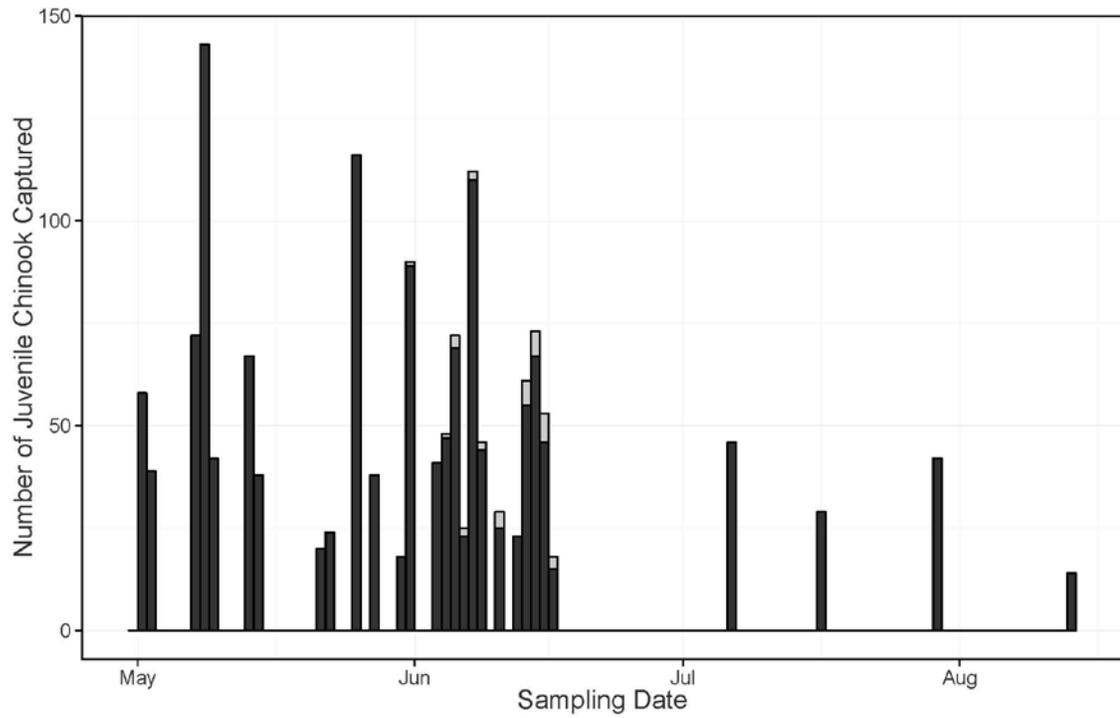
The two sampling groups, freshwater fish and saltwater challenged fish, were analyzed separately for sodium concentration, as they were for the gill NKA activity analysis. A saltwater challenge is expected to increase sodium levels in the fish tissues and blood. I first performed linear regression analyses to determine if body size predicted plasma sodium concentrations in both sampling groups. I then performed an ANCOVA for the effect of sampling period with fork length as a covariate. If there was a significant effect of either sampling date or sampling period, I performed Tukey's post hoc pairwise test to determine which dates differed significantly from each other. Any interactions that were significant, I performed a post hoc pairwise test for slope differences between sampling periods (R package "phia").

# RESULTS

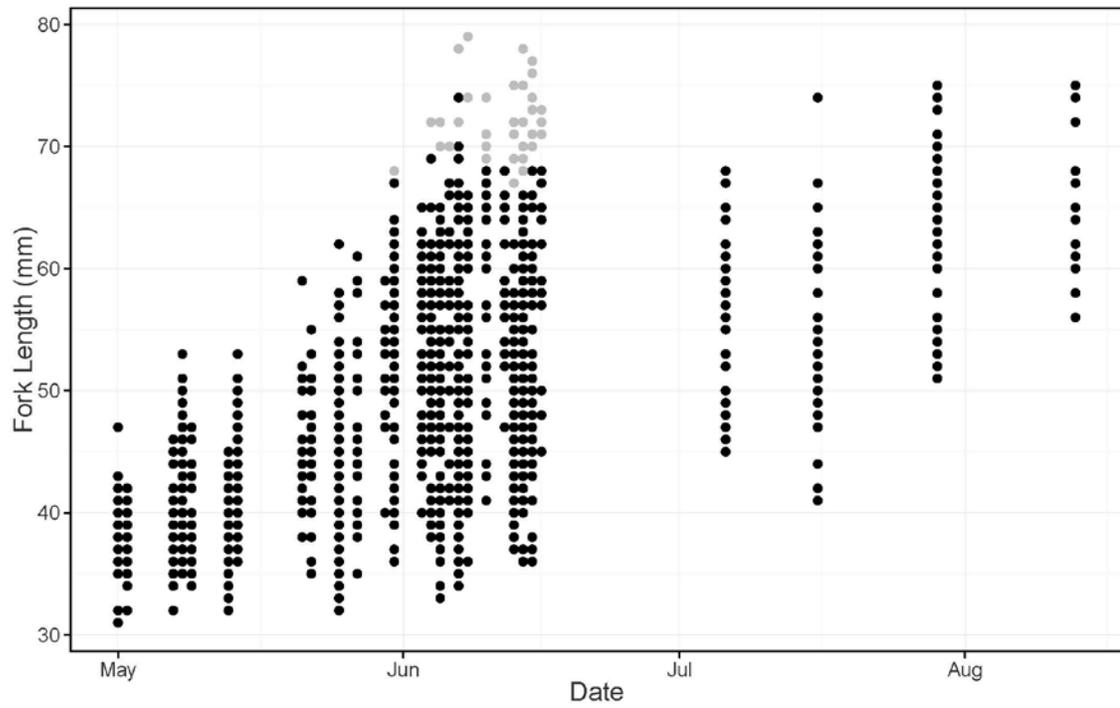
## PART 1. FIELD STUDY

Juvenile Chinook salmon were captured in the Pine River throughout the sampling period from May 1 to August 13 (N = 1429, Figure 13). All were categorized as subyearlings based on their fork length, except for one fish that was positively classified as a yearling due to its size (99 mm FL) and scale circuli patterning (a distinct annual check in circuli). This yearling fish was not used in any of the analyses. The average fork length increased over the sampling season (Figure 14) from  $37.5 \pm 2.7$  mm (sd) on the first day of sampling (May 1) to  $44 \pm 5.3$  mm on May 14, to  $53.8 \pm 7.0$  mm on June 3. On July 29, mean fork length was  $62.1 \pm 6.8$  mm.

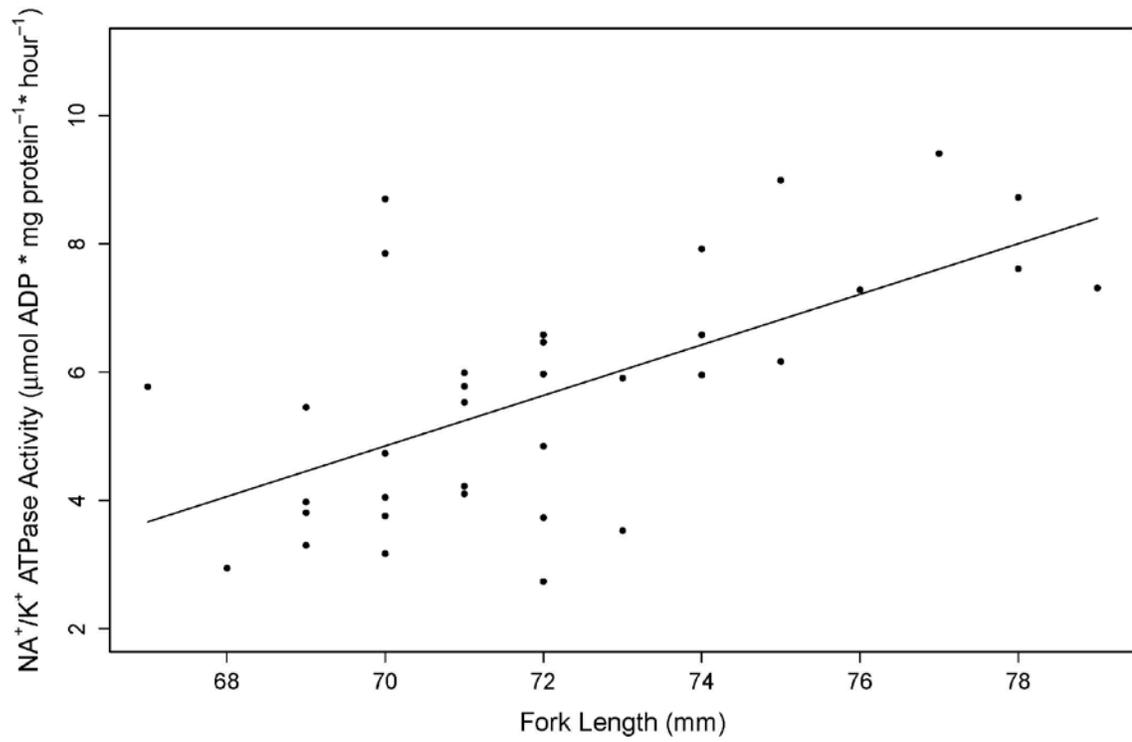
Overall, there is a trend towards larger fish late in the season, but most size classes were captured throughout the season. In total, 35 juvenile Chinook salmon captured between May 31 and June 16 were assayed for smoltification indices. These individuals ranged from 67 to 79 mm fork length (mean = 72 mm) and 3.32 to 5.30 g mass (mean = 3.97 g). Fork length was a significant predictor of NKA enzyme activity in a linear regression model ( $R^2 = 0.39$ ,  $p < 0.001$ , Figure 15). Plasma sodium concentration was not significantly predicted by FL in a linear regression model ( $R^2 = 0.01$ ,  $p = 0.58$ ).



**Figure 13.** Bar plot showing the number of juvenile Chinook salmon captured on each sampling date throughout the spring, 2013. Grey bars represent individuals lethally sampled for smoltification assays.



**Figure 14.** Fork Length for all individual Chinook salmon captured in the Pine River, spring 2013. Grey points indicate individuals that were lethally sampled for smoltification trait assays.

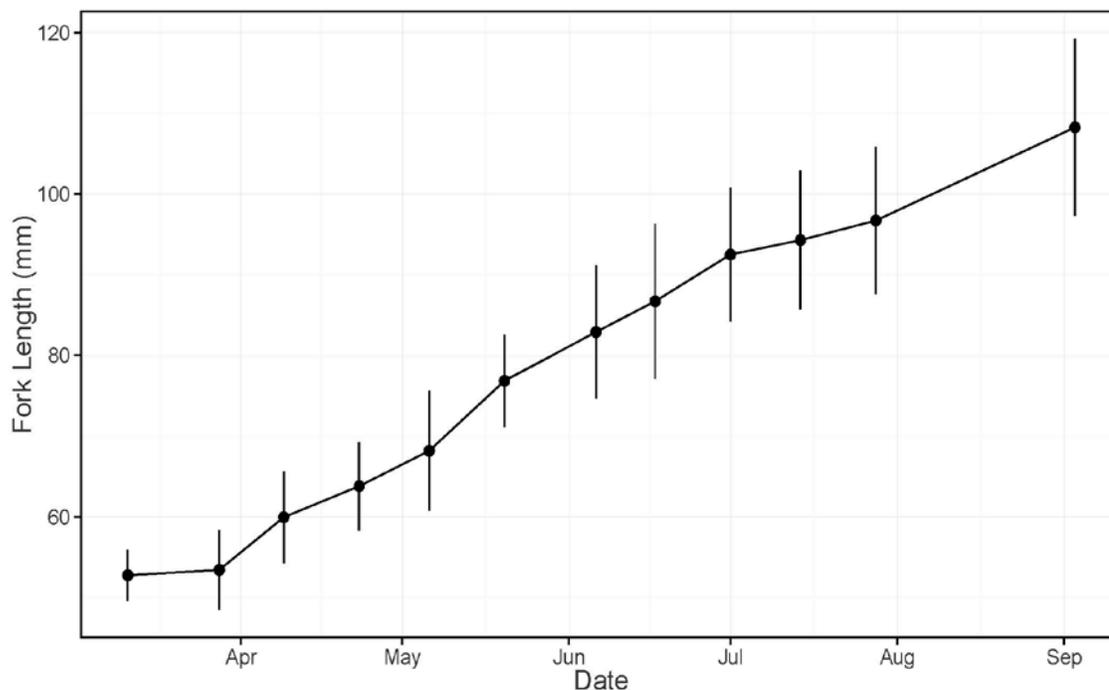


**Figure 15.** Plot of NKA enzyme activity by fork length in juvenile Chinook salmon captured in the Pine River, spring 2013. Regression line is included.  $R^2 = 0.39$ .

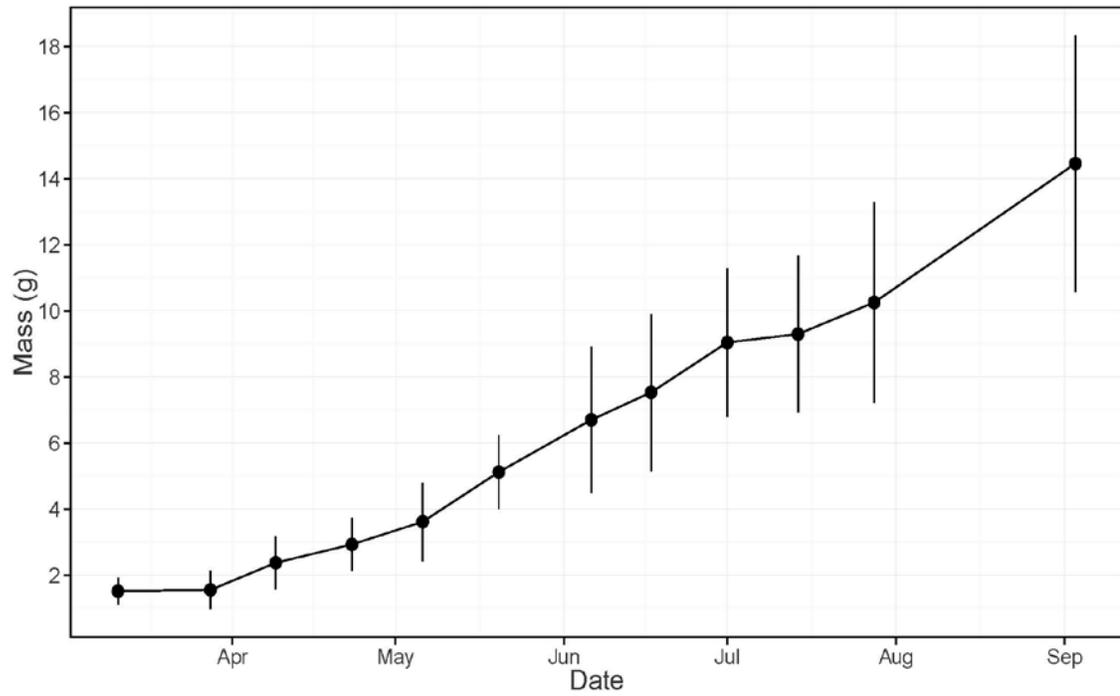
## **PART 2. HATCHERY STUDY**

### **Fish Growth**

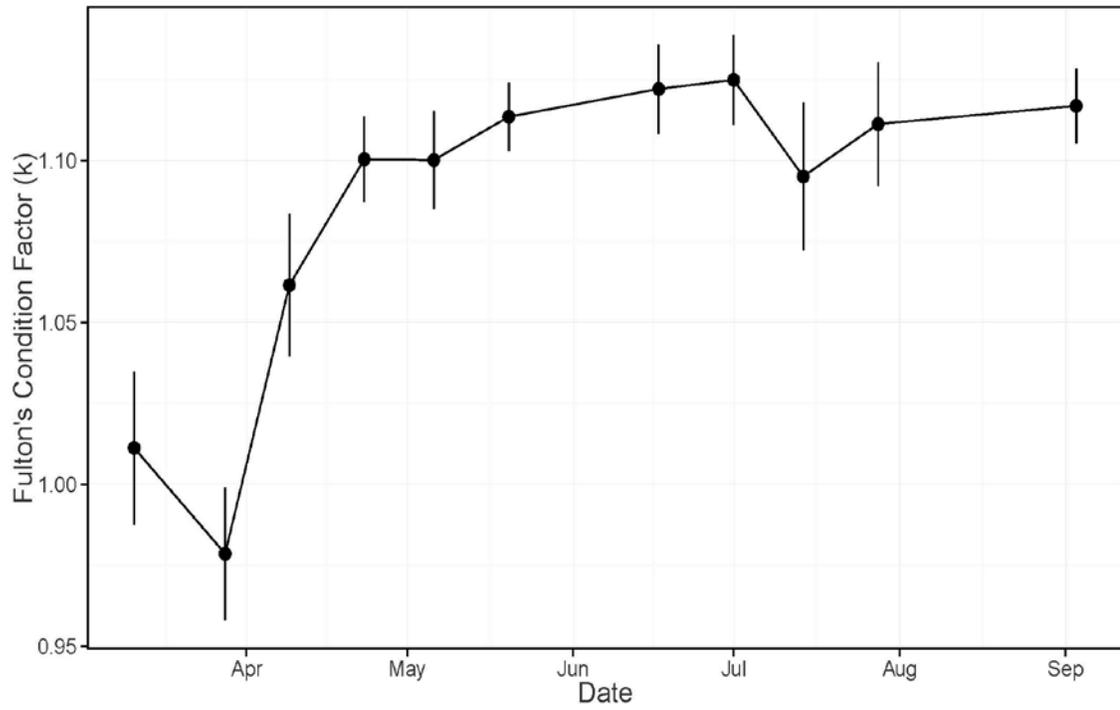
There was much variation in growth among fish in the experiment. All fish used in this study were from eggs fertilized on the same day (October 16, 2013) and they were all from groups that showed 50% hatching status between November 25 - 27 2013. The youngest fish used in the study were 106 days old and the oldest were 283 days old. Mean FL increased from  $39.5 \pm 2.6$  mm (sd) on January 22 to  $108.6 \pm 10.9$  mm on September 4 (Figure 16). Mass increased from  $0.600 \pm 0.153$  g on January 22 to  $14.460 \pm 3.878$  g (Figure 17). Fulton's condition factor (K) increased from January 22 ( $0.949 \pm 0.096$ ) to April 9 ( $1.060 \pm 0.137$ ). Thereafter, there was no significant change in condition factor throughout the experiment (Figure 18).



**Figure 16.** Fork length of sampled fish (mean  $\pm$  sd, n = 21-40) over the sampling period, March 11 – September 3 2014.



**Figure 17.** Mass of sampled fish (mean  $\pm$  sd, n = 21-40) over the sampling period, March 11 – September 3 2014.



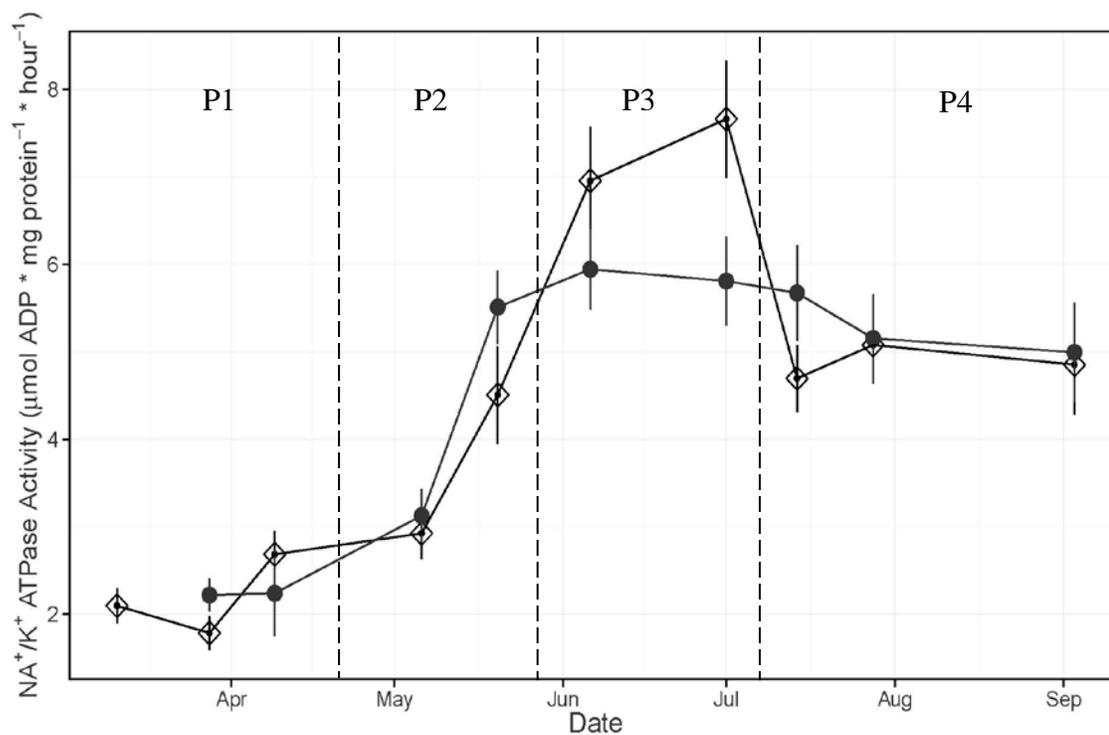
**Figure 18.** Fulton's condition factor (K) of sampled fish (mean  $\pm$  sd, n = 21-40) over the sampling period, March 11 – September 3 2014.

## ***Sodium Potassium ATPase Enzyme Activity***

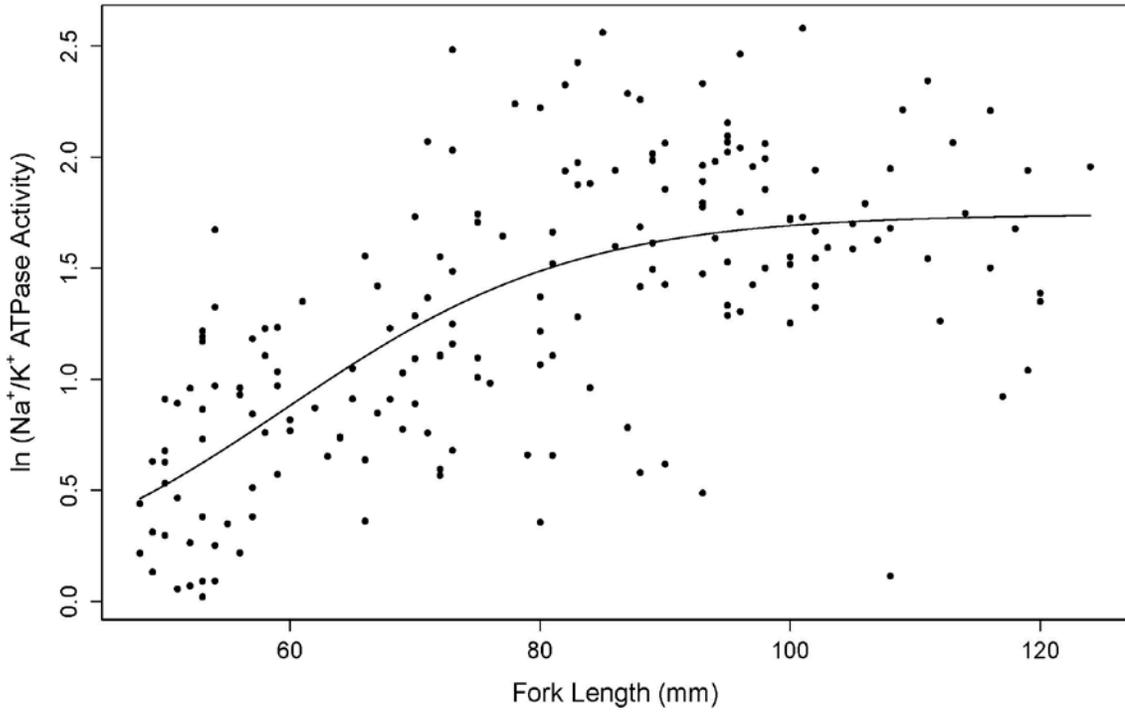
In both the freshwater fish and the fish subjected to the 24 hour saltwater challenge, there was a significant increase in NKA enzyme activity in the spring. In fresh water there was an increase in activity on May 20, followed by a peak reached on July 1, and a precipitous decline on the following sampling date, July 14 (Figure 19). For saltwater fish, there was again a rise on May 20 and subsequent peak. Unlike the freshwater fish, the NKA enzyme activity levels in saltwater fish remained relatively constant around the peak level throughout the rest of the study. The peak activity levels reached by the freshwater fish was significantly higher ( $7.66 \pm 0.67$  S.E.M.  $\mu\text{moles ADP/mg protein/hour}$ ) than the peak activity levels of the saltwater fish ( $5.94 \pm 0.46$  S.E.M.  $\mu\text{moles ADP/mg protein/hour}$ ), ( $t = 2.11$ ,  $p = 0.04$ ).

Body size was the best predictor of NKA enzyme activity levels across the sampling season. A logistic regression model (Figure 20 and Figure 21) was chosen as a better fitting model than a linear regression model using Aikake's Information Criteria test (AIC). This was true in both fresh water (Figure 20,  $\text{AIC}_{\text{logistic}} = 237$ ,  $\text{df} = 4$ ;  $\text{AIC}_{\text{linear}} = 259$ ,  $\text{df} = 3$ ) and in salt water (Figure 21,  $\text{AIC}_{\text{logistic}} = 206$ ,  $\text{df} = 4$ ;  $\text{AIC}_{\text{linear}} = 209$ ,  $\text{df} = 3$ ).

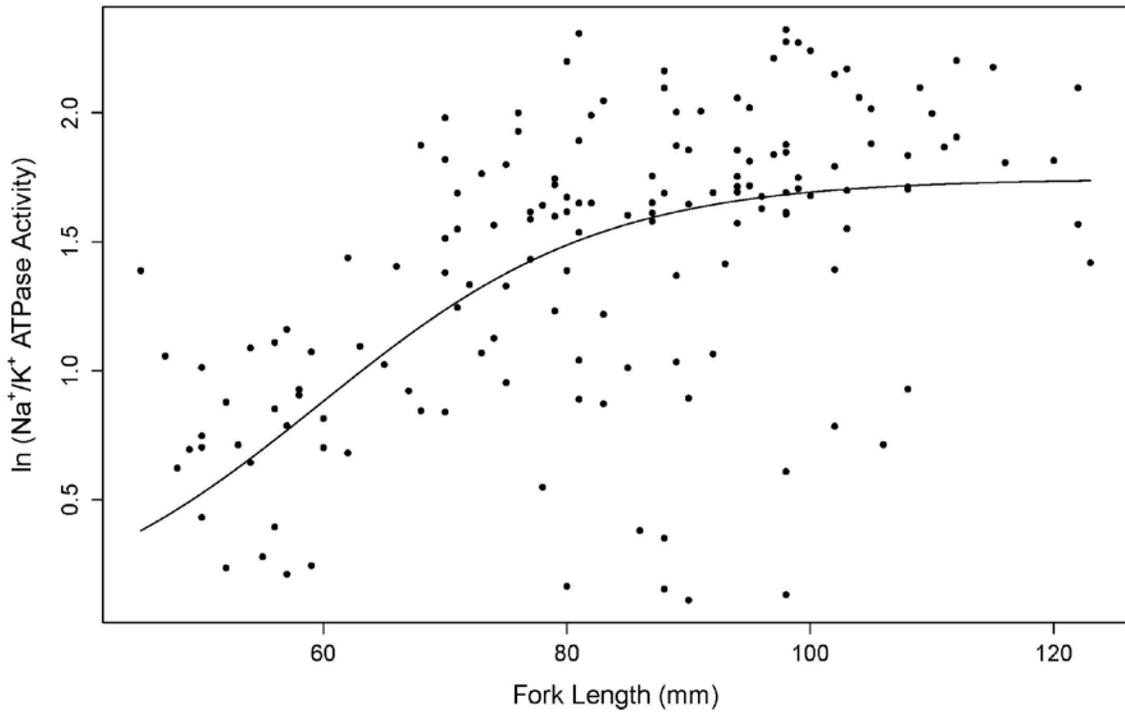
In both sample groups, there was a significant effect of sampling period on NKA enzyme activity when controlling for fork length in an ANCOVA model (Table 1 and Table 2). The interaction term was significant in freshwater fish, indicating that the effect of fork length differed among sampling periods. Individual linear regression analyses for each of the four sampling periods showed that during most time periods in both sampling groups, body size was significantly correlated with NKA enzyme activity (Table 3). In fresh water during the peak NKA enzyme activity period (p3), the slope of the regression line was not significant. In salt water during the first period (p1), the slope of the regression line was not significant. This indicates that body size was not a significant predictor of NKA enzyme activity during these periods. The pattern of increased NKA enzyme activity when controlling for body size can be further illustrated in Figure 22 and Figure 23. There is a peak during the smoltification window, p3, followed by a decline thereafter.



**Figure 19.** Branchial sodium potassium ATPase (NKA) enzyme activity of juvenile Chinook salmon raised in fresh water and sampled from March 11 to September 3 2014. Values are mean  $\pm$  SE. Open diamonds represent fish from fresh water ( $n = 16-20$ ) and close circles represent fish from the salt water treatment ( $n = 6-20$ ). The four sampling periods that were pooled together for analyses are identified and labeled.



**Figure 20.** Logistic regression plot of NKA enzyme activity (natural logarithm transformed) by fork length for juvenile Chinook salmon in fresh water.  $n = 187$ . Logistic regression curve included ( $\ln(\text{NKA activity}) = 1.74 e^{-(-5.18 + 0.087 * \text{Fork Length})}$ ). Asymptote = 1.74; Inflection point = 59.7 mm.



**Figure 21.** Logistic regression plot of NKA enzyme activity (natural logarithm transformed) by fork length for juvenile Chinook salmon after 24 hour saltwater challenge.  $n = 154$ . Logistic regression curve included ( $\ln(\text{NKA activity}) = 1.81 e^{-(3.5 + 0.061 * \text{Fork Length})}$ ). Asymptote = 1.81; Inflection point = 56.9 mm.

**Table 1.** ANCOVA table for the model testing the effect of sampling period (p1, p2, p3, p4) and fork length on NKA enzyme activity in juvenile Chinook salmon in fresh water in the hatchery study. (\* Denotes significant effects)

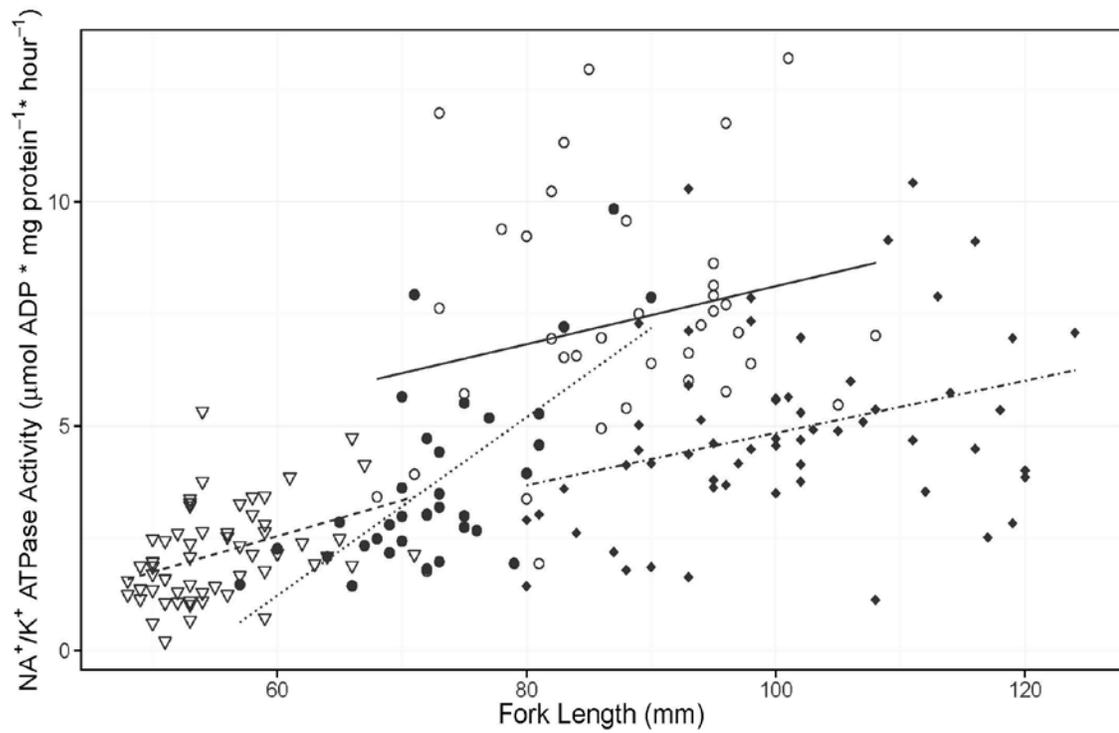
	df	sum sq	mean sq	F-value	P-value
Sampling Period	3	40.6	13.5	64.8	<0.0001 *
Fork Length	1	6.3	6.3	30.3	<0.0001 *
Sampling Period * Fork Length	3	2.4	0.81	3.9	<0.01 *
Residuals	181	37.7	0.21		

**Table 2.** ANCOVA table for the model testing the effect of sampling period (p1, p2, p3, p4) and fork length on NKA enzyme activity in juvenile Chinook salmon challenged in salt water in the hatchery study. (\* Denotes significant effects)

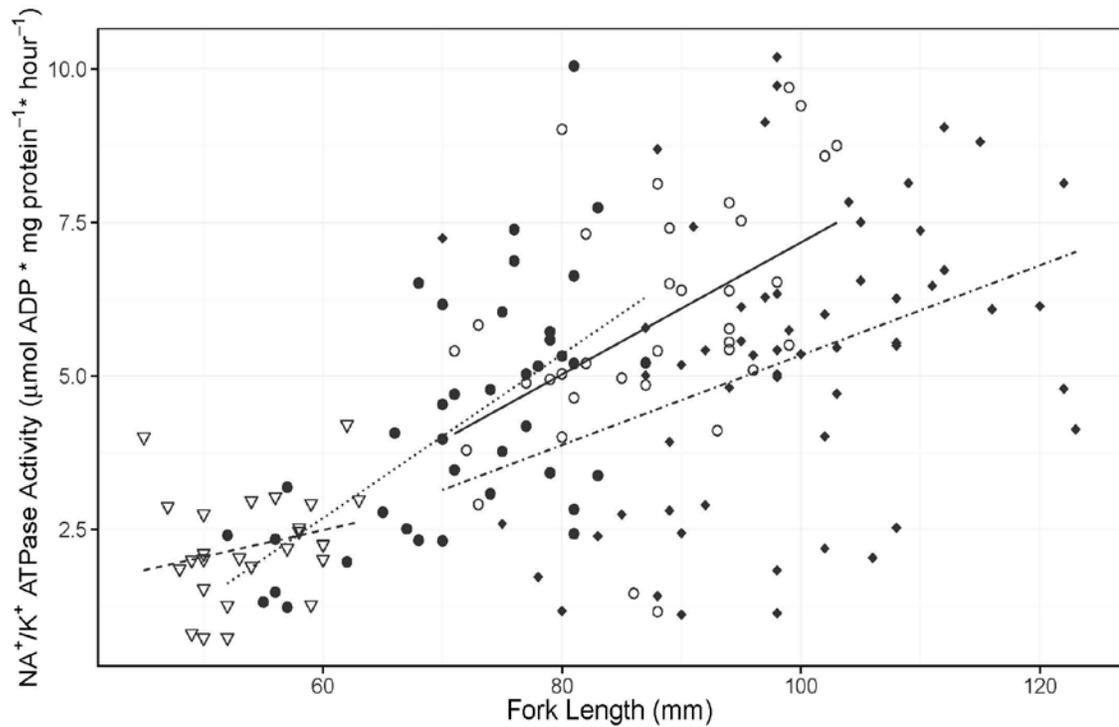
	df	sum sq	mean sq	F-value	P-value
Sampling Period	3	16.1	5.4	25.15	<0.0001 *
Fork Length	1	7.7	7.7	36.30	<0.0001 *
Sampling Period * Fork Length	3	0.84	0.28	1.32	0.272
Residuals	149	31.8	0.21		

**Table 3.** Results from regression analyses of NKA enzyme activity by fork length for juvenile Chinook salmon from both the saltwater and freshwater groups for each sampling date. (\* Denotes significant effects)

Sampling Period	Salt Water				Fresh Water			
	n	slope	R <sup>2</sup>	P	n	slope	R <sup>2</sup>	P
P1	25	0.04	0.06	0.236	59	0.08	0.16	0.001 *
P2	39	0.13	0.37	<0.001 *	34	0.20	0.46	<0.001 *
P3	35	0.11	0.23	0.004 *	37	0.06	0.05	0.168
P4	58	0.07	0.13	0.005 *	59	0.06	0.10	0.01 *



**Figure 22.** Plot of NKA enzyme activity by fork length for juvenile Chinook salmon in fresh water from the hatchery study. Open triangles represent fish from the p1 sampling period (n = 59). Closed circles represent fish from the p2 period (n = 34). Open circles represent fish from the p3 period (n = 37). Solid diamonds represent fish from the p4 sampling period (n = 59). Regression lines are included for each sampling period (p1 = dashed; p2 = dotted; p3 = solid; p4 = dot-dash).



**Figure 23.** Plot of NKA enzyme activity by fork length for juvenile Chinook salmon after 24 hour saltwater exposure in the hatchery study. Open triangles represent fish from the p1 sampling period (n = 25). Closed circles represent fish from the p2 period (n = 39). Open circles represent fish from the p3 period (n = 35). Solid diamonds represent fish from the p4 sampling period (n = 58). Regression lines are included for each sampling period (p1 = dashed; p2 = dotted; p3 = solid; p4 = dot-dash).

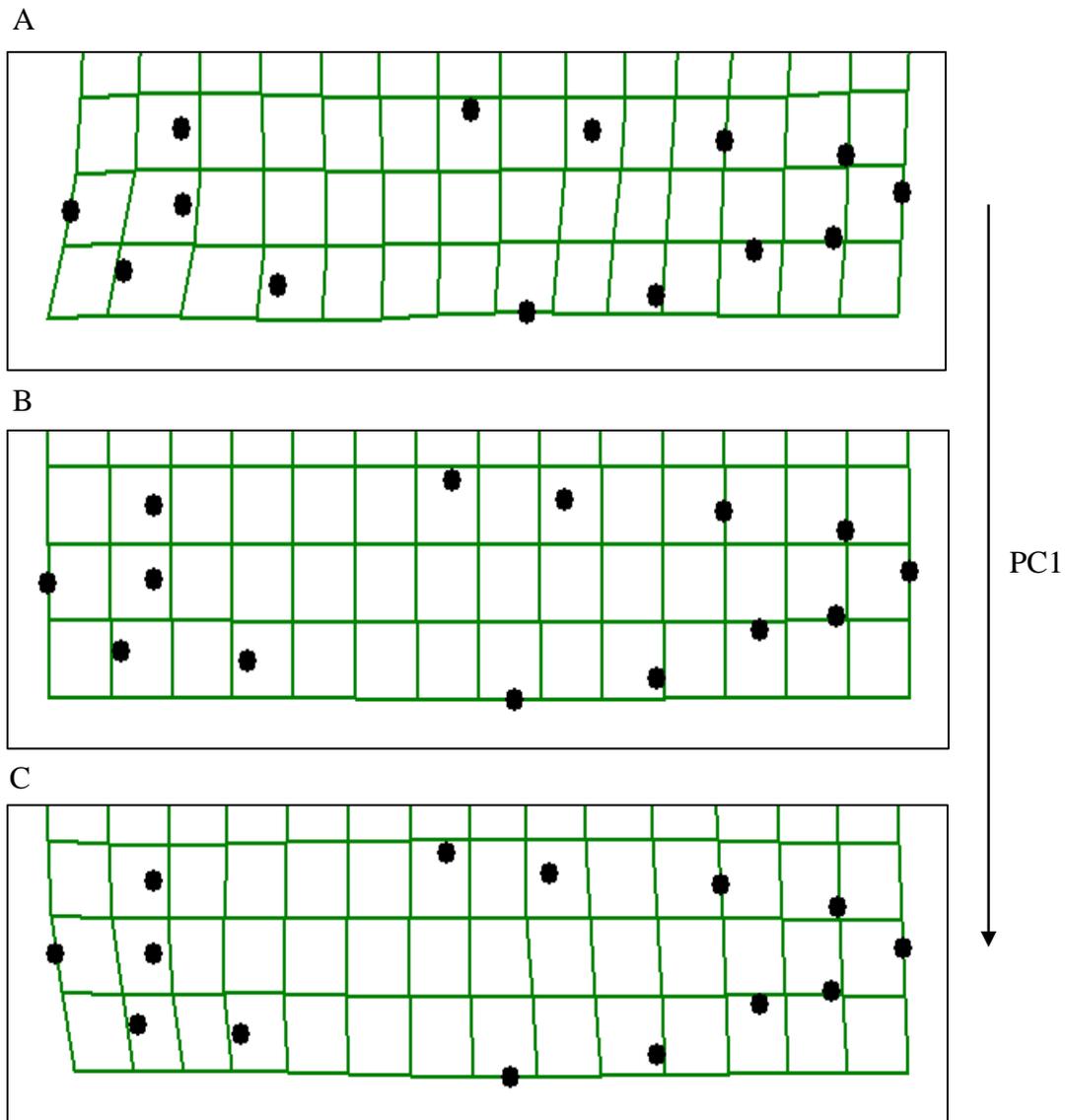
## **Body Shape Analysis**

In the thin-plate spline warp analysis (TPSW) of body shape, there were two principle warp components that had eigenvalues greater than one and therefore considered relevant for the study. These two principle warp components (PC1 and PC2) explained 25% and 19% of the variance in individual body shape, respectively. PC1 generally corresponds to a change in shape along the length of the body (Figure 24, positive PC1 is a shortened relative length and negative PC1 corresponds to an elongated caudal peduncle and body). This is seen in a warp from the consensus body shape in two regions of the body. First, in the head region, the mouth and anterior-most portion of the fish warps forward and anteriorly in the negative PC1 space and posteriorly in the positive PC1 space. Second, there is a warp in the body region underneath the dorsal fin and in the caudal peduncle. In the negative PC1 space, fish exhibit a warp from the reference configuration towards the caudal end of the fish. This means an elongation of the caudal peduncle. In the positive PC1 space, there is a warp in the opposite direction towards the anterior.

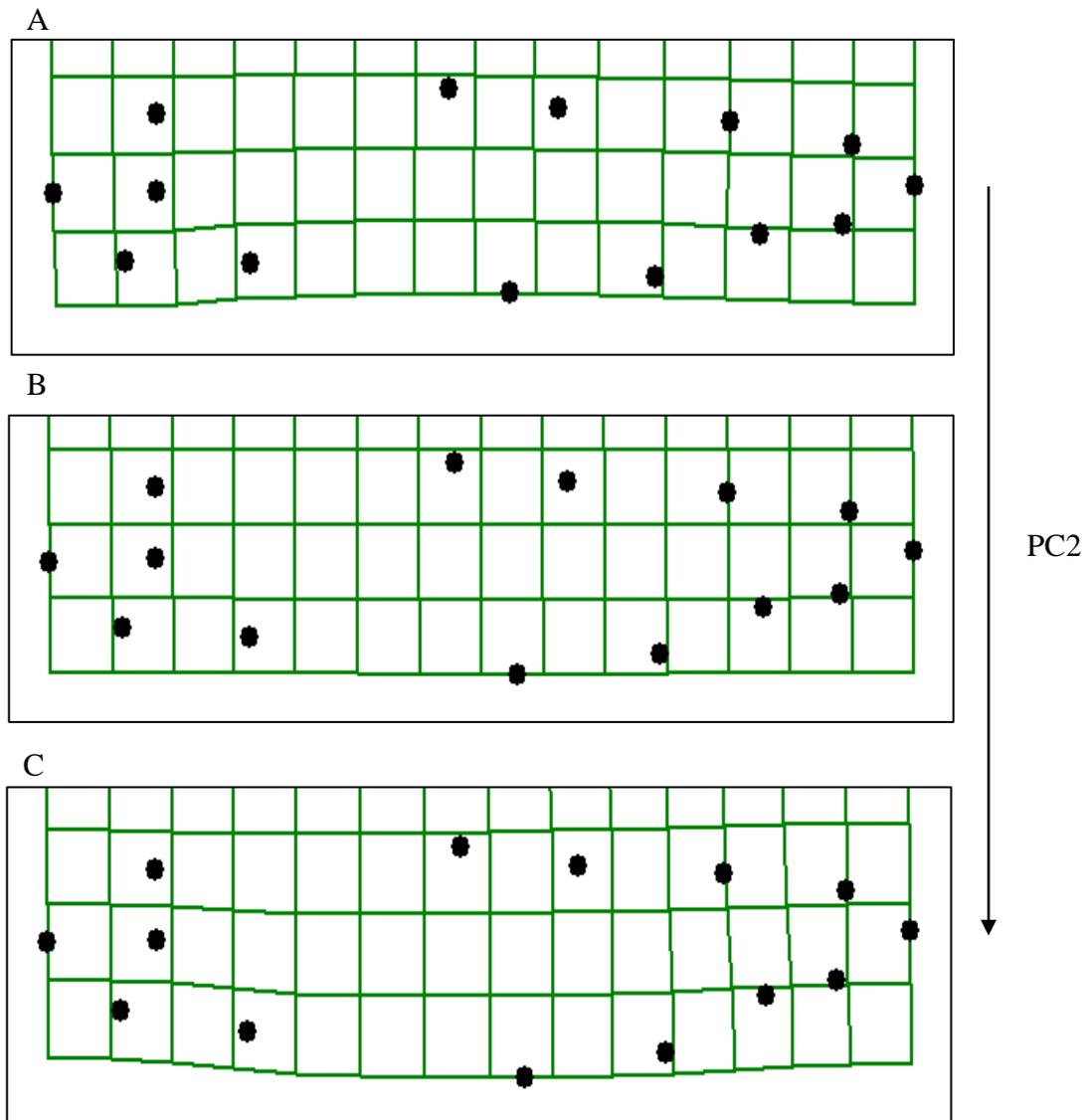
PC2 from the TPSW corresponds to a deepening of the body in the dorso-ventral axis (Figure 25, positive PC2 scores correspond to a deeper body). This is especially pronounced in the body region underneath the dorsal fin. In the positive PC2 space, the stomach of the fish is extended ventrally, giving a deeper body relative to the reference shape. In the negative space, the warp of the ventral side is in the dorsal direction.

PC1 and PC2 from the TPSW were significantly predicted by fish body size. I found that a logistic regression model was a better predictor than a linear regression model using AIC scores for both PC1 (Figure 26,  $AIC_{\text{logistic}} = -3655$ ,  $df = 4$ ;  $AIC_{\text{linear}} = 3610$ ,  $df = 3$ ) and PC2 (Figure 27,  $AIC_{\text{logistic}} = -3502$ ,  $df = 4$ ;  $AIC_{\text{linear}} = -3406$ ,  $df = 3$ ). Both logistic models were found to be significant using the Hosmer-Lemeshow test ( $\chi^2_{\text{PC1}} = -1871$ ,  $p = 1$ ;  $\chi^2_{\text{PC2}} = -197585$ ,  $p = 1$ ). The relationship between PC1 and fork length indicates that there is a relative elongation of the body independent to the actual body lengthening during growth. All of the body shapes are standardized to a calculated reference shape and centre.

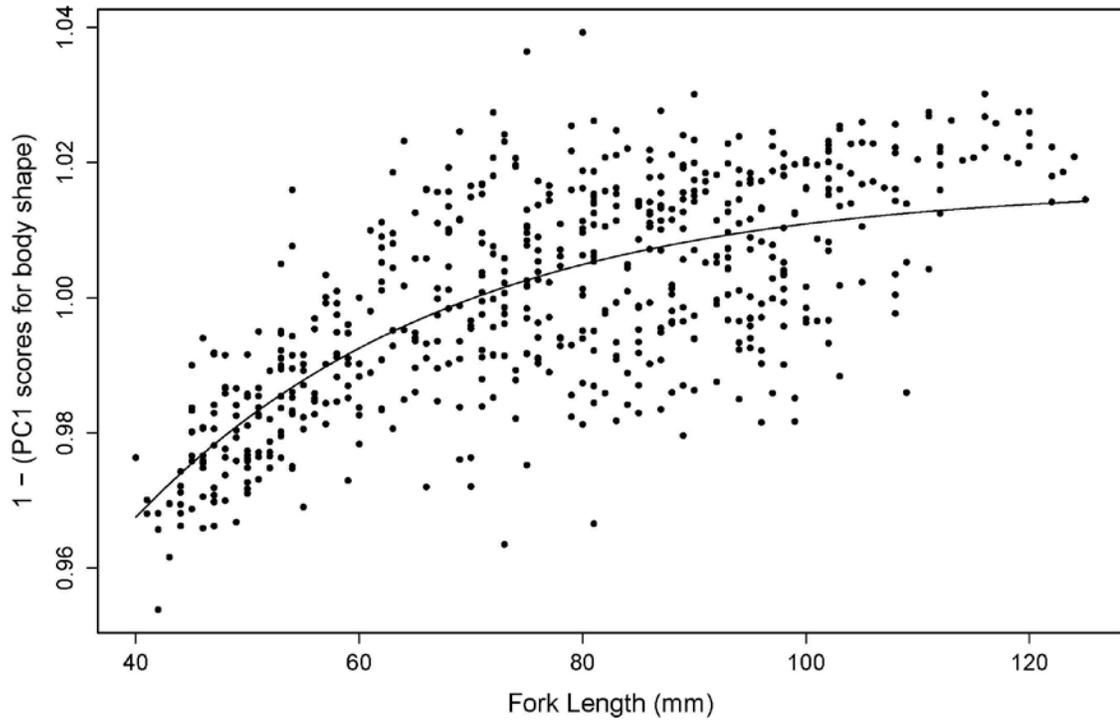
Sampling period was significant in explaining PC1 scores when controlled for body size in an ANCOVA model ( $F_{1,3} = 228$ ,  $P < 0.0001$ ,  $n = 518$ ). Over the entire study, there was a general decrease in PC1 scores, but during the two middle sampling periods there was a lot of variation between sampling dates (Figure 28). There was a significant increase on July 1 (corresponding to a less elongated body shape), which is also the sampling date where the peak of mean NKA enzyme activity occurs and the last sampling date in the p3 sampling period. There was also a significant difference in PC2 between sampling periods in an ANOVA model ( $F_{1,3} = 16.21$ ,  $P < 0.0001$ ,  $n = 518$ ). The seasonal progression for PC2 scores was non-linear (Figure 29). Generally, there was a large decrease in late April, a steady rise, and a decline in July 1.



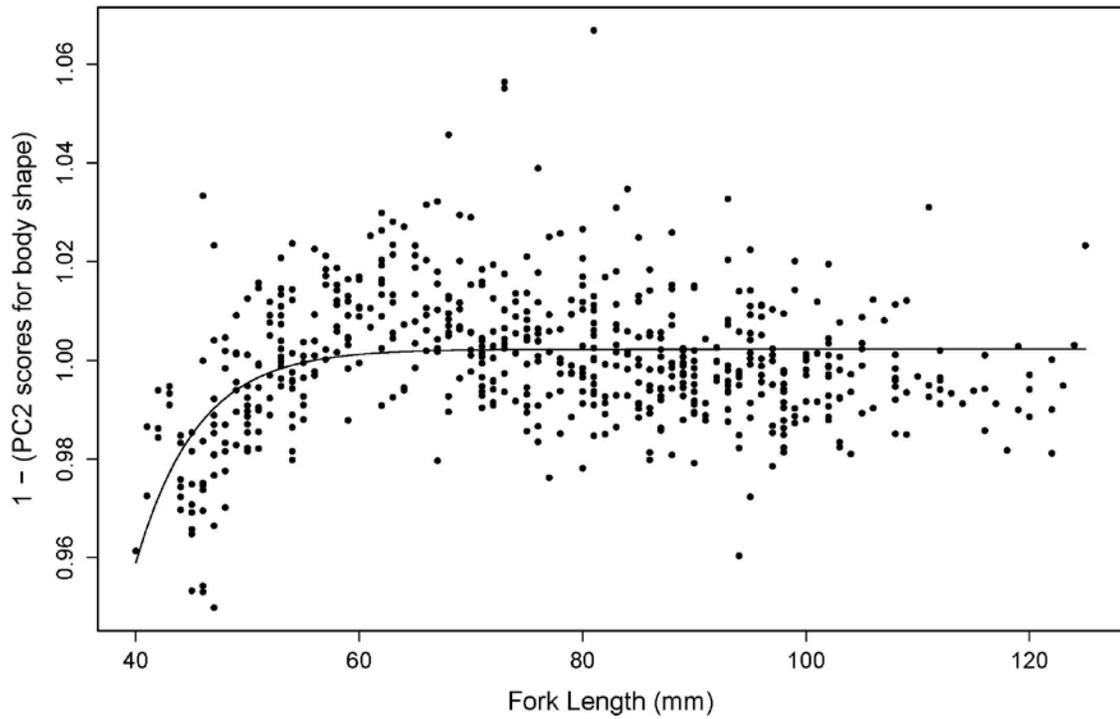
**Figure 24.** Relative warp diagrams showing the shape of a juvenile Chinook salmon along the PC1 axis. PC2 scores for all three are 0. Each black circle represents one landmark used in the TPSW analysis. Diagram B represents the reference body shape as the PC scores are 0 for both PC1 and PC2. Diagram A represents a fish with a PC1 score of -0.02. The face is elongated and the mid-body is warped anteriorly, leading to an elongation of the caudal end. Diagram C represents a fish with a PC1 score of +0.02. In this fish, the face is warped in the caudal direction and the mid-body is also warped caudally. This leads to a deeper bodied, shorter fish.  $n = 603$ .



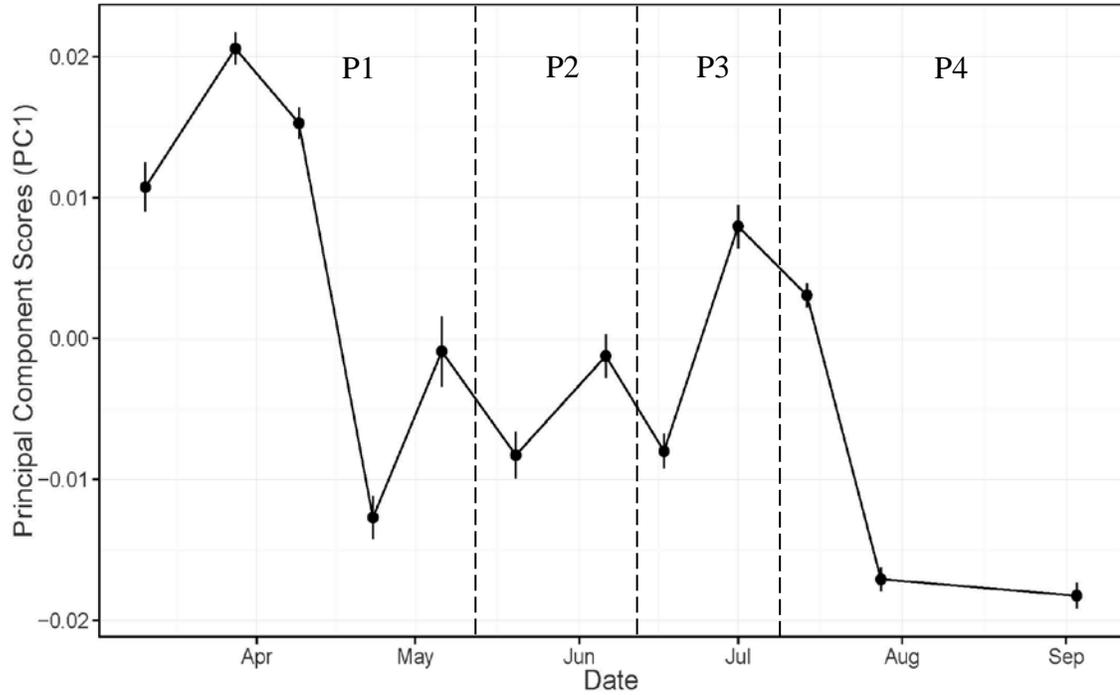
**Figure 25.** Relative warp diagrams showing the shape of a juvenile Chinook salmon along the PC2 axis. PC1 scores for all three are 0. Each black circle represents one landmark used in the TPSW analysis. Diagram B represents the reference body shape as the PC scores are 0 for both PC1 and PC2. Diagram A represents a fish with a PC2 score of -0.02. There is a general shortening of the body width (dorso-ventral axis) under the dorsal fin compared to the reference fish shape. Diagram C represents a fish with a PC2 score of +0.02. In this fish, the body is wider in the dorso-ventral axis under the dorsal fin leading to a fatter fish than the reference fish.  $n = 603$ .



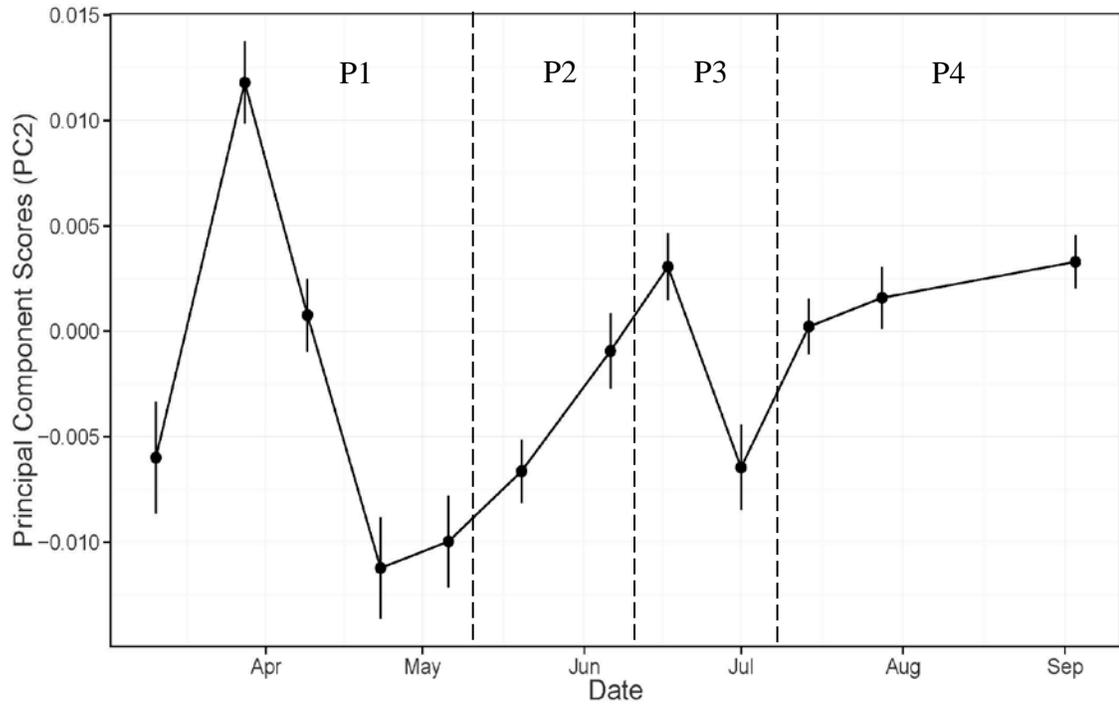
**Figure 26.** Logistic regression plot of PC1 scores from the TPSW analysis and fork length for juvenile Chinook salmon the hatchery study. Logistic regression curve included ( $1 - \text{PC1 scores} = 1.01 e^{-(1.5 + 0.037 * \text{Fork Length})}$ ). Asymptote = 1.01; Inflection point = -40.6 mm. Higher PC1 scores correspond to an elongated body relative to size. n = 591.



**Figure 27.** Logistic regression plot of PC2 scores from the thin-plate spline warp analysis and fork length for juvenile Chinook salmon the hatchery study. Logistic regression curve included ( $1 - \text{PC1 scores} = 1.00 e^{-(-4.4 + 0.018 * \text{Fork Length})}$ ). Asymptote = 1.01; Inflection point = 23.4 mm. Higher PC1 scores generally correspond to a thinner body size. n = 591.



**Figure 28.** Plot of principal component scores (PC1) from thin-plate spline warp analysis (mean  $\pm$  SE) for Chinook salmon in the hatchery study by sampling date. Negative PC1 scores correspond to a more elongated body shape along the antero-posterior axis than the reference body shape.

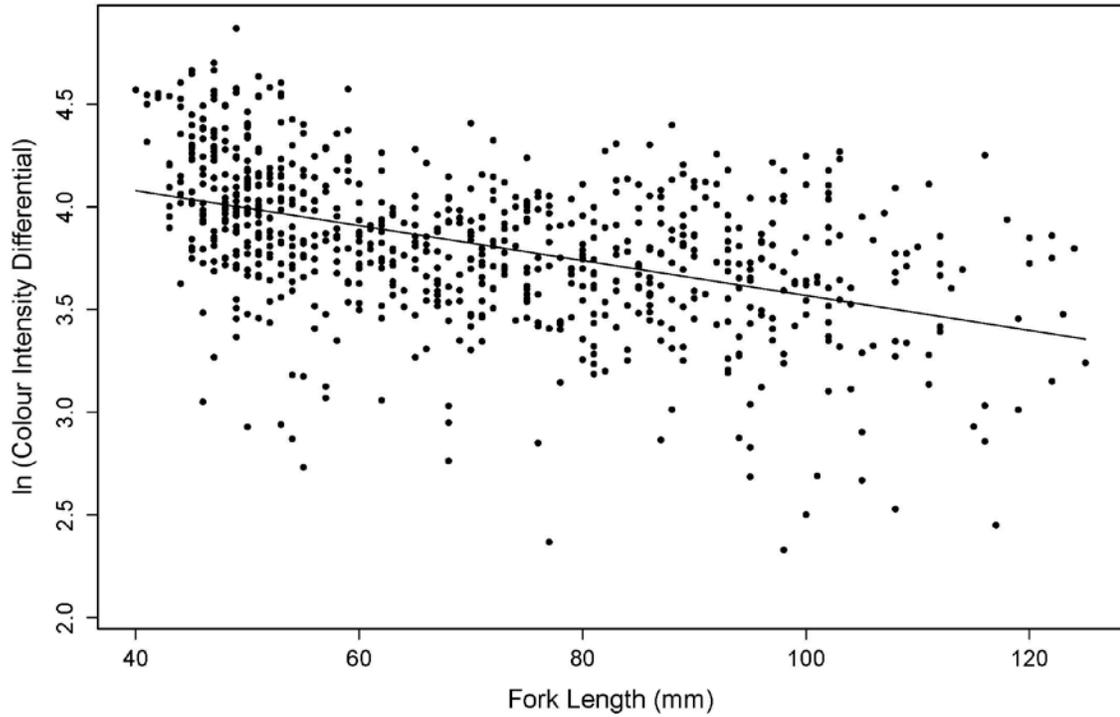


**Figure 29.** Plot of principal component scores (PC2) from thin-plate spline warp analysis (mean  $\pm$  SE) by sampling date for Chinook salmon in the hatchery study. Positive PC2 scores correspond to a wider body depth in the dorso-ventral axis.

## ***Body Colour Analysis***

Body colour showed a high degree of variation amongst individual Chinook salmon in my hatchery study. The mean intensity score of the space between parr marks was  $83.4 \pm 12.8$  (sd) and the mean score of the parr marks was  $58.3 \pm 14.5$ . Body colour differential scores in the ranged from the greatest light-dark differential of 130.3 to the lowest differential of -0.35 (mean =  $48.7 \pm 17.8$ ).

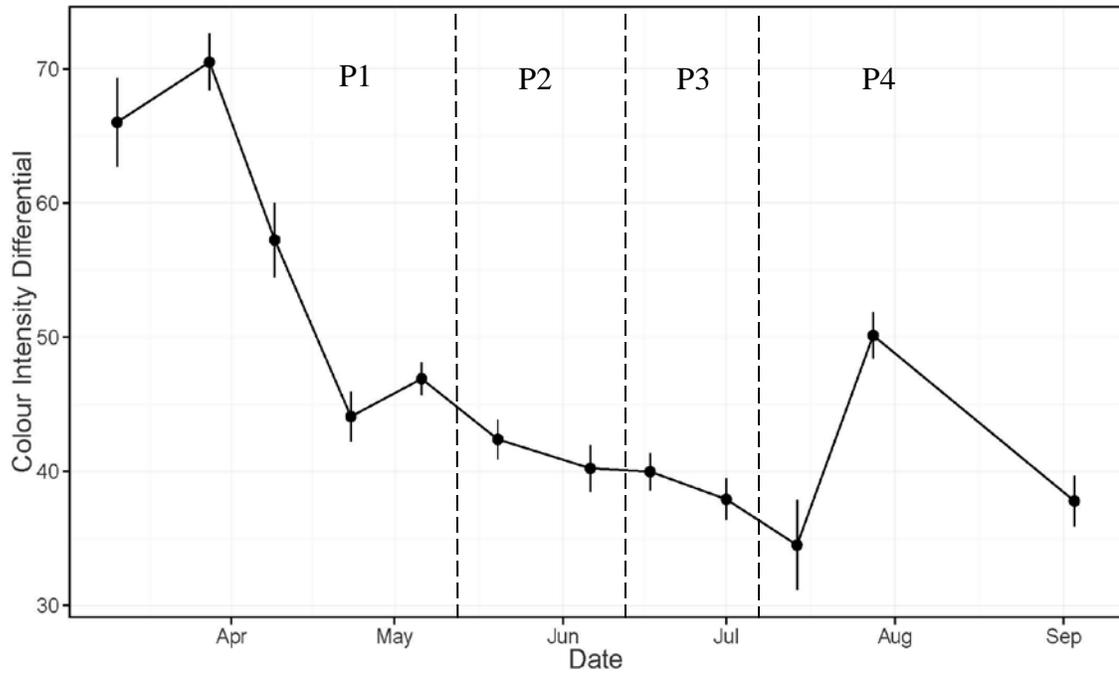
Body silvering of juvenile Chinook salmon was negatively correlated to fork length, meaning that larger fish were more silvered than smaller fish (Figure 30,  $r = -0.47$ ,  $P > 0.0001$ ). Sampling period, fork length and the interaction term all had a significant effect on body colour in an ANCOVA model (Table 4). A pairwise test for interaction differences showed that p1 and p4 differed significantly from the other sampling periods ( $p = 0.02$ ,  $>0.0001$ ) but the two middle periods, p2 and p3, did not have significantly different slopes ( $p = 0.79$ ). This is consistent with the pattern seen in Figure 31 where after p3, there is a large increase in dark colouration. While the seasonal pattern in body colour appears relatively linear, there is a sharp increase on July 28 that is inconsistent with the general pattern.



**Figure 30.** Plot of the natural logarithm of the colour intensity differential in juvenile Chinook salmon skin (light – dark) by fork length. Regression line is included ( $R^2 = 0.21$ ,  $n = 768$ ).

**Table 4.** ANCOVA table for the model testing the effect of sampling date and covariate fork length on colour intensity differential in juvenile Chinook salmon from the hatchery study. (\* Denotes significant effects)

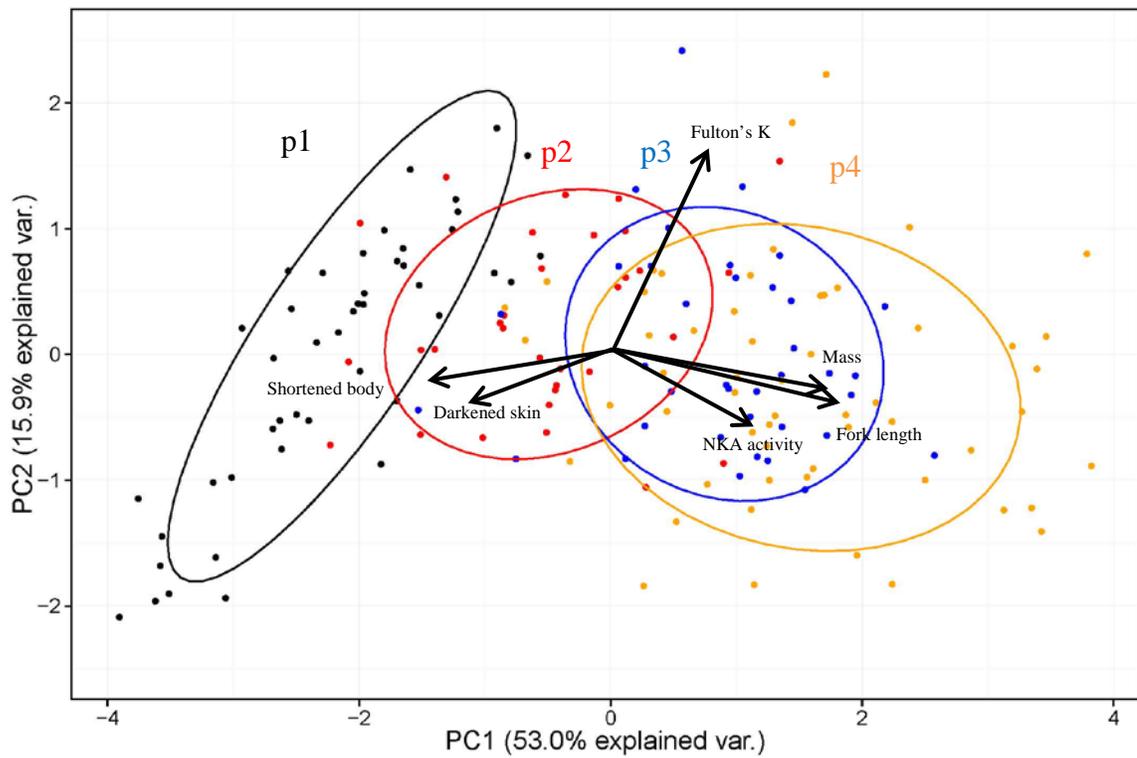
	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F-value</b>	<b>P-value</b>
Sampling Period	3	55212	18404	86.97	<0.0001 *
Fork Length	1	14956	14956	70.67	<0.0001 *
Sampling Period * Fork Length	3	12269	4090	19.33	<0.0001 *
Residuals	523	110674	212		



**Figure 31.** Plot of colour intensity differential scores of juvenile Chinook salmon in this study by sampling date (mean  $\pm$  se). There is a significant decrease over the season, indicating a silvering of body skin.  $n = 648$ .

### ***Using All Measured Variables to Assess Smoltification Status***

The principal component analysis was effective in explaining the variation in smoltification traits of individuals in freshwater from my hatchery study (Figure 32). PC1 captured 53% (Eigenvalue = 3.30) of the variance in the individuals from this study. The variables that loaded highly on PC1 were fork length, mass, NKA enzyme activity, and body shape (Table 5). The body shape variable is the PC1 from the TPSW analysis and corresponds to a shortened relative body shape. PC2 captured 15.9% of the variance (Eigenvalue = 1.14). The variables that loaded highly on PC2 were condition factor and body colour, along with NKA enzyme activity and plasma sodium (Table 5). Body colour is the differential colour intensity score and corresponds to a darkened body shape. The other components were not used because they fell below the 1.0 eigenvalue threshold (E.g., PC3 = 0.92, PC4 = 0.63). There is a pattern in the PCA plot (Figure 32) showing a shift in individuals over time along the PC1 axis. The first sampling period (p1) does not overlap with either of the last two sampling periods (p3, p4) and barely overlaps with p2. There is a significant difference in PC1 between sampling periods ( $F_{3,147} = 161.8$ ,  $p < 0.0001$ ). All four sampling periods were significantly different from each other, as shown in Tukey's HSD pairwise comparison test ( $p < 0.001$  for all comparisons). The PC2 axis does not capture the difference in sampling periods as evidenced from the fact that the variation along this axis remains relatively constant.



**Figure 32.** Plot of the first two dimensions of a principle component analysis of six smoltification traits in freshwater fish from the hatchery study. The four colours represent the sampling periods (p1, p2, p3, p4). The ellipses show the 68% probability regions of each sampling period. Arrows indicate the loadings of each variable in two dimensional space.  $n = 173$ .

**Table 5.** Loadings for the PCA analysis of six variables explaining the variance in individuals from the freshwater sampling group in the hatchery study. The values indicate the correlation of the variables to the principle components. PC1 captures 53.0% of the total variance and PC2 captures 15.9% of the variance. n = 173

<b>Variable</b>	<b>Principle Component 1</b>	<b>Principle Component 2</b>
Fork Length	0.526	-0.11
Mass	0.505	-0.061
Condition Factor (k)	0.108	0.827
NKA Activity	0.337	-0.206
Darkened Body Colour	-0.26	-0.329
Body Shape PC1	-0.341	-0.273

## ***Plasma Sodium Concentration***

Plasma sodium concentrations increased in juvenile Chinook salmon during the sampling season in both the freshwater and saltwater fish (Figure 33). This increase was not linear but a similar pattern was seen in both groups. In both groups, there was a significant decline in plasma sodium concentrations on July 1. This decline was from 148.2 mmol/l to 135.4 mmol/l ( $t = 2.54$ ,  $p = 0.018$ ) in freshwater and from 166.4 mmol/l to 135.3 mmol/l ( $t = 4.35$ ,  $p = <0.001$ ) in saltwater fish. This corresponds to the peak of NKA enzyme activity in both sampling groups. There was also a large, significant increase in plasma sodium at the next sampling date in both groups (July 14). This large increase corresponded to the beginning of the last sampling period, p4.

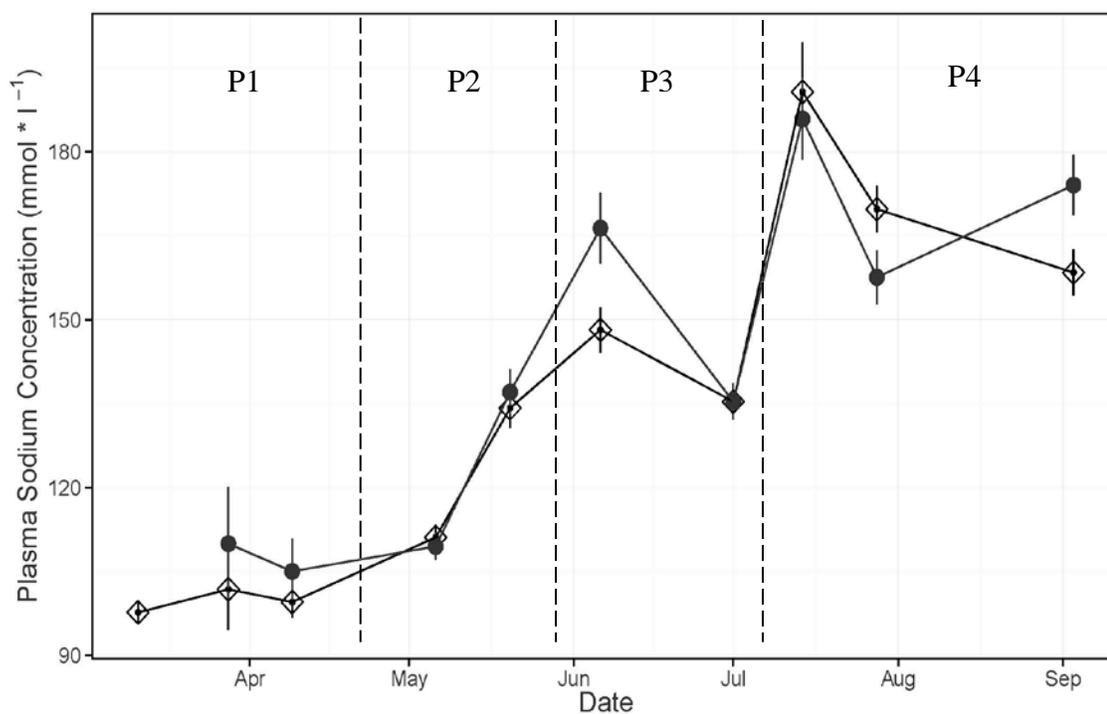
In both sampling groups, body size was a good predictor of plasma sodium concentrations. Fork length predicted 48% of the variation in plasma sodium for freshwater fish (Figure 34) and 46% of the variation in saltwater fish (Figure 35).

When controlling for fish body size, there was a significant effect of sampling period on plasma sodium concentration in freshwater fish (Table 6). Tukey's HSD post hoc pairwise comparison test revealed that all four sampling periods differed significantly from each other ( $p < 0.001$  for all comparisons). Specifically, all periods had significantly higher plasma sodium concentrations than the periods before them. Body size was not a significant predictor of plasma sodium concentration in freshwater fish.

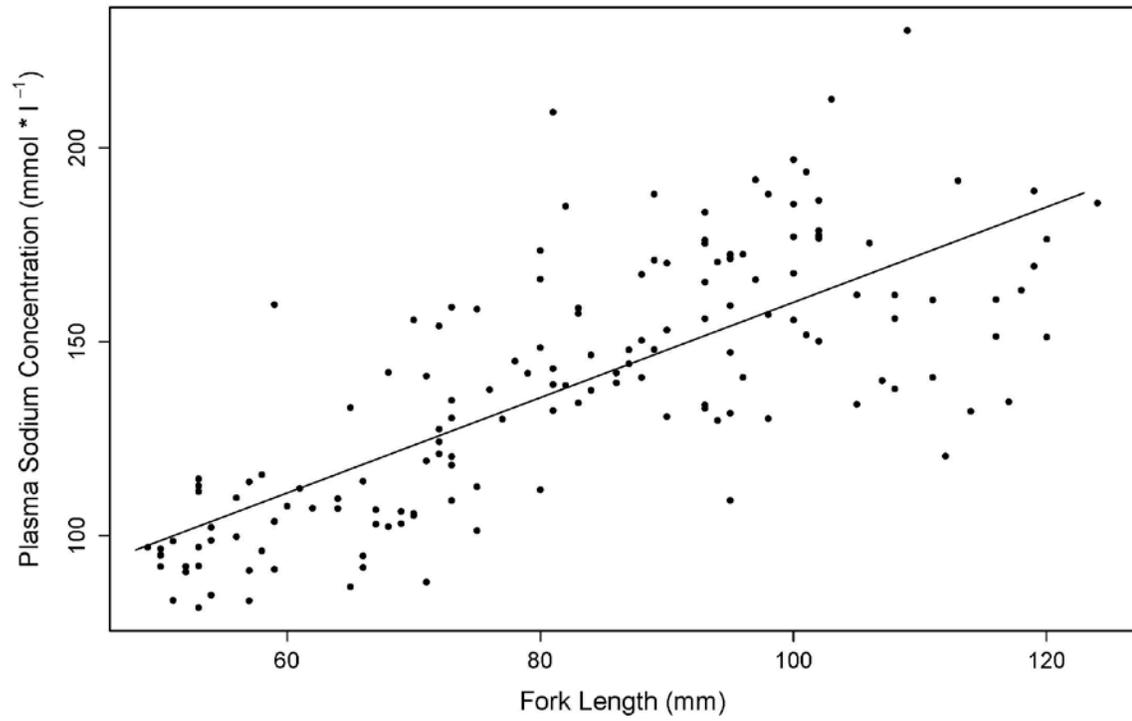
For fish from the saltwater challenge, sampling period and fork length had a significant effect on plasma sodium concentrations (Table 7). There was also a significant effect of the interaction between sampling period and fork length. Tukey's HSD post hoc pairwise comparison test revealed that all four sampling periods differed significantly from each other, except p1 and p2 ( $p < 0.001$  for all significant comparisons). As for the freshwater group, all periods had significantly higher plasma sodium concentrations than the periods before them.

A post hoc test for differences in slopes of the regression models for saltwater fish (plasma sodium by fork length) between sampling dates showed that there was a

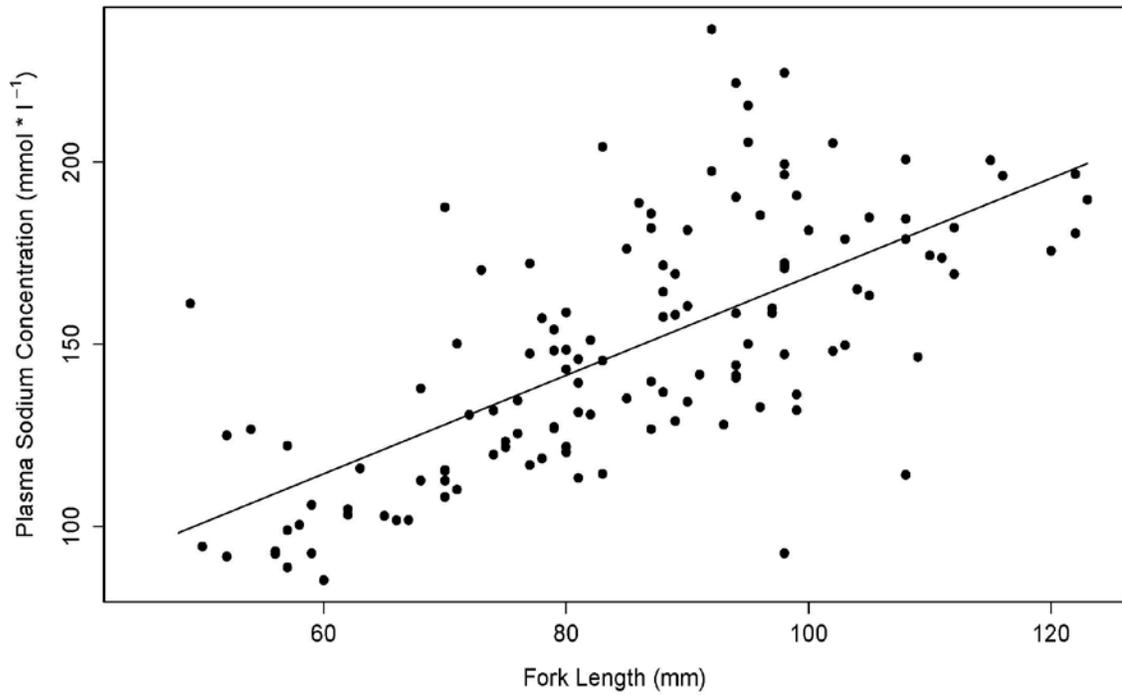
significant difference in the slopes of p1 and p4, with p4 having the greater slope ( $p = 0.04$ ). p2 and p3 were not significantly different from each other ( $p = 0.31$ ) and all of the other pairwise comparisons were marginally insignificant ( $0.05 < p < 0.08$ ). Figure 36 shows the lack of slope difference between sampling periods in freshwater fish and Figure 37 shows the slope differences discussed above in saltwater fish.



**Figure 33.** Plot of plasma sodium concentration in juvenile Chinook salmon in fresh water (open diamonds,  $n = 16-20$ ) and salt water (closed circles,  $n = 6-20$ ) over the hatchery study from March 11 to September 3. Values are mean  $\pm$  SE. The four sampling periods used for analysis are separated by dashed lines.



**Figure 34.** Plot of plasma sodium concentration by fork length in Chinook salmon in fresh water. Regression line is included.  $R^2 = 0.48$ ,  $n = 155$ .



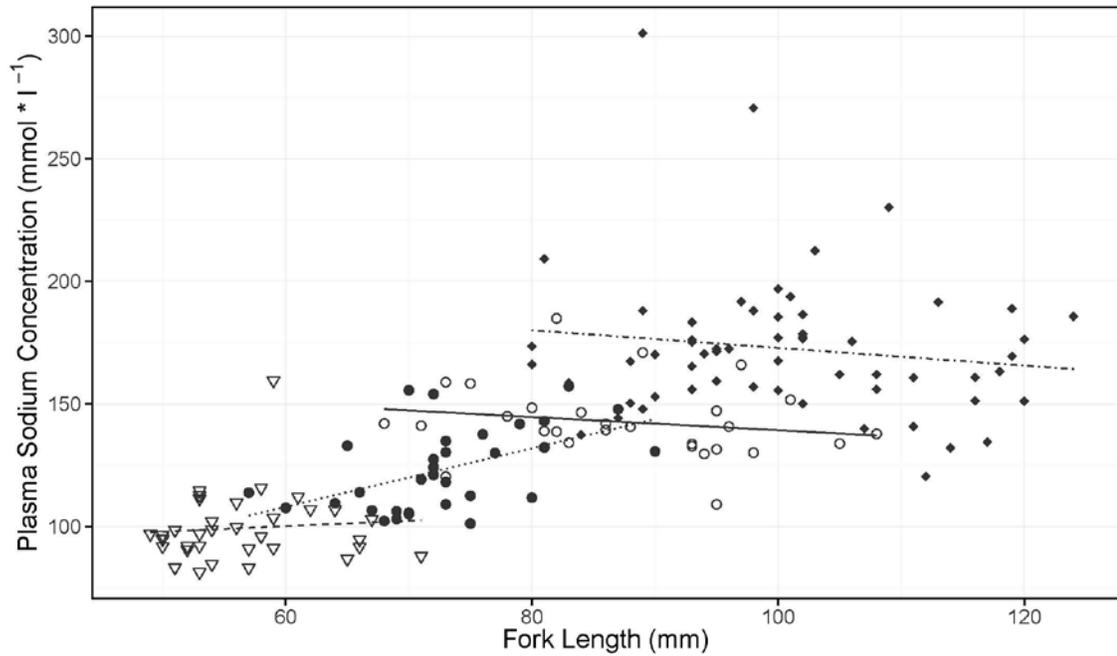
**Figure 35.** Plot of plasma sodium concentration by fork length in Chinook salmon following the 24 hour saltwater challenge. Regression line is included.  $R^2 = 0.46$ ,  $n = 129$ .

**Table 6.** ANCOVA table for the model testing the effect of sampling date and covariate fork length on plasma sodium in juvenile Chinook salmon in fresh water from the hatchery study. n = 155. (\* Denotes significant effects)

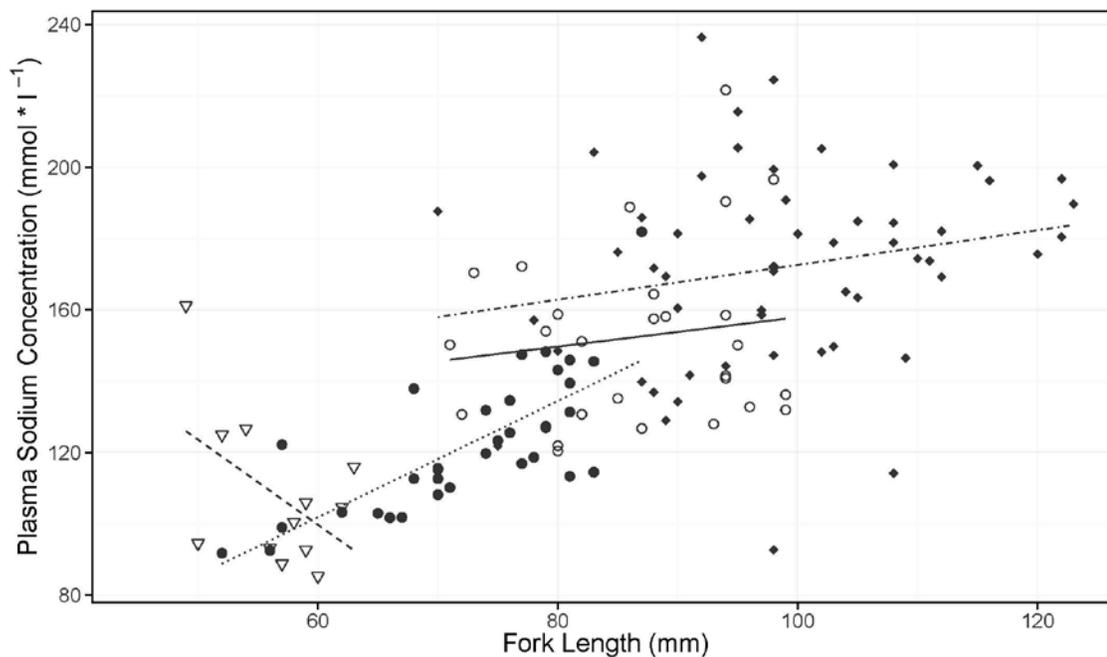
	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Sampling Period	3	131287	43762	93.12	<0.0001 *
Fork Length	1	137	173	0.29	0.59
Sampling Period * Fork Length	3	3337	1112	2.37	0.07
Residuals	147	69084	470		

**Table 7.** ANCOVA table for the model testing the effect of sampling date and covariate fork length on plasma sodium in juvenile Chinook salmon following a 24 hour saltwater challenge in the hatchery study. n = 129. (\* Denotes significant effects)

	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Sampling Period	3	74156	24719	46.07	<0.0001 *
Fork Length	1	5257	5257	9.80	0.002 *
Sampling Period * Fork Length	3	4772	1591	2.97	0.035 *
Residuals	121	64922	537		



**Figure 36.** Plot of plasma sodium concentration by fork length for juvenile Chinook salmon in fresh water from the hatchery study. Open triangles represent fish from the p1 sampling period (n = 36). Closed circles represent fish from the p2 period (n = 32). Open circles represent fish from the p3 period (n = 28). Solid diamonds represent fish from the p4 sampling period (n = 59). Regression lines are included for each sampling period (p1 = dashed; p2 = dotted; p3 = solid; p4 = dot-dash).



**Figure 37.** Plot of plasma sodium concentration by fork length for juvenile Chinook salmon after 24 hour saltwater challenge in the hatchery study. Open triangles represent fish from the p1 sampling period (n = 12). Closed circles represent fish from the p2 period (n = 34). Open circles represent fish from the p3 period (n = 27). Solid diamonds represent fish from the p4 sampling period (n = 56). Regression lines are included for each sampling period (p1 = dashed; p2 = dotted; p3 = solid; p4 = dot-dash).

## DISCUSSION

### ***Smoltification Timing in an Adfluvial Chinook Salmon Population***

I found that adfluvial Chinook salmon undergo smoltification in fresh water in a similar time course to populations of anadromous Pacific salmon. The pattern of smoltification across salmonid species has been shown to be remarkably similar in the months leading up to the achievement of smolt status and downstream migration. The general pattern of increased NKA enzyme activity followed by a subsequent decrease (Figure 19) is seen in all studies focusing on smolting salmonids in both hatchery studies (Whitman 1987, Shrimpton et al. 1994a, McCormick et al. 2009) and in studies of wild populations (Tiffan et al. 2000, Bystriansky et al. 2007a). My study was the first to quantify smoltification traits in an adfluvial population of Chinook salmon.

There were some differences in the seasonal pattern of NKA enzyme activity in my study population from the Sydenham River, including the dates of peak activity. This was true when compared to another Chinook population originating from the same ancestral broodstock as the Great Lakes Chinook salmon. In a Master's research study using juveniles from the University of Washington population, Whitman (1987) found that there was a peak in NKA activity on May 12 1982 in the hatchery (Figure 1). The date of peak NKA activity for the Sydenham River population in my study was July 1 2014.

The differences between these dates could be due to a number of factors, the most obvious of which being the size and growth trajectories of the fish used in the experiments. Growth rates and growth hormone (GH) levels during early development are known to affect smoltification timing, with faster growing individuals undergoing smoltification earlier in the season (Björnsson 1997, McCormick et al. 1998). The University of Washington fish were raised on an accelerated growth regime. On February 27 1982, the mean mass of juveniles was 2.5 g, compared to 1.2 g for my study fish on February 28 2014. By May 13 1982, the mass of Washington fish increased rapidly to 13.3 g whereas individuals from my study had a mean mass of 4.4 g on May 20 2014.

Growth and body size are known to affect smoltification timing in ocean-type Chinook salmon, which generally out-migrate as subyearlings. There is evidence that increased water temperatures during development can lead to an advancement of smoltification, even as much as a full year (Holtby et al. 1989). The University of Washington fish were raised at temperatures that increased from around 11 °C in January to around 14 °C in May. In my study, the water temperature remained constant at 12 °C (Figure 8). These differences in temperature may appear trivial but small temperature differences can have large influences on juvenile fish growth and development over time (Brett et al. 1969, Neuheimer and Taggart 2007). For comparisons between populations, it is therefore important to either raise the fish in a common garden study at controlled temperatures or compare with studies using similar temperature regimes.

In addition to different temperature regimes, there may be inherent growth differences between the populations, such as were seen between naturalized populations of ocean-type Chinook salmon introduced into New Zealand's Southern Island (Unwin et al. 2000). They found that when raised under common garden conditions, juvenile Chinook salmon from cold water populations had slower growth. Slower growth is more consistent with the stream-type life history strategy. Whether the environmental conditions in the Great Lakes contributed to evolutionary divergence in growth rates has yet to be studied. A further research study using a common garden experiment with individuals from the Green River population and Great Lakes populations, as proposed in my original study design, would help to determine this.

There was also a large difference in the intensity of NKA enzyme activity between my study and the University of Washington study. The University of Washington juveniles had a peak mean activity level of 33.2  $\mu\text{moles ADP/mg protein/hour}$  compared to 7.7  $\mu\text{moles ADP/mg protein/hour}$  in my study fish. These differences could be due to a number of factors. It could be disparate laboratory techniques leading to over or under estimation of actual NKA activity. The technique that I used was from a protocol developed by McCormick (1993), a full six years after the Whitman study was published. McCormick's technique and its specific variations are now standard protocol for NKA enzyme activity assays and other researchers have found NKA enzyme activity levels

similar to those in my study using the same protocol. For example, juvenile Chinook salmon from the upper Fraser River, BC raised in a hatchery had a peak mean NKA enzyme activity of 8  $\mu$ moles ADP/mg protein/hour (Sykes and Shrimpton 2010). Unlike in my study, these fish were raised using stream water with naturally increasing temperatures across the spring-summer season to a maximum of 12°C.

NKA enzyme activity is generally regarded as a good indicator of smoltification status and allows for comparison between studies, but comparison of the actual values and dates between studies can be flawed. Moreover, there can be drastic differences between study years in the same populations. For example, Zaugg (1982) found that there was a drastic difference in the time it took for the mean NKA enzyme activity to double in a population of coho salmon raised in an outdoor hatchery setting. This doubling of enzyme activity occurred in two weeks in 1978, but it took over a month for it to double in 1979. Combining NKA enzyme activity data both in fresh water and when exposed to salt water may be the best way to assess smoltification status of individual fish.

### ***Timing of Seawater Tolerance***

When challenged in salt water, juvenile Chinook salmon were able to maintain their plasma ion concentrations at similar concentrations relative to individuals in fresh water (Figure 37). The pattern of NKA enzyme activity was also found to be similar between fresh water and salt water groups (Figure 19). This is consistent with other studies that show a correlation between timing of increased NKA enzyme activity in fresh water and seawater tolerance (Richards et al. 2003, Nilsen et al. 2007, McCormick et al. 2009). Increased NKA enzyme activity and decreased plasma ions are an indication of seawater tolerance. To prepare for salt water, individuals must be able to efficiently excrete excess salt ions from their gills as they are taking water across the filaments. The correlation between the rise in NKA enzyme activity and the decrease in plasma sodium concentrations on July 1 indicates that ionoregulation is highest during this sampling period.

I found that there was lower NKA enzyme activity in saltwater challenged juveniles than in freshwater fish during the period p3 (May 20 – July 1). This is not consistent with data

from previous smoltification studies. For example, Shrimpton et al. (1994a) found elevated NKA enzyme activity in juveniles exposed to salt water over individuals in fresh water in three consecutive years. The lower maximum NKA enzyme activity seen in saltwater challenged fish from my study could be due to treatment stress. I used a saltwater challenge protocol from (Blackburne and Clarke 1987) that has been used in many studies since (Grant et al. 2010, Aykanat et al. 2011). I attempted to decrease the possible stress from the treatment by using a lower salt concentration (24 ppt versus 30 ppt), as proposed by Flores and Shrimpton (2012). However, because the treatment apparatus had to fit inside five gallon plastic buckets, the small size of the containers I used in my study may have contributed to the stress and lowered activity. Other studies have used a separate tank and water supply system to run their saltwater treatments (Flores and Shrimpton 2012). If the fish were stressed, they were not stressed to the point of mortality, as I only witnessed two very small individuals die during the treatments. All fish appeared to be swimming normally and showed no external signs of stress. A further study examining the effect of container size may help in future studies.

### ***The Effect of Body Size on Smoltification***

During the peak smoltification period, individual body size does not appear to strongly influence smolt status. Mine is the first study to examine the relationship between size and smoltification status over the season. One of the major motivations for my study was to determine how growth would affect smoltification. In fresh water, there was no significant linear relationship between body size and NKA enzyme activity during the period of highest NKA activity (p3, Table 3). During every other period in fresh water and also in the three latest periods in salt water, body size was a significant predictor of NKA enzyme activity in fresh water. During these periods, there were higher coefficients of determination in the relationship between size and NKA activity (e.g., p2,  $R^2 = 0.46$ ,  $P < 0.001$ ) compared to the period of highest NKA activity (p3,  $R^2 = 0.05$ ,  $P = 0.17$ ). Previous studies have shown a relationship between smoltification status and out-migration timing (e.g., Tiffan et al. 2000). Under this model, it would appear that fish elevate their smolt status to out-migrate at a specific time of year, regardless of their body

size at that time. This provides evidence that calendar date is perhaps more important than body size in affecting smolt status.

Alternatively, there may be a threshold body size for smoltification and that all individuals at the smoltification window had reached this threshold. I found some evidence for a threshold body size for smoltification as seen in other smoltification studies, although it appears to have a weak effect. There appears to be a body size at which most individuals undergo smoltification. From the logistic model showing the relationship between NKA enzyme activity and fork length in fresh water (Figure 20), the inflection point was found at approximately 60 mm (NKA enzyme activity = 2.6  $\mu$ moles ADP/mg protein/hour). The asymptote is reached at approximately 80 mm fork length and an ATPase activity of 5.7  $\mu$ moles ADP/mg protein/hour. The inflection point can be considered as the threshold fork length beyond which most individuals will smolt. Negus (2003) determined that 71 mm was the threshold fork length for smoltification in hatchery raised Chinook salmon from an adfluvial population in Minnesota. She did this by calculating the minimum fork length at which fish reached the NKA enzyme activity target of 11  $\mu$ moles ADP/mg protein/hour outlined by the United States Geological Survey (USGS) in previous studies. However, it is difficult to disentangle size thresholds from photoperiod thresholds because of the coincidental and correlated progression of body size and day length.

### ***Changes in Body Shape During Smoltification***

My results suggest that the elongation of the body that takes place in juvenile salmonids may be a linked part of smoltification and growth. NKA enzyme activity was significantly correlated with PC1 (i.e., body elongation) in the thin spline body shape analysis ( $r = -0.33$ ,  $p < 0.001$ , Figure 24). This shows that individuals that have reached a high physiological smolt status tended to have an elongated body shape. Fork length was also significantly related to body shape in my study, as seen in the logistic regression models for both PC1 (Figure 26) and PC2 (Figure 27). This indicates that morphological differentiation occurs concurrently with both growth and the seasonal progression of smoltification.

An elongated body shape is considered adaptive for migrating juvenile salmonids. There appears to be selection for elongate bodies in species and populations that have longer migration distances. Atlantic salmon in New Brunswick from tributaries with higher velocity and that are further from the sea (132.6 km) have more elongate bodies than those from a river nearer to the sea (42.5 km) and with lower flows (Riddell and Leggett 1981). Growth rates were found to be similar between them, providing evidence that this is an adaptation independent of body size or growth. In the Pine River, Ontario, Chinook salmon migrate approximately 73 km to and from Georgian Bay. In the Sydenham River, Chinook salmon migrate only 1 km. These are short migratory distances for Chinook salmon (Waples et al. 2004). It is still unknown where these juveniles spend their time once they reach Georgian Bay and how much selection pressure there is on swimming ability in this system. A study investigating the relative fitness advantages of body shape transformations during smoltification would be prudent.

### ***Body Colour Changes During Smoltification***

Juvenile Chinook salmon in my hatchery study became more silver as smoltification progressed. Silvering increased with both body size (Figure 30) and photoperiod (Figure 31). The results of my study show the peak body silvering occurring in the sampling date immediately after peak NKA was reached (July 15). It is difficult to compare these data with other studies, except as a general pattern, because there are many ways to quantify skin colouration and the equipment and software used can affect measurements. In juvenile Chinook salmon from the University of Washington, peak body silvering occurred during the same two weeks as peak NKA enzyme activity (Whitman 1987). This means that the increased body silvering and the reduction of parr marks are occurring alongside other smoltification traits and can be used when modelling the progression of smoltification over time.

### ***The Interrelationship of Smoltification Traits***

The suite of measured smoltification traits were correlated with body size and changed over the season. The PCA analysis shows this progression over the four time periods in

the hatchery study (Figure 32). This is the first set of data showing the interconnected nature of many smoltification traits. The assumption of many researchers that these traits change together has thus been validated in a formal quantitative analysis. The plot also demonstrates that smoltification is not a straightforward process whereby at one time point an individual is a pre-smolt and in the next, they have become a smolt. There is a gradual progression and overlap between periods. This is why smoltification studies are complex. Most researchers agree that smoltification traits are tightly coupled and this is why some of the traits may persist under relaxed selection for individual smoltification traits (McCormick et al. 1998, Björnsson and Bradley 2007).

If all of the smoltification traits are tightly coupled, this may explain why evidence showing adaptation of physiological traits for saltwater preparedness (i.e., NKA enzyme activity) in adfluvial environments is sparse and contradictory. If smoltification traits were coupled during smolting and development, I would not expect there to be a deviation in the patterns of development of Great Lakes Chinook salmon because there is still selection to migrate downstream as part of their life cycle. Many of the transformations are adaptive for downstream migration, predator avoidance, and increased proficiency as predators. This means that in any future studies investigating adaptation of individual smoltification traits, the costs and benefits of the trait must be closely examined.

### ***The Effect of Hatchery Rearing and Unexpected Mortalities***

Using hatchery raised fish in a study investigating smoltification and out-migration timing of individuals in the wild has consequences. During my study, a large number of fish (approximately 1,200) were unexpectedly dying in two of the treatment tanks in February and March. The symptoms matched the symptoms of a common disease in salmonids, bacterial kidney disease (BKD) caused by the bacterium *Renibacterium salmoninarum*. These external symptoms included red spots indicative of muscle ulcers, bloated abdomens, and occasionally popped eyes or exophthalmia (Fisheries and Oceans Canada 2010). Because of this, in my study I only used fish from the hatchery tank that did not show external signs of BKD. This meant that I was unable to obtain data on more

than one temperature treatment. It is still unclear, however, whether the individuals that I used to collect data in this study had BKD even though they did not show symptoms. I am confident that even if they had the disease and survived, this did not affect the data in my study considerably. Mesa et al. (1999) found that there was no difference in NKA enzyme activity between individuals diagnosed with BKD and those that were raised free of the disease. Unfortunately, the bacterium is difficult to treat and control when it infects fish raised at high densities (Fisheries and Oceans Canada 2010). It also appears that at least one Great Lakes Chinook salmon population has greater resistance to BKD than individuals from the ancestral population (Purcell et al. 2014). The Sydenham River population may have some BKD resistance and the resistance may be most effective at higher temperatures (12°C), but this is unclear at this point.

There are other issues with hatchery research and studies using individuals from hatchery supplemented populations that need to be considered. For example, hatchery raised juveniles have been shown to be less tolerant of seawater exposure than wild juveniles. Upon transition to sea water, plasma concentrations of both sodium and chloride rise initially before acclimation. In coho, it has been shown that these levels reach pre-exposure levels after seven days and that hatchery fish had higher levels both immediately after exposure and after the seven day acclimation period (Shrimpton et al. 1994a). Wild fish had a significantly higher density of ionocytes in their gills at all stages of the saltwater challenge. This indicates that when compared with wild fish, hatchery-raised individuals may be less adapted for efficient transfer to salt water. It is therefore possible that the decrease in NKA enzyme activity in fish subjected to the 24 hour saltwater challenge is due to effects of hatchery rearing itself. This could be explicitly tested using a saltwater challenge of juveniles from a naturalized population, such as that in the Pine River.

### ***Smoltification and Out-migration in the Field***

I found that juveniles from the naturalized population in the Pine River were indeed undergoing smoltification and migrating downstream at approximately the same time as individuals from the hatchery study. The NKA enzyme activity levels in the sampled

individuals showed similar levels to hatchery juveniles at similar body sizes (mean NKA activity levels =  $5.7 \mu\text{moles ADP/mg protein/hour}$ , mean FL = 72 mm). In fact, the mean NKA enzyme activity level in the field was the same as the asymptote from the logistic regression model in the hatchery study (Figure 20) which was reached at 80 mm fork length. These data are important for studies of smoltification timing because most of the research has been performed in the hatchery on populations that have been bred in captivity for generations (Björnsson and Bradley 2007). When dealing with complex environmental factors such as photoperiod, water temperature, and water flow, it is important to match the observed phenomena in the hatchery with those observed in wild fish. My study was able to capture natural photoperiod but other parameters were not simulated. The pressures and cues that lead to out-migration timing are still not fully understood.

While I have been discussing subyearling out-migration as the life history strategy for ocean-type Chinook salmon, this is not strictly true for all of these fish. In many Columbia River populations of ocean-type Chinook, more juveniles are migrating as yearlings than ever before (Williams et al. 2008). The researchers suggest this is due to the effect of dams which can lead to, among other things, lower flows in the estuary and altered selection regimes. In my field survey, I encountered one individual that was identified as a yearling juvenile. Local fishers have told me that they observe yearlings in the Pine River in the spring (Nottawasaga Steelheaders, personal communication). A subsequent field survey using a smolt trap capturing all outmigrating juveniles would help determine the proportion in this population. I hypothesize that there are not many yearlings in the system because of the very high water temperatures. In my summer surveys in 2013, I recorded temperatures of  $28 \text{ }^\circ\text{C}$  in the Pine River. Juvenile Chinook salmon prefer cold water temperatures and their growth is limited in high temperatures (Richter and Kolmes 2005). The increased temperatures of the Great Lakes rivers could lead to timing shifts in these Chinook salmon populations.

## ***Relationship Between NKA Enzyme Activity and Out-migration***

There is a particular period during their development at which juvenile salmonids reach their peak physiological ability to osmoregulate in sea water. This is known as the smolt window and it is theoretically the time when downstream migration would confer the greatest benefits of seawater adaptability (McCormick et al. 1998). There is plenty of evidence that juveniles released from stocking hatcheries with higher smolt status have higher survival rates (Negus 2003), migrate faster with greater migration success (Norrgård et al. 2014), and most importantly, have increased adult return rates (Tiffan et al. 2000). Wild studies are lacking in this area but it can be assumed that we would observe the same pattern in wild populations with no hatchery supplementation. Depending on the migration distance, NKA enzyme activity is known to increase during the downstream migration in anadromous populations (Negus 2003, Holecek 2012). In systems with short migration distances, such as the two rivers from my study, preparedness for the downstream environment at the time of the onset of migration is particularly important. In these adfluvial systems, however, there is no selection for saltwater preparedness alone so any insights into whether NKA enzyme activity continues to increase or if there is a decline as seen in the hatchery fish are necessary. A future study on naturalized Great Lakes populations could involve sampling individuals in the rivers, partway on the river towards the Georgian Bay, and in the near-shore of the Georgian Bay.

Assessing smoltification in juvenile rearing habitat can perhaps be misleading in some populations. High NKA enzyme activity is not a prerequisite for downstream migration, as demonstrated in Chinook salmon by (Ewing et al. 1980). When introduced to higher river flows, individuals migrated downstream without elevated levels of NKA activity. The relationship between smoltification timing and the timing of out-migration in natural populations is complex and is still yet to be fully explained. This should be a focus of future studies, as other researchers have pointed out (Björnsson and Bradley 2007).

## ***Conservation of Natural and Introduced Populations***

The integration of evolution and ecology in fisheries management is becoming more prominent in current research (Fraser 2013). Many historically important salmonid populations are threatened or in decline in their native range (Gustafson et al. 2007). Introduced salmonid populations have also become commercially important in many areas, including in the Laurentian Great Lakes. For example, Chinook salmon are considered the most valuable sportfish in the Great Lakes and there are no plans to cease stocking programs on both the U.S. and Canadian sides of the lakes (Melstrom and Lupi 2013). This means that these fish will continue to be a major part of Great Lakes ecology. Understanding the scope for adaptation of successfully introduced Pacific salmon can inform conservation efforts of native populations in a changing global climate.

Warming global temperatures along with supplemental breeding programs may be contributing to decreases in fitness in many wild populations. There is evidence that declines in returning Pacific salmon from hatchery supplemented populations may be due to failure of the release timing of the juveniles to coincide with near-shore marine phytoplankton blooms (Chittenden et al. 2010). This is an example of the match-mismatch hypothesis, which states that when the conditions affecting the optimal environmental window change in their timing, organisms may not be able to change their own timing in the short term (Cushing 1969). For example, increased water temperature can affect the timing of valuable food sources for salmonids, in both rivers (Schindler et al. 2005) and in the downstream habitats (Chittenden et al. 2010). Combining my data on the timing of out-migration with data on the timing of available food in Georgian Bay and deteriorating river conditions would test for a match between out-migration and optimal environmental conditions. There should be selection for early migrating individuals as long as the river conditions decline and food availability in the Georgian Bay matches up with this timing.

## ***Future Research Considerations***

The large question driving my research was whether the naturalized populations of Great Lakes Pacific salmon have become locally adapted to their new environment since introduction. Specifically, have they shifted their timing of smoltification to coincide with generally warmer river temperatures and the absence of a saltwater life history stage? Differentiation between genetic change and phenotypic plasticity in studies of adaptation is often difficult (Merilä and Hendry 2014). While most research on range shifts of organisms due to climate change attributes them to phenotypic plasticity, there is evidence of genetic change as well. Examples include the red squirrel of Yukon (*Tamiascurus hudsonicus*), and the European blackcap (*Sylvia atricapilla*) (Bradshaw and Holzapfel 2006).

For studies of adaptation in Great Lakes Chinook salmon, it would be prudent to examine the source population in Green River, Washington to assess the amount of genetic variation in smoltification timing in that population. Furthermore, researchers should perform a common garden study testing for divergence between populations in smoltification traits such as peak NKA enzyme activity in fresh water. There is evidence of divergence in physiological traits and a genetic basis for physiological capacity in disparate environments (Eliason et al. 2011, Muñoz et al. 2014). A next step would be to compare juveniles from different populations to determine if there is evidence of local adaptation of physiological traits based on environmental differences in photoperiod, migration distances, and salinity.

There is tantalizing evidence of rapid adaptation of smoltification traits in other salmonids from adfluvial systems. For example, two studies found depression of the NKA alpha isoform switching during smoltification in populations that diverged from anadromous populations (Nilsen et al. 2007, Aykanat et al. 2011). Both of these hatchery studies found that during the smolt window, the adfluvial populations showed a lower expression level of the saltwater adapted isoform ( $\alpha_{1b}$ ) than the anadromous populations. There was also a concomitant increase in the expression of the freshwater adapted isoform ( $\alpha_{1a}$ ) over the anadromous population. There is contradictory evidence, however,

from an adfluvial population of Atlantic salmon in Finland. Here they found no difference in isoform switching from their anadromous ancestral population in a hatchery setting (Piironen et al. 2013). This conflicting evidence of rapid adaptation means that more research is required into the possible adaptation of smoltification timing and intensity for populations that no longer migrate to a saltwater environment.

Saltwater preparedness is a good place to look for adaptation or divergence in salmonid populations introduced into adfluvial systems. There is expected to be a cost to preparatory physiological transformations when the environment that they are adaptive for is no longer part of their ontogeny. In my study, similar to other smoltification studies, physiological smoltification indices decreased after reaching a peak level. This is evidenced by the drop in mean NKA enzyme activity levels from approximately 8  $\mu\text{moles ADP/mg protein/hour}$  to approximately 5  $\mu\text{moles ADP/mg protein/hour}$ . This is a possible indication that there is a cost to maintaining this high physiological preparedness and they cannot maintain it throughout the season. To date, there are no studies investigating the costs of mistimed smoltification or maintaining the osmoregulatory mechanisms for saltwater tolerance when individuals do not migrate to a salt water environment. Other researchers have implied this hypothesis of a cost to smoltification as well and they should be important when discussing adaptation of individuals from populations introduced into adfluvial environments (Seppanen et al. 2009, Piironen et al. 2013).

A follow-up study is necessary to examine the fitness differences (e.g., migration speed, juvenile survival, or adult return rate) between individuals with depressed saltwater preparedness (e.g., NKA enzyme activity, NKA  $\alpha_{1b}$  isoform expression) and individuals showing normal levels. Comparing traits between populations in a common garden study is an effective way to test for divergence between them. A reciprocal transplant study of adfluvial and anadromous fish may begin to determine if there is indeed a benefit to reduced saltwater adaptability in adfluvial populations. However, this may be difficult in a wild setting due to disease concerns. The fitness benefits of decreased saltwater preparedness could be related to decreased osmotic stress in the lakes, leading to reduced depredation and increased feeding. This would allow the individual to allocate its

resources to the other parts of smoltification such as swimming ability and growth. This would hence decrease the  $\mu/g$  during this important ontogenetic shift.

## ***Conclusion***

My study offers the first set of data for studies on the timing of smoltification in an introduced adfluvial population and the effect of body size and photoperiod on this timing. My results supported the hypothesis that juvenile Chinook salmon from naturalized populations in the Great Lakes undergo smoltification in a similar time course to individuals from native populations. There were, however, differences in the dates and individual growth in my study population. The juveniles from the Great Lakes population reached peak NKA enzyme activity later than juveniles from a population from the same ancestral broodstock. Data from field capture of juveniles corroborated the timing of smoltification and out-migration found in the hatchery study.

I also found evidence to support the hypothesis that there is selective pressure for individuals to undergo smoltification and out-migrate at a specific time period, independent of body size. During the most important smoltification period, body size did not have a significant effect on NKA enzyme activity. Individuals, therefore appear to be making the decision to smolt and out-migrate during a specific smoltification window and not simply upon reaching a threshold size. Further investigation into the costs and benefits of smoltification by a specific time period is necessary to fully answer the questions of local adaptation of Pacific salmon introduced into adfluvial environments.

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## APPENDICES

### Appendix A. Animal Use Protocol

**AUP Number:** 2013-033

**PI Name:** Morbey, Yolanda

**AUP Title:** Evolutionary Biology And Ecology Of Salmon

**Approval Date:** 08/07/2013

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "Evolutionary Biology And Ecology Of Salmon" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2013-033::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura  
on behalf of the Animal Use Subcommittee  
University Council on Animal Care

## Appendix B. Permit to Sample Juveniles and Collect Gametes



Ontario

Ministry of  
Natural ResourcesMinistère des  
Richesses  
naturelles

### Licence to Collect Fish for Scientific Purposes

### Permis pour faire la collecte de poissons à des fins scientifiques

Licence No. N° de permis	1073155
Local Reference No. N° de référence local	71
Issuer Account No. N° de compte du délivreur de permis	7190610

This licence is issued under Part I of the Fish Licensing Regulation made under the Fish and Wildlife Conservation Act, 1997 to:

Ce permis est délivré en vertu de la Partie I du règlement sur la délivrance de permis de pêche formulé conformément à la Loi sur la protection du poisson et de la faune de 1997 à:

<i>Name of Licensee</i> Nom du titulaire du permis	<i>Last Name / Nom de famille</i> Ms. MORBEY	<i>First Name / Prénom</i> YOLANDA	<i>Middle Name / Second Prénom</i> ELIZABETH
<i>Name of Business/Organization/Affiliation (if applicable) / Nom de l'entreprise/de l'organisme/de l'affiliation (le cas échéant)</i> DEPT OF BIOLOGY, WESTERN UNIVERSITY			
<i>Mailing address of Licensee</i> <i>Adresse postale du titulaire du permis</i>	<i>Street Name &amp; No./PO Box/RR#/Cdn. Del./N° rue/C.P./R.R./poste restante</i> 1151 RICHMOND STREET NORTH		
	<i>City/Town/Municipality / Ville/Village/municipalité</i> LONDON	<i>Province/State Province/État</i> ON	<i>Postal Code/Zip Code Code Postal/Zip</i> N6A 5B7

to collect the species, size and quantities of fish from the waters as set out below.

Pour faire la collecte des espèces suivantes (stade et nombre indiqués ci-dessous):

Species Espèces	Eggs Ouf	Juvenile Fretin	Adults Adulte	Numbers Nombre	Name of Waterbody Nom de l'étendue d'eau
VARIOUS SPECIES PRESENT	X	X	X		PINE RIVER, NORTH OF EVERETT, TOSORONTIO TWP

Yes/Oui  Additional species/Waterbody list attached / Liste d'espèces/d'étendue d'eau additionnelles ci-jointe

*Purpose of collection*  
*But de la collecte* SUPPORT THE COLLECTION OF FISH AND SPAWN FOR A GRADUATE STUDENTS THESIS PROJECT.

<i>Licence Dates</i> <i>Dates du permis</i>	<i>Effective Date / Date d'entrée en vigueur</i> (YYYY-MM-DD) 2013-04-22	<i>Expiry Date / Date d'expiration</i> (YYYY-MM-DD) 2013-11-30
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*Licence conditions* This licence is subject to the conditions contained in Schedule A if included. / Ce permis doit respecter les conditions de l'annexe A si celle-ci est jointe.

*Conditions du permis* Yes/Oui  No/Non  Schedule A included. / Annexe A ci-jointe

<i>Issued by (please print)</i> <i>Délivré par (veuillez écrire en caractères d'imprimerie)</i> JOHN T. KUS, AREA SUPERVISOR	<i>Signature of Issuer / Signature du délivreur</i>	<i>Date of Issue/Date de délivrance</i> (YYYY-MM-DD) 2013-04-09
<i>Signature of Licensee / Signature du titulaire du permis</i>		<i>Date</i> (YYYY-MM-DD) 2013-04-09

Personal information contained on this form is collected under the authority of the Fish and Wildlife Conservation Act, 1997 and will be used for the purpose of licensing, identification, enforcement, resource management and customer service surveys. Please direct further inquiries to the District Manager of the MNR issuing district.

Les renseignements personnels dans ce formulaire sont recueillis conformément à la Loi sur la protection du poisson et de la faune, 1997 et ils seront utilisés aux fins de délivrance de permis, d'identification, d'application des règlements, de gestion des ressources et de sondage sur les services à la clientèle. Veuillez communiquer avec le chef du district du MNR qui délivre le permis si vous avez des questions.

## Appendix C. Disease Testing Results – University of Washington Chinook Salmon

Washington Animal Disease Diagnostic Laboratory  
P.O. Box 647034, Pullman, WA. 99164-7034

**Laboratory Results Report**

WADDL Number 2013-13014

Fish source Dr. George Sanders University of Washington Department of Comparative Medicine			Address or location of fish source University of Washington Research and Teaching Hatchery 1122 NE Boat Street Seattle, WA 98105												
Fish sampled	Sample Date 11 Nov 2013		Pathogens detected properties consistent with <sup>1</sup>												
Species	Age	Sample size <sup>2,3,4,5,6</sup>	E H N	I H N	I P N	O M V	S V C	V H S	I S A	A S L	Y E R	R E N	C M S	M Y X	P S R
<i>Oncorhynchus tshawytscha</i>	Adult	5 k/s	-	+*	-	-	-	-							
		5 i													
		5 hc													

<sup>1</sup>EHN - Epizootic Hematopoietic Necrosis Virus  
IHN - Infectious Hematopoietic Necrosis Virus  
IPN - Infectious Pancreatic Necrosis Virus  
OMV - *Oncorhynchus masou* virus  
SVC - Spring Viremia of Carp  
VHS - Viral Hemorrhagic Septicemia Virus  
ISA - Infectious Salmon Anemia Virus

ASL - *Aeromonas salmonicida*  
YER - *Yersinia ruckeri*  
REN - *Renibacterium salmoninarum*  
CMS - *Ceratomyxa shasta*  
MYX - *Myxobolus cerebralis*  
PSR - *Piscirickettsia salmonis*  
TET - *Tetracapsula bryosalmonae*

<sup>2</sup>c = culture media

<sup>3</sup>k/s = kidney/spleen

<sup>4</sup>hc = head cores

<sup>5</sup>i = intestinal scrapings

<sup>6</sup>ov = ovarian fluid

\* See summary

+ Agent detected, properties consistent with

- Agent not detected

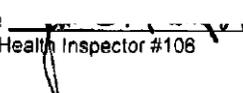
O No test performed

**Laboratory findings summary:**

\*Infectious Hematopoietic Necrosis Virus (IHNV) was detected in the one 5-fish pool of kidney/spleen tissue that was submitted by cell culture. The cell culture sample also produced an amplicon of appropriate size following polymerase chain reaction (PCR) using primers specific for IHNV.

No other significant pathogens were detected.

Fish at the above facility were inspected in accordance with procedures outlined in the OIE *Diagnostic Manual for Aquatic Animal Diseases*, Sixth Edition, 2009.

Laboratory official's signature   
Jim B. Thompson, AFS Fish Health Inspector #108

Date 11 December 2013

# CURRICULUM VITAE

## **Steve Sharron**

### **Education**

**Master's of Science (M.Sc.)**, April 2015, Biology,  
*Western University, London, ON*

**Bachelor's of Science (BSc) with honours**, August 2012, Biological Sciences  
*Simon Fraser University, Burnaby, BC*

**Associate of Science Degree (ASc)**, August 2009, Biology, *Langara College,*  
*Vancouver, BC*

### **Work Experience**

**Teaching Assistant**, 2013-2015, Western University, London, ON

**Field Research Assistant**, Fall 2012, Western University, London, ON

**Field Research Assistant**, Summer 2012, Simon Fraser University, Burnaby, BC

**Laboratory Research Assistant**, 2011- 2012, Simon Fraser University, Burnaby, BC

**Field Research Assistant**, Summer 2011, Simon Fraser University, Burnaby, BC

**Building Maintenance Employee**, 2009-2011, Pacific Asset Management, North  
Vancouver, BC

### **Publications and Presentations**

*"Fish out of Salt Water: Smoltification in subyearling Chinook Salmon from the Laurentian Great Lakes."* **Steve Sharron**, *M.Sc. thesis, 2015.*

"Using scale characteristics and water temperature to reconstruct growth rates of juvenile steelhead (*Oncorhynchus mykiss*)."

*"Fish out of Salt Water: Smoltification in subyearling Chinook Salmon from the Laurentian Great Lakes."* **Steve Sharron**, *Presentation to the Nottawasaga Steelheaders Association, November 2014.*

"Smolting under time constraints: is there a seasonal decline in smoltification size of subyearling Chinook salmon?" **Steve Sharron** and Yolanda Morbey. Presentation at the Annual Meeting of the American Fisheries Society 2014. Quebec City, QC. Summer 2014

"Why I study rapid adaptation in salmon." **Steve Sharron**. Presentation at the Western Biology Graduate Research Forum. London, ON. Fall 2013

"Rapid adaptation of smoltification traits in anadromous Pacific salmon introduced into an adfluvial environment." **Steve Sharron** and Yolanda Morbey. Presented at the Ontario Ecology, Ethology, and Evolution Colloquium. London ON. Spring 2013

"Marine Mammals and Their Adaptations." **Steve Sharron**. Lecture given to 4<sup>th</sup> year Marine Environments class. Fall 2013 and Fall 2014.

### ***Certifications and Courses***

**Class I and II electrofishing certificate in Ontario.** 2013.

**ROM Fish Identification Course.** 2013. Finished 3-day course of Ontario Freshwater Fishes

**PADI Advanced Open Water SCUBA diving certification.** 2006. Over 100 logged dives. Gained extensive boat and water safety training and experience along the way.

**Valid Ontario Pleasure Craft Operator Card.** 2012. Obtained to pilot a 15' Boston Whaler-style aluminum fishing boat.

**First Aid and CPR training.** 2013. Learned valuable skills for dealing with medical emergencies.

**OH&S supervisor training.** 2013. Western University course required for laboratory supervisors.

**Advanced Statistics Course.** 2013. UWO graduate course focusing on multivariate statistical methods useful for fisheries research. Proficient in the use of R statistical software.

### ***Professional Affiliations***

**College of Applied Biology.** Biologist in Training since 2014 and preparing to obtain a Registered Professional Biologist (RPBio) designation.

**American Fisheries Society.** Member of international society and the Ontario chapter since 2012. Presented at the 2014 Annual Meeting in Quebec City.

**Canadian Society for Ecology and Evolution.** Member since 2012. Membership allows me to interact with other researchers interested in evolutionary ecology research.