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Title: Effects of Current Hatchery Practices on Salmon Odor Recognition and Responses

Abstract approved:

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David L.G. Noakes

Salmon hatcheries have been utilized throughout the Pacific Northwest for many decades to mitigate losses, supplement natural spawning populations, and to provide tribal, sport and commercial fishing opportunities. Currently, there is substantial debate on their efficacy and the potential threats hatchery-reared salmonids pose to natural populations. These concerns have lead to a large body of scientific investigation. The potential impacts hatchery-reared salmon may have on natural spawning populations can take many forms, such as competition, predation, and genetic introgression. Many of these issues arise from straying of hatchery-reared salmon from target return locations into areas where naturally spawning populations of salmon occur. In my thesis, I considered two questions related to hatchery practices and straying of hatchery-reared salmon. First, I addressed whether exposure to unfamiliar conspecifics during incubation (embryonic development) affects the population recognition responses of emergent steelhead (*Oncorhynchus mykiss*). I then examined whether dissolved free amino acids (DFAAs, chemicals believed to be important for natal stream identification in salmonids) are significantly altered as

water sourced from a river passes through a salmon hatchery. The results of my first study suggested that newly emergent steelhead showed no preference for water conditioned by either familiar (fish from the same population) or unfamiliar (fish from geographically separate population) populations. These results suggest that exposure to unfamiliar or unrelated population specific odors during incubation in a hatchery may not have any significant effects on population recognition responses. The data from my second study suggested that both hatchery and river water DFAA profiles are very similar. I hypothesize that straying observed in hatchery-reared salmon may be due in part to a lack of a unique, distinguishable odor profile that hatchery-reared salmon might use to differentiate between target return locations and other potential spawning grounds.

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Effects of Current Hatchery Practices on Salmon Odor Recognition and Responses

by
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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Joseph R. Lemanski, Author

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CONTRIBUTION OF AUTHORS

David Noakes, Andrew Dittman, and Marc Johnson provided expert advice in the development of the experimental design, data analysis and editing of all chapters. Ryan Couture and Joseph O'Neil contributed to the experimental design, materials and construction of experimental equipment at the Oregon Hatchery Research Center of chapter 2. Hiroshi Ueda and Ernest Chen provided professional guidance in the experimental design and methodology of chapter 3.

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CHAPTER 1: GENERAL INTRODUCTION

For centuries, salmon have been revered for their ability to migrate long distances and return to natal rivers and streams as spawning adults (Lichatowich 1999). This incredible feat has been intensely studied over the last century, and it has been shown that home stream identification (homing) in anadromous salmonids is guided by olfaction (Hasler & Scholz 1983). Juvenile salmonids preparing to out-migrate to the ocean imprint upon the odor signature of their natal stream during the parr-smolt transformation (Hasler & Scholz 1983). When salmon return to freshwater as spawning adults, they recall the odor signature imprinted upon as juveniles to identify their natal rivers and streams.

While the vast majority of returning adults navigate to their natal rivers with incredible accuracy and precision (homing), a small proportion of individuals return to and spawn in non-natal areas. This is referred to as straying. Straying by salmon is often regarded negatively, but straying provides salmon populations with an ecological and evolutionary "bet-hedging" strategy that allows for the diversification of a population's gene pool, the colonization of new viable habitat, and maintenance of "reservoir" populations so that entire populations are not eliminated as a result of environmental catastrophes (Quinn 1984; Westley *et al.* 2013; Fleming 2014).

Due to the highly predictable timing and location of salmon migrations, salmon have been fundamentally important to the indigenous peoples of the Pacific Northwest (hereafter; PNW) and continue to hold social, cultural, dietary and economic importance in this region (Craig and Hacker 1940; Lichatowich 1999; Schwarcz *et al*. 2014). Beginning in the late-1800s, increased harvest and anthropogenic habitat changes (e.g. gold mining, fur trapping, cattle grazing, timber harvest) resulted in dramatic declines in salmon populations across the PNW (Lichatowich 1999). More recently, extensive

hydropower development has limited salmon production in this region (Raymond 1979; NPPC 1986; Raymond 1988; Harrison 2008). It has been estimated that 10-16 million Chinook salmon (*Oncorhynchus tshawytscha*) returned annually to the Columbia River drainage before the construction of hydroelectric, diversion and flood control dams (NPPC 1986). Over the last 100 years, the number of returning salmon has been reduced to 5-7% of the estimated historical returns (calculated from values made available by fpc.org).

Declines in specific salmon populations prompted the National Marine Fisheries Service to list several evolutionary significant units throughout the PNW as threatened or endangered (Waples 1991). While many threats faced by salmon have been addressed over the last 100 years, salmon populations across the PNW have struggled to return to their historical numbers. Salmon hatcheries are a tool that are very effective for producing juvenile salmon at mortality rates much lower than observed in nature, and are used extensively across the PNW today to mitigate losses, supplement natural spawning populations, and to provide tribal, sport and commercial fishing opportunities (Naish *et al.* 2008). While producing and rearing salmon in hatcheries has become highly efficient over the last 100 years, hatcheries are still imperfect systems that can have undesired impacts on naturally occurring populations. These impacts are due, in part, to negative interactions with stray hatchery fish including competition, genetic introgression and reduced reproductive success (Waples 1999; Brannon *et al.* 2004; Araki *et al.* 2007a,b; Araki *et al* 2008). .

Stray rates of hatchery-reared salmon vary among species and populations and it is still unclear whether hatchery-reared salmon stray more frequently than naturally

produced salmon (Waples 1999; Quinn 1993; 2005; Westley *et al.* 2013). Compounding concerns about the potential impacts of straying by hatchery-reared fish is a lack of understanding of the causes for straying, along with the difficulty of detecting strays and quantifying stray rates in natural salmon populations. For these reasons, understanding and minimizing straying by hatchery fish is important for reducing the deleterious effects of these interactions and promoting the preservation, restoration, and conservation of natural salmonid populations across the PNW

Hatcheries and hatchery-reared salmon are often utilized for research purposes to focus on various aspects of salmon behavior, physiology, genetics, and ontogeny because making similar observations in the natural environment can be challenging. To better understand how hatchery-reared salmon might affect natural salmon populations, many studies have also focused on the impacts that rearing environment can have on these characteristics (Berejikian *et al.* 1996; Flagg *et al.* 2000; Weber and Fausch 2003; Lee and Berejikian 2008). However, the causes for straying, and the specific odors salmon utilize for homing have not yet been clearly identified. Furthermore, which hatchery practices and operations might influence straying (or the failure to identify target return locations) in hatchery-reared salmon has not been investigated entirely. Filling these knowledge gaps could provide managers with valuable tools to reduce straying by hatchery-reared salmon and thereby minimize interactions with naturally produced salmon. My thesis is concerned with certain factors that may affect homing and straying in hatchery-reared salmon.

My thesis considers two competing homing hypotheses (that may be operating simultaneously) and focuses on how certain hatchery practices and operations might

affect odor recognition and behavioral responses that could influence homing and straying as adults. The second chapter focuses on the question of whether exposing steelhead (*Oncorhynchus mykiss*) during incubation to water borne chemicals (presumed to be odors that are detected by the olfactory system) from a geographically separate population of conspecifics affects the behavioral responses of newly emerged steelhead to water conditioned by members of their own population versus members of a separate population. The third chapter considers the hypothesis that salmon utilize dissolved-free amino acids for natal stream identification. I tested whether hatchery equipment and operations significantly altered this profile from the hatchery's river water source. The fourth and final chapter provides a general discussion and conclusion on the implications of my studies. My thesis is intended to contribute to the improvement of hatchery operations and practices so as to reduce the impacts of hatchery-reared salmon on natural populations.

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CHAPTER 2: EFFECTS OF COMMON INCUBATION ON OLFACTORY POPULATION RECOGNITION IN JUVENILE STEELHEAD (*Oncorhynchus mykiss*)

Abstract:

The olfactory recognition of conspecifics has been studied extensively in fishes, and has been shown to be a widespread ability across salmonids. While many studies have considered how common rearing and familiarity of juveniles can alter recognition behavior, there is no published information on potential effects of common incubation of embryos. I used y-maze behavioral assays to evaluate whether population recognition in newly emerged steelhead (*Oncorhynchus mykiss*) is affected by earlier exposure to chemical cues from conspecifics. My results showed no test group preferred water conditioned by either test population, or unconditioned river water. Exposure to a geographically separate population of conspecifics during incubation (common incubation) had no effect on subsequent water preference of the fish. Population-specific odors do not appear to be relevant for embryos or young juveniles (at least in this experimental paradigm), suggesting population recognition might develop or be learned over a longer period of time, possibly after total yolk absorption.

Introduction:

Conspecific recognition by olfaction has been studied extensively in fishes, and a number of experiments have tested whether salmonids possess the ability to distinguish between groups of fish at varying levels of relatedness (i.e. kin, population, species) (Griffiths 2003). Hans Nordeng (1971; 1977) hypothesized that salmon homing is based on an ability to recognize and respond to conspecific odors. This hypothesis requires that adult salmon returning to their natal spawning location are able to recognize and respond to population-specific pheromones released by juveniles (Nordeng 1971; 1977). Døving

et al. (1974, 1980) observed that the olfactory system of Arctic char (*Salvelinus alpinus*) discriminated population level differences in odors, and suggested that skin mucus, amino acids and/or bile acids are potential odorants used in population recognition. However, the chemical (or set of chemicals) used for population recognition remain(s) unknown.

Several studies have focused on the olfactory ability of migratory adult salmonids to distinguish between populations since they are actively homing to their natal rivers. Quinn *et al*. (1983) showed that adult coho salmon (*O. kisutch*) preferred water conditioned by conspecifics compared to unconditioned water. Furthermore, both juvenile and adult coho salmon preferred water conditioned by members of their own population compared to water conditioned by an unfamiliar population (Quinn and Tolson 1986). Groot *et al.* (1986) tested the behavioral responses of adult sockeye salmon (*O. nerka*) from two separate populations. They showed that adult sockeye salmon preferred water conditioned by members of their own population, but they also observed varying magnitudes of preference between different stocks of fish.

Juvenile salmonids are also able to distinguish relatedness using olfactory cues. Quinn and Hara (1986) showed that juvenile coho salmon are capable of distinguishing water conditioned by siblings and non-siblings, preferring water conditioned by siblings. Common rearing within a hatchery affected their ability to distinguish between familiar and unfamiliar (i.e. reared together or separately) groups of juvenile fish. Similarly, Courtenay *et al.* (1997) showed that some populations of juvenile coho salmon distinguished between water conditioned by members of their own population versus an unfamiliar population and common rearing of juveniles affected preferential responses of

fish as well. Several other studies have shown that juvenile salmon are capable of using olfactory recognition to distinguish between conspecific/heterospecific populations, and varying degrees of relatedness or kinship (Stabell 1987; Quinn and Busack 1985; Olsen 1989; reviewed in Griffiths 2003). However, the early development of olfactory recognition capabilities in salmonids has not been studied, and it is not clear at what developmental stage fish might first show recognition responses.

Steelhead (*O. mykiss*) differ from other Pacific salmon in a number of important ways. They are native to the Pacific Northwest (PNW), but unlike other Pacific salmon they are iteroparous. They also have very distinct facultative alternative life history patterns as anadromous steelhead and freshwater resident rainbow trout. Steelhead are important from ecological, management, economic and tribal perspectives throughout their native range. Little is known about the olfactory recognition capabilities of steelhead. However, Brown and Brown (1992; 1993; 1996) investigated the olfactory recognition capabilities of juvenile rainbow trout. Brown and Brown (1992) performed y-maze behavioral testing with rainbow trout where test fish spent significantly more time in water conditioned by kin than water conditioned by non-kin, and preferred conspecific odors Further studies suggested that altruistic behavior resulting from kin recognition might be displayed as reduced aggressive behavior towards closely related individuals and greater partitioning of feeding territories with closely related individuals.

Steelhead are produced in hatcheries throughout the PNW and effects of hatchery-produced steelhead on natural populations is a particular concern for the Oregon Department of Fisheries and Wildlife (ODFW) and other agencies tasked with the production and management of hatchery-reared salmon and steelhead. Hatcheries

occasionally incubate and rear several groups of fish at one location while each group's intended release location may be different. This practice might expose juveniles to odors from several different conspecific populations during incubation and rearing. If unintended exposure to different population-specific odors during incubation and rearing affects or alters which population juveniles perceive as their own, this could potentially affect homing behavior later in life as adults (increase straying). My experiment was designed to address how exposure to unfamiliar population-specific odors during incubation (hereafter, common incubation) in a hatchery setting might affect preferential responses to population-specific odors (implying recognition) of juvenile steelhead.

In an effort to understand the function of olfactory population recognition, and potential roles in homing (and straying), I examined population-specific olfactory recognition in young steelhead. Several studies of conspecific recognition by juvenile salmonids have been performed with fish incubated and reared in a setting characteristic of a hatchery (Quinn and Busack 1985; Quinn & Hara 1986; Courtenay *et al.* 1997; Courtenay *et al.* 2001). Furthermore, many of those studies have tested various aspects of conspecific odor recognition in juvenile salmonids following exogenous feeding and common rearing. However, some have found exposure to digestion products of familiar and unfamiliar groups of fish produced behaviors that are not characteristic of olfactory recognition, which may be explained by confounding factors such as similar diets (Bryant & Atema 1987; Courtenay *et al.* 1997; Rajakaruna & Brown 2006). Whether preferential responses of newly emergent salmonids are affected by exposure to familiar and unfamiliar odors prior to emergence remains to be explored.

Dittman *et al.* (2015) demonstrated that juvenile salmon are capable of discriminating between water sources at the swim-up stage (transition from embryo to juvenile) of their life cycle (Noakes & Godin 1988), a point during ontogeny when the olfactory receptor cells of rainbow trout are partially developed and sensitive to some chemical odors (Zielinski & Hara 1988). Dittman *et al*. (2015) showed that juvenile salmon tested in y-mazes immediately following emergence, but before exogenous feeding, can discriminate between two water sources based upon the olfactory signature of those sources. They further showed that newly-emerged Chinook salmon prefer surface water to well water, and preferences were altered by previous exposure, demonstrating that embryonic salmon are capable of learning the chemical signature of their incubation water source prior to emergence. I followed their methods to test juvenile steelhead with behavioral y-maze assays at the swim-up stage (following emergence, before exogenous feeding).

Using behavioral attraction assays, I tested the null-hypothesis:

H_o: common incubation will have no effect on preferential responses to population-specific odors by juvenile steelhead.

Testing this hypothesis could yield two possible results. First, steelhead exposed to odors from a different steelhead population prior to emergence might show the same behavioral responses to population-specific odors whether they had previously experienced these odors or not, failing to reject my null hypothesis. Alternatively, I would reject my null hypothesis if groups previously exposed to odors from a different population display a different behavioral response, either attractive or repulsive, relative to the responses of fish that never experienced the different population (suggesting

recognition has been altered by common incubation with another population). Either result would have implications for understanding the development of olfactory-mediated population recognition capabilities in salmonids, as well as the management of salmon in a hatchery setting. The first result would suggest that common incubation has no direct effect on population recognition, and would be unlikely to affect the homing behavior of these fish as adults, assuming population recognition is needed for successful homing. The second result would suggest that common incubation does have an effect on population recognition, and the implications of this effect would need to be studied further, potentially throughout an entire life cycle. Full life cycle studies could provide a better understand of how common incubation might affect olfactory population recognition at later life stages such as sub-yearlngs, smolts and adults. Testing at these later life stages could determine how social behaviors might be affected, and how any effects might relate to homing (and straying) as adults.

Materials and Methods:

I. Test Fish and Incubation

The Siletz and Alsea rivers are both located along Oregon's central coast, separated by 57 km of coastline (straight-line distance). Both drainages support naturally spawning populations of steelhead as well as hatchery-reared steelhead (Matt Frank, ODFW, personal communication). Genetic evidence suggests that naturally spawning Siletz River and Alsea River steelhead populations are genetically distinct from one another (Will Hemstrom, personal communication). Based on this genetic information and the purpose of this study, the offspring of F1-generation adult hatchery steelhead

from these populations were used for behavioral testing, and believed to be sufficiently different from one another genetically. For this study, four pairs of adult steelhead were collected from each of two locations 1) the North Fork Alsea Hatchery (ODFW facility in Alsea, Oregon; Figure 2.1) and 2) the Siletz Falls fish trap on the Siletz River, Oregon (ODFW facility).

All fish were spawned at the North Fork Alsea Hatchery by ODFW personnel following their standard production procedures. Fertilized eggs from single-mate spawning pairs were disinfected and transported to the Oregon Hatchery Research Center (Figure 2.1) where embryos from each population (i.e. North Fork Alsea steelhead (NF-Alsea) and Siletz steelhead) remained in separate incubation stacks until they reached the eyed egg stage. All holding and incubation procedures followed standard ODFW procedures used at the OHRC (Ryan Couture, personal communication). Once the embryos reached the eyed egg stage, equal numbers of individuals (1,548) from each of the four families from each population were pooled to create a population pool (Alsea pool, Siletz pool; Figure 2.2). Each population pool was then split into two equal sized experimental groups; NF-Alsea 1, NF-Alsea 2, Siletz 1 and Siletz 2 (Figure 2.2).

Water for these experiments was taken from Carnes Creek, a small tributary of Fall Creek near the OHRC that contains no anadromous salmonids or conspecifics (Ryan Couture, personal communication), thus preventing any embryonic exposure to conspecific odors that might confound behavioral responses during testing. Heath trays containing embryos of the subpopulation groups were arranged in incubation stacks so one group from each population (NF-Alsea1 and Siletz 1) would receive unconditioned Carnes Creek water (control), while the remaining subpopulation groups (NF-Alsea 2 and

Siletz 2) were placed directly below a tray containing embryos from the other population, thus experiencing Carnes Creek water conditioned by odorants from embryos of the other population (experimental) (Figure 2.3). Holding the embryos in this fashion for the remainder of incubation allowed me to determine if common incubation affects the population recognition abilities of the experimental groups. All procedures for obtaining and handling fish were approved by the Oregon State University Institutional Animal Care Committee (ACUP #4286).

II. Behavioral Testing

Behavioral responses (water preference) of juvenile steelhead were tested in eight identical juvenile y-mazes (70 cm long x 20 cm wide x 15.25 cm deep; Figure 2.4). Each set of four y-mazes were supplied by two 56.75 liter polyethylene head tanks containing conditioned or unconditioned Carnes Creek water at a constant rate of 3 L min^{-1} to each y-maze (1.5 L min^{-1} for each arm of y-maze) and held at a constant depth of 6 cm (Figure 2.5). The downstream end of each y-maze was blocked by a metal screen with 2 mm holes to retain individuals in the maze. Fish were initially retained in a starting area at the downstream ends of the two arms of each y-maze by a mesh gate (Dittman et al. 2015). That gate was lifted after the initial acclimation period to allow the fish to move freely into either arm of the y-maze. Details of the materials and construction for the y-maze systems are provided in Appendix 1.

Water preference testing of juvenile steelhead was conducted between emergence and total yolk absorption. This time frame was chosen to reduce the number of odors test fish were subjected to and prevent exposure/assumption (i.e. odors created as a result of diet) to odors that could result from exogenous feeding potentially confounding results.

Three different combinations of water treatment were used to test for juvenile steelhead preferences. The three water sources were Carnes Creek water conditioned by individuals of the Alsea population (ACW), Carnes Creek water conditioned by individuals of the Siletz population (SCW), and unconditioned Carnes Creek water (UCW).

Each experimental group of steelhead was tested for water preference with the following water combinations each day of testing: ACW vs. SCW, ACW vs. UCW and SCW vs. UCW (32 trials/water combination for a total of 96 trials). To create the conditioned water(s) for the y-maze testing, 30 individuals from a population were removed from incubation trays via dip net that was handled wearing nitrile gloves to prevent the transfer of any potential human chemicals from skin contact. Individuals were placed in a porous container that was submerged in the head tank directly under the flow of water in effort to allow maximum odor accumulation (conditioning). Fish placed in header tanks for water conditioning acclimated for a minimum of 5 minutes before each set of trials began. Head tanks, circle tanks, and pumps used to move Carnes Creek water into head tanks were drained and rinsed with well water 3 times per day following the testing of all experimental groups to a specific test water combination. All groups of fish were tested in each testing scenario at different times of the day to account for any potential diurnal changes in behavior (order of daily testing scenarios provided in Table 2.1).

To determine preference of juvenile steelhead for one water source over another, 10 individuals were placed in the downstream acclimation area of a y-maze with the gate closed. The entire testing area, including water header tanks and y-mazes were shrouded in opaque black plastic (polyethylene) sheeting to reduce light. Test fish were allowed to

acclimate for 10 minutes, the gate was lifted remotely with minimal disturbance to the fish, and then the fish were allowed to swim freely for 50 minutes. After the 50-minute free-swimming period, the gates were closed and the numbers of fish in each arm of the y-maze were recorded. Following each trial, fish were removed and euthanized in MS-222 (ACUP #4286), and y-mazes were flushed and rinsed thoroughly with well water between trials. When switching head tanks from one water condition to another, y-mazes, head tanks, and pumps used to move Carnes Creek water into head tanks were drained and rinsed thoroughly with well water.

III. Data Analysis:

For my analyses, I used a point system that only considered which arm of each y-maze had more fish at the end of the trial to determine an overall preferential response. A preference point was given to a water treatment when more fish were in the arm of the maze containing that water source. Fish that did not make a decision, or trials that had an equal number of responses were considered to display "no preference" and were not included in the analyses. I analyzed the point data in two ways 1) pairwise comparisons of each experimental group to each test scenario, and 2) compared each experimental group's responses to the ACW vs. UCW and SCW vs. UCW test scenario. I performed Chi-Square Tests for Homogeneity to compare the proportion of preferential responses of each experimental group in each testing scenario. (Table 2.2). Chi-Square Tests for Homogeneity were also performed for my second analysis which compared the proportions of preferential responses for each experimental group to the ACW vs. UCW and SCW vs. UCW test scenario (Table 2.3) These analyses allowed me to make treatment and control group comparisons, within population comparisons, between

population comparisons, and within experimental group comparisons. These comparisons allowed me to identify whether any experimental group of fish had a significant preference for any particular water source in any testing scenario, and whether common incubation affects/alters the preferential responses of juvenile steelhead to population-specific odors.

Results

For my first analysis, my results show no significant differences in the preferential responses within populations, between populations, or between control and treatment groups (Chi-Square Test for Homogeneity, Critical Value = 0.05, Degrees of Freedom = 1; Table 2.2). Interestingly, one significant result was produced in my second analysis when comparing experimental group Alsea-1's preferential responses to the ACW vs. UCW and SCW vs. UCW test scenario's (Chi-Square Test for Homogeneity, Critical Value = 0.05, Degrees of Freedom = 1; Table 2.3). While this observation produced a significant result, when compared to the results from my first analysis for the same testing scenario, no significant result was found. This suggests that, although more Alsea-1 fish responded preferentially to a particular water source, no consistent pattern of preference was observed when compared to other experimental groups in those test scenarios. Furthermore, twelve assays were performed in this study, and there was a 46% chance of observing at least one significant result (under $\alpha = 0.05$) simply by chance. A common procedure is to adjust the critical value (α) to account for multiple comparisons, through methods such as a Bonferroni correction (Bland & Altman 1995); in this case the critical value would be adjusted to $\alpha = 0.004$, and the single significant difference observed would be insignificant at this adjusted α (p-value = 0.007, Chi-Square Test for

Homogeneity, Degrees of Freedom = 1; Table 2.3). Overall, I conclude that the only "significant result" observed in this experiment was simply an artifact of multiple testing, and I found no evidence to suggest common incubation affects water preference recently emerged steelhead.

Discussion

My first objective in this study was to determine whether juvenile steelhead show chemically-based population recognition behavior immediately following emergence. My results show no such population recognition responses. My second objective was to determine if there were alterations to population recognition behavior from exposing embryos to unfamiliar population odors during incubation. I found no evidence to suggest that the preferential responses of juvenile steelhead to conditioned water sources are affected by common incubation. The overarching finding of this study is that there is no indication common incubation alters preferential responses of the fish to water from their own population or populations they were exposed to during incubation.

It has been shown that the capability of recognizing fish of varying relatedness through olfaction is widespread across salmonids (Brown and Brown 1992; Griffiths 2003). Salmonids may use various odors (chemical cues) to recognize fish of varying genetic relatedness, but how this function directly relates to homing is still unclear (Døving *et al.* 1974, 1980; Quinn and Busack 1985; Quinn and Hara 1986; Quinn and Tolson 1986). Previous studies of these responses have typically tested juvenile salmonids after the initiation of exogenous feeding, and it is clear that "population-specific odors" can be affected by the feed or byproducts from digestion or metabolism of the feed (Courtney *et al*. 1997; Rajakaruna & Brown 2006). Furthermore, odor

concentration in test water, and/or odors eliciting antipredator behavior from handling of fish should be accounted for, or recognized, when performing behavioral testing of this nature (Brown and Smith 1997; Courtenay *et al.* 1997; Quinn and Busack 1985; Quinn and Hara 1986). After accounting for several potentially confounding factors listed above, I chose to test fish in this experiment at the time of total yolk absorption (i.e. at the transition from the embryonic to the juvenile phase of life history, before exogenous feeding).

The most intuitive and biologically relevant explanation for my results is that the chemical recognition capabilities and responses of salmonids develop at a later stage of life. This explanation is consistent with the observations of Courtenay *et al.* (2001) who showed that the behavioral responses of coho salmon fry to kin-specific odors were not altered or affected by exposure during incubation. To determine whether recognition capabilities develop later in life (or from longer periods of exposure) tests comparing the behavioral responses of salmonids at selected times during ontogeny, together with detailed studies of the neurological responses of the olfactory receptor cells to population-specific odors should be performed. Detailed molecular genetic studies of olfactory receptors of salmonids (Johnson & Banks 2011; Johnstone *et al*. 2011, 2012) are likely to be the most productive approach to resolve the question of the timing and tuning of salmonid olfactory receptors. Studies of this nature would provide detailed information on the expression of olfactory recognition related genes, and how these gene expressions affect population recognition capabilities.

Another possible explanation for the behavior that I observed could be that population-specific odors might not be relevant to embryonic steelhead. These odors may

not elicit behavioral responses at this time in their life and may require more/longer exposure for learning these odors, or may not yet be relevant to them (Tang-Martinez 2001). Dittman *et al.* (2015) showed that Chinook salmon are capable of discriminating between different sources of water and they show an innate preference for surface water compared to well water during yolk absorption. My study is the first to examine steelhead olfactory population recognition at this early life stage. Testing juveniles at later life stages following exposure to unfamiliar populations during incubation could provide a positive control to determine whether fish will respond later in life with this same test setup. Information on whether the behavioral test setup used in this study was a factor that limited the behavioral responses of test fish would be useful in fully interpreting the results observed in my study, and could provide useful insight for improving the use of juvenile y-maze testing. It may be that the test fish used in this study were very capable of recognizing population-specific odors, but the test setup used in this study inhibited their responses to these odors in some way.

A great deal remains to be learned of the basic mechanisms, the ecological significance, evolutionary history, and the role olfactory recognition might play in homing and straying in Pacific salmon and steelhead (Keefer & Caudill 2012). There are a few important conclusions to be taken from my study. Many hatcheries utilize incubation stacks, and very little information has been published on potential effects of common incubation. My study is the first to address this concern at this early life stage, and this study provides evidence that common incubation does not appear to have any immediate effects on population recognition capabilities in juvenile steelhead. This is important for future studies testing similar principles at later life stages because it is

possible that population or even conspecific odors may be learned by juveniles early in life and may be of relevance to them at a later time (Tang-Martinez 2001). Future studies should focus on precisely when in ontogeny olfactory population recognition behavior emerges, and determine the points in ontogeny previous to this behavior when species- and population specific odors are learned.

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Figures and Tables

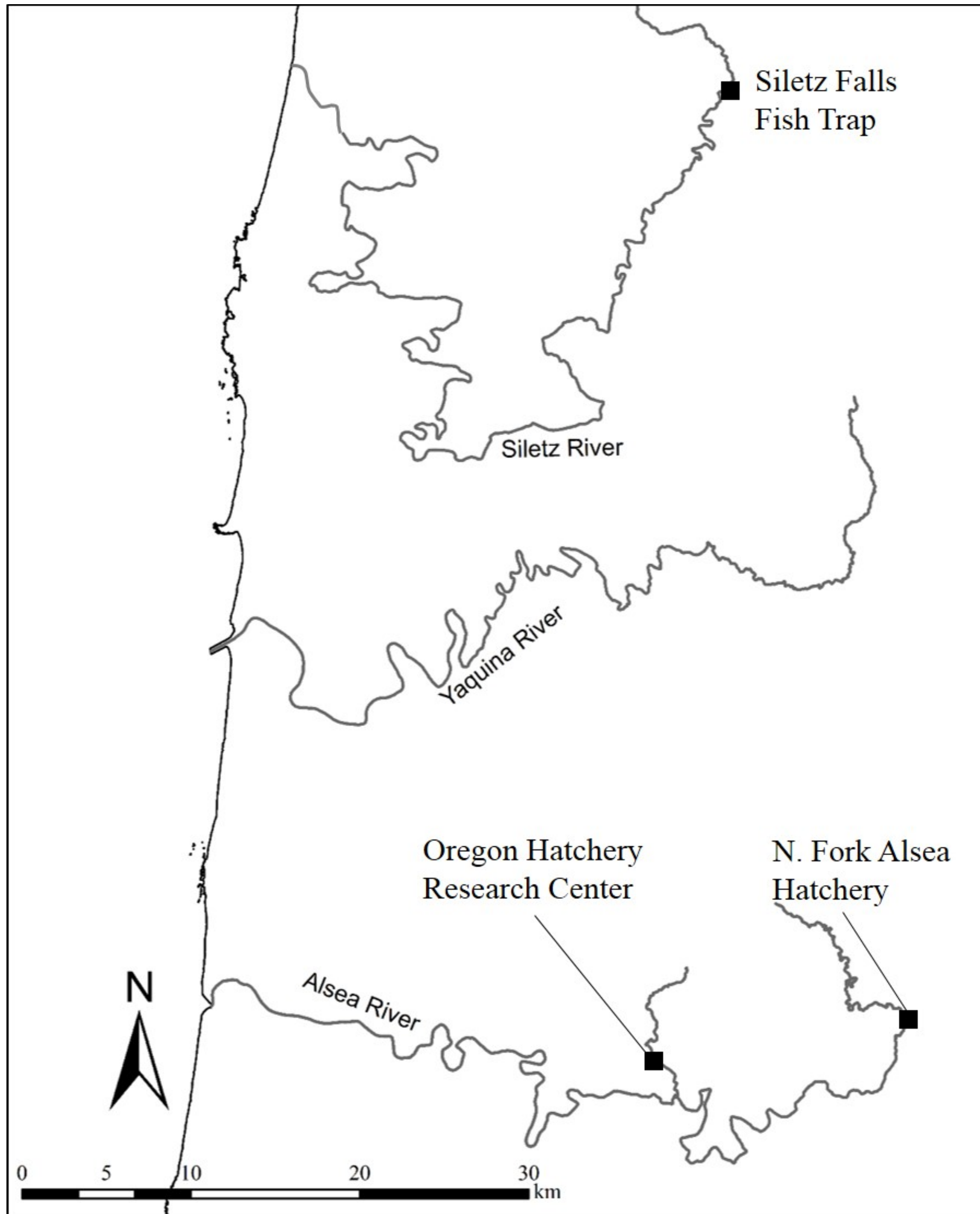

Figure 2.1. Map of collection, spawning, incubation and experimentation sites. Fish collected from the Siletz River fish trap (44° 51.762'N, 123° 43.990'W) were transported to the North Fork Alsea Hatchery (44° 25.283'N, 123° 33.893'W) for spawning. All groups of fertilized eggs were transported the same day to the Oregon Hatchery Research Center (44° 25.283'N, 123° 33.893'W) where they remained until emergence and behavioral testing.

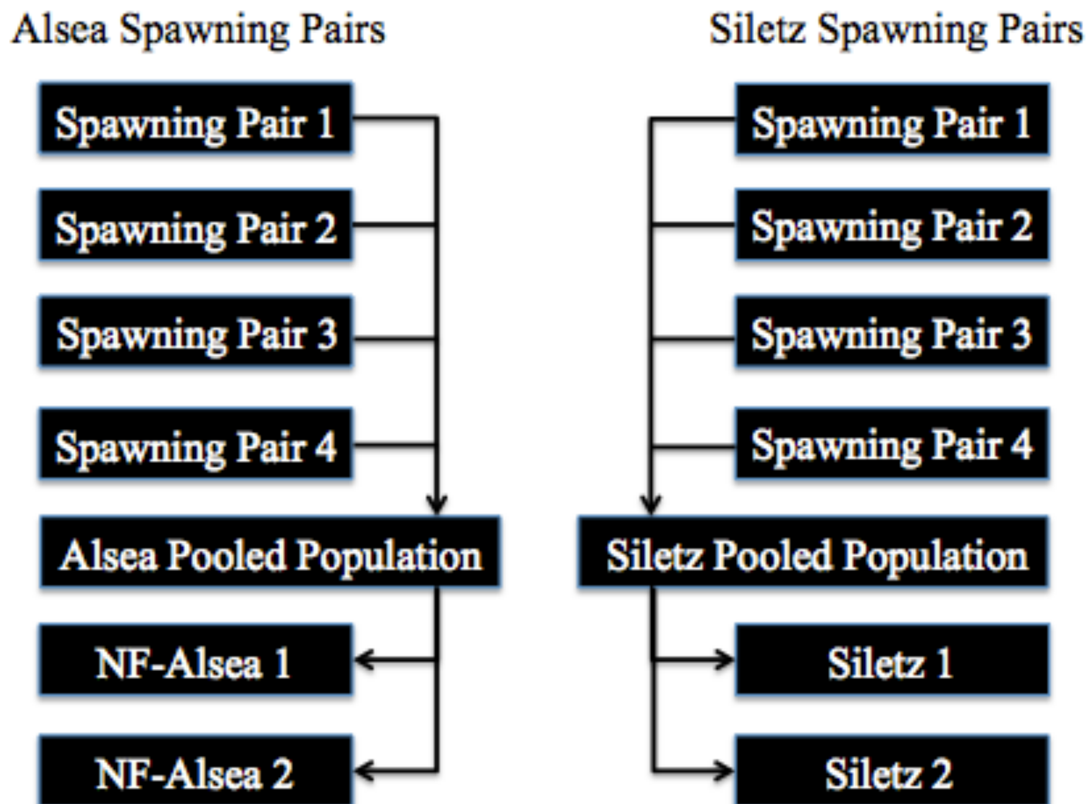

Figure 2.2. Diagram depicting the formation of experimental groups. Offspring of spawning pairs were used to create pooled populations with an equal number of individuals from each family (1,548 individuals per family, 6,192 individuals per pooled population). Pooled populations were subsequently divided into two groups for separate treatments (3,096 individuals per experimental group, four experimental groups in total).

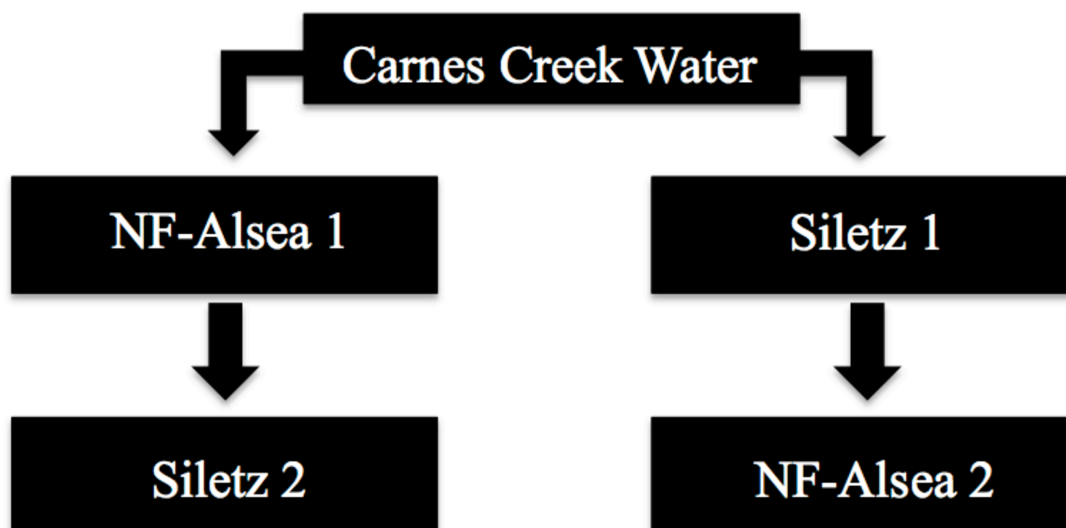

Figure 2.3. Diagram depicting the incubation configuration of treatment groups. Treatment groups NF-Alsea 1 and Siletz 1 were placed on the top rack of the incubation stacks and exposed to Carnes Creek water containing no conspecific odors. Treatment group NF-Alsea 2 was placed below Siletz 1 while Siletz 2 was placed below NF-Alsea 1 so each of these exposure groups received Carnes Creek water conditioned by the populations placed above them.

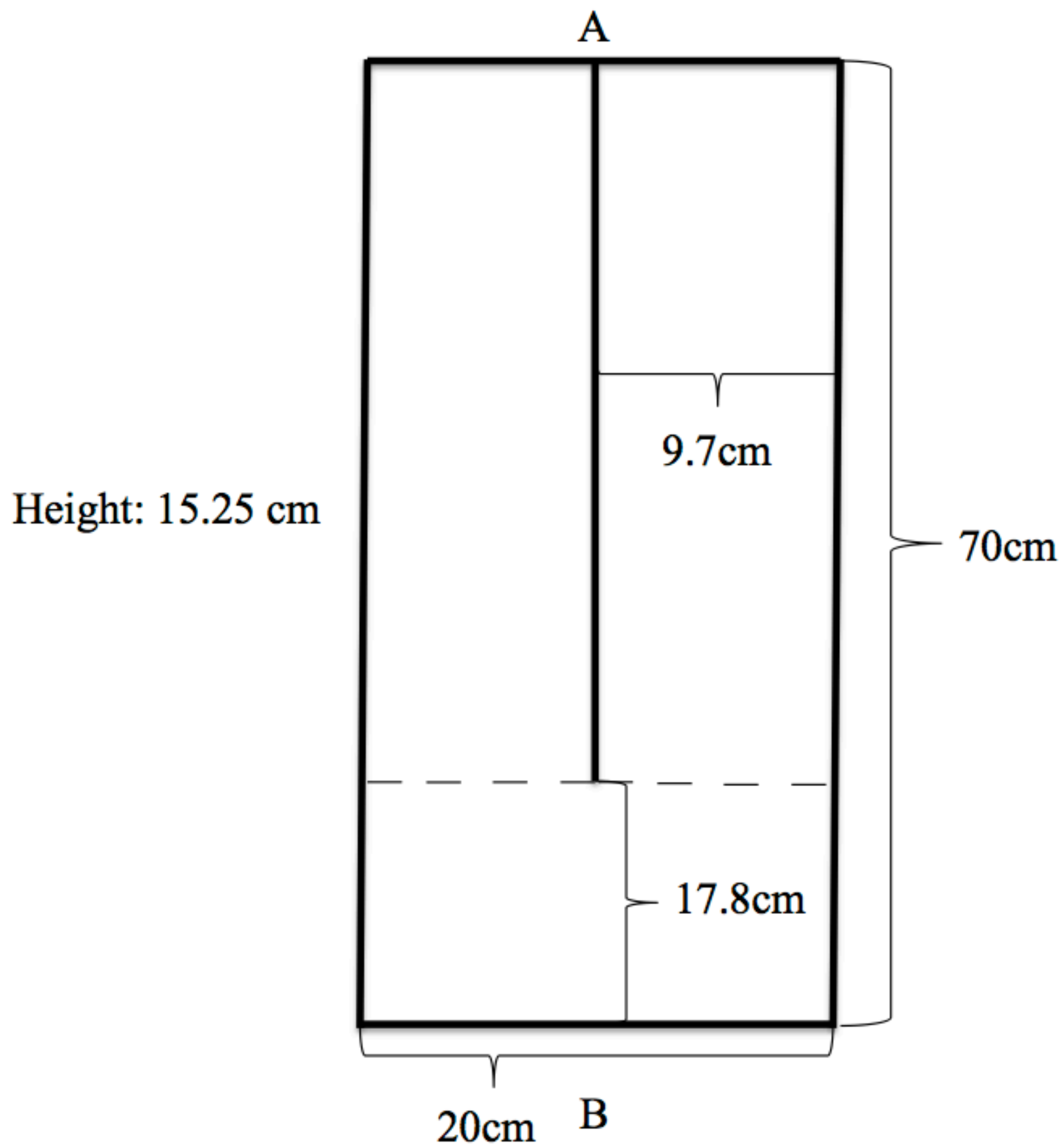

Figure 2.4. Individual y-maze dimensions and design (materials list found in Appendix 1). Test water flowed from the upstream end (labeled A) to the downstream end (labeled B) of the y-maze. Dashed lines represent the location of the movable aluminum 2mm perf-plate gates.

Figure 2.5. Y-mazes and experimental equipment setup (materials list found in Appendix 1). Photo courtesy of Joseph O’Neil.

Table 2.1.

Daily order of behavioral testing scenarios. All behavioral trials performed from May 23rd, 2014 to May 26th, 2014.

	Testing Date			
Time	4/23/14	4/24/14	4/25/14	4/26/14
6am-12pm	ACW vs. SCW	ACW vs. UCW	SCW vs. UCW	ACW vs. SCW
12pm-6pm	ACW vs. UCW	ACW vs. SCW	ACW vs. UCW	SCW vs. UCW
6pm-12am	SCW vs. UCW	SCW vs. UCW	ACW vs. SCW	ACW vs. UCW

Table 2.2.

Pairwise comparisons of each experimental group in each testing scenario. Numeric values represent p-values for Chi-Square Tests for Homogeneity; degrees of freedom = 1.

	Test Scenario		
Comparison	ACW vs. SCW	ACW vs. UCW	SCW vs. UCW
A1 vs. A2	0.44	0.33	0.10
S1 vs. S2	0.31	0.71	0.59
A1 vs. S1	0.64	0.16	0.18
A2 vs. S2	0.19	0.46	0.41
A1 vs. S2	0.55	0.08	0.44
A2 vs. S1	0.77	0.69	0.78

Table 2.3.

Pairwise comparisons of preferential responses in two testing scenarios for each experimental group. Numeric values represent p-values for Chi-Square Tests for Homogeneity; degrees of freedom = 1 (* denotes significant result; critical value = 0.05).

Subpopulation	ACW/UCW vs. SCW/UCW
Alsea 1	0.01*
Alsea 2	0.94
Siletz 1	0.97
Siletz 2	0.89

CHAPTER 3: CHARATERIZATION OF DISSOLVED FREE AMINO ACIDS IN SALMON HATCHERY WATER

Abstract:

Over the last decade, considerable effort has been directed towards understanding the role that dissolved free amino acids (DFAA) serve in salmon navigation as related to identification of natal rivers (homing). Using ultra high performance liquid chromatography, I identified the DFAA composition (types and percent composition) of river water before and after it passed through a research hatchery. Hatcheries are often designated as target return locations (i.e. fishways), and straying of hatchery salmon to unintended locations might be due in part to highly similar DFAA signatures of hatchery fishway water and unintended locations for return (i.e. spawning grounds upstream of hatcheries). I analyzed DFAA compositions at three locations within the hatchery and three locations in the river that provides the hatchery with water for operations. The composition of DFAAs within this hatchery system did not differ significantly from the composition of DFAAs in river water. This raises the possibility that some of the straying observed in hatchery-reared salmon might result from their inability to distinguish between hatchery water and river water. If salmon utilize DFAAs for homing to natal spawning sites, alteration of DFAA composition in hatchery system water during key imprinting and homing periods may be a possible technique to improve return rates of hatchery-reared salmon to their hatcheries.

Introduction:

Salmon hatcheries have been utilized in the Pacific Northwest for many decades to produce salmon for tribal, commercial and recreational fishing as well as for conservation and recovery efforts (Naish *et al*. 2008). Recently, questions have been

raised about the imprinting and homing abilities of hatchery-reared salmonids relative to their naturally spawning counterparts and the ecological and genetic impacts that straying hatchery-reared salmonids may have on these populations (Waples 1991; Quinn 1993; Naish *et al*. 2008; Westley *et al.* 2013). Araki *et al*. (2007a) demonstrated that the reproductive success of naturally reproducing steelhead (*Oncorhynchus mykiss*) could be reduced substantially following interbreeding with steelhead reared in captivity for only one generation. Additionally, Araki *et al.* (2007b) showed that fish that reared for many generations in a traditional hatchery had a lower reproductive success than naturally spawning fish as well as fish from a supplementation hatchery (fewer generations removed from naturally reproducing ancestors). Therefore, managers have focused efforts to limit or eliminate interactions between hatchery-reared salmon and naturally spawning salmon to prevent such interactions that can result in these detrimental effects.

One cause of interactions between hatchery and natural salmon populations is straying by hatchery-reared salmon from their hatchery or their intended return locations (e.g. collection facilities). It is commonly accepted that salmon imprint on their parent stream's chemical signature as juveniles, and identify this stream through olfaction while homing as adults (Hasler & Wisby 1951; Wisby & Hasler 1954; Hasler & Scholz 1983; Dittman & Quinn 1996). One potential explanation for straying of hatchery-reared salmon is that the chemical nature of their targeted return location is chemically similar to that of nearby spawning habitat for naturally reproducing salmon populations. While the chemical or set of chemicals salmon use for imprinting and homing has not been definitively identified, dissolved free amino acids (DFAAs) have been studied extensively as potential home stream odorants, and there is increasing evidence that these

chemicals are relevant to salmonid imprinting, homing and home stream identification (Shoji *et al.* 2000; Shoji *et al.* 2003; Ueda & Tsukamoto 2014).

The salmon olfactory system is particularly sensitive to a range of chemicals including bile acids, skin mucus, steroids, prostaglandins, and DFAAs (Døving *et al*., 1980; Hara 1973; Hara *et al*. 1984; Hara 1994). Masu salmon (*O. masou*) showed similar olfactory responses to natural stream water and water containing DFAAs designed to resemble this stream water (Shoji et al. 2000). Yamamoto et al. (2010) showed that the olfactory responses of juvenile lacustrine sockeye salmon to L-proline and L-glutamic acid were much higher in fish exposed to these amino acids during the parr-smolt transformation (PST). Using electroolfactogram testing (EOG) they demonstrated that juvenile sockeye salmon exposed to a particular amino acid during PST longer than 1 week produced EOG responses much greater than juvenile exposed during that same period for any period of time less than 1 week. Furthermore, behavioral testing suggested artificially imprinted juveniles were not only capable of discriminating between two water sources based on the composition of DFAAs, but they also preferred water that contained the particular amino acid they imprinted upon during PST. Yamamoto *et al.* (2013) expanded upon this idea by demonstrating that adult chum (*O. keta*) salmon are capable of discriminating between two water sources based on the composition of DFAAs, and that they prefer artificial stream water that resembles the composition of DFAAs present during juvenile imprinting periods (spring) as well as adult homing periods (fall) over a control water source. No preference between the artificial and homing water sources was observed.

Many hatcheries utilize filtration and settling ponds to improve the overall quality of river water that comes into their hatchery system by allowing large particulate matter, silt, sand, algae, and other constituents responsible for contributing at least some portion of the DFAAs within aquatic and riverine systems to settle out (Ishizawa *et al.* 2010). Settling ponds are relatively stagnant and open to the environment providing an ideal (and practical) location for the accumulation of organic and inorganic materials while allowing vegetation, algae, fungi, biofilms and invertebrates to thrive before water is filtered and used for hatchery operations. By altering the composition of organisms present within a river, it is possible that the DFAA signature of river water used for incubation and rearing in salmon hatcheries could be altered. However, the degree to which the DFAA signature is altered remains unknown.

The Oregon Department of Fish and Wildlife (ODFW) manages hatcheries with the objective of minimizing the impacts hatchery-reared salmon have on naturally spawning populations of salmon such as competition, predation, and genetic introgression (ODFW 2010). Limited data are available on the stray rates of naturally produced salmon, and whether hatchery-reared salmon truly stray more frequently than those produced naturally remains unknown (Quinn 1993; Waples 1999). However, with the information available on straying of hatchery-reared salmon and the negative impacts associated with resulting interactions with naturally reproducing populations, any amount of straying by hatchery-reared salmon could result in negative impacts on naturally reproducing populations. Therefore, ODFW and agencies tasked with conserving and protecting naturally spawning populations of salmon have made reducing straying in hatchery-reared salmon a major priority. The purpose of my study is to determine if a set of

organic chemicals that are potentially crucial for natal stream identification (DFAAs) are significantly altered by hatchery equipment and processes. The results from this study will help provide an explanation for the highly variable stray rates observed in hatchery-reared salmon to targeted return locations such as hatcheries and hatchery fishways. Based on the results of Ishizawa *et al.* (2010), who suggested that the DFAA profile of a river system do not change over a 24-hour incubation period (i.e. molar % of each individual DFAA stays constant), I expected no change to occur at the hatchery sampling locations because water had a maximum residence time of 2.5 hours in the hatchery, (Ryan Couture, OHRC Facility Manager, personal communication).

While there are a few potential sites in which the types, concentrations and proportions (hereafter; profile) of DFAAs could change within a hatchery setting, I tested the null hypothesis:

H_o: the DFAA profile of river water is not significantly different from river water that has passed through a hatchery (hatchery water).

One possible result from this study would be that the DFAA profiles are not significantly different between river and hatchery samples. This result would provide a potential explanation for a portion of observed straying in salmon that are reared at hatcheries utilizing a fishway as a target return location. The second potential outcome would be that the DFAA profiles are significantly different between hatchery sites and river sites. This result would suggest that the DFAA hypothesis may not provide any potential explanation for the stray rates observed in hatchery-reared salmon, and that observed stray rates are unlikely attributed to the difference in DFAA profiles between hatchery water and river water.

Materials and Methods:

I. Sampling Locations and Sample Collection

This study was conducted at the Oregon Hatchery Research Center (OHRC), a facility that utilizes natural stream water and infrastructure (settling pond, filtration system) similar to those found in most production hatcheries in the PNW. I analyzed water collected from the OHRC and Fall Creek (OHRC's main operation water source) for dissolved free amino acids (DFAA). Three replicate water samples were collected from three locations within the OHRC hatchery and three locations in Fall Creek (Figure 3.1). The Fall Creek sites were just above the hatchery water intake system (site 1), approximately midway between the hatchery water intake system and the outflow pipe (site 2), and immediately downstream of the outflow pipe (site 3) where any change in the DFAA profile of river water would likely be attributed to hatchery outflow water. Fall Creek sites were chosen to identify normal river DFAA levels, which would identify whether OHRC's outflow has an influence on the Fall Creek DFAA profile immediately downstream. The sites within the OHRC were the outflow of the settling pond (site 4), outflow of the 37 µm drum filter (site 5), and water coming from the inflow pipe that supplies the raceways, rearing tanks and incubation stacks (site 6).

The three hatchery sites were chosen to collect samples representative of hatchery water as a whole, and include different points that might influence the hatchery water DFAA profile such as the silting and filtration. Water samples from these sites within the OHRC facility are subject to the normal hatchery operations, but have not circulated through rearing tanks, raceways or incubation stacks due to the possibility that the

presence of fish may alter DFAA profiles. Effects from the presence or concentration of fish and feed on the DFAA profiles of hatchery water were not considered in this study.

I collected all water samples during the morning of 15 May 2014. Water flowing through the hatchery has a longer residence time than in Fall Creek, so samples were collected first from the river sites, working downstream from site 1 (approximately 5 minutes between locations). Then, hatchery water samples were collected starting at site 4. This rapid collection procedure eliminated any temporally dynamic factors that might influence the composition of DFAAs such as turbidity, change in discharge, temperature, or amount of light. River samples were collected along the thalweg approximately 30 cm below the surface of the water. Hatchery water samples were taken in the main current of flow at each site following the protocol of Chen and Welker (2013) (Appendix 2).

All sampling materials were handled using nitrile gloves, changing gloves at each location to prevent contamination or cross-contamination of samples. At each location, a 0.5L wide mouth Nalgene bottle was rinsed thoroughly with sampling location water and then lowered into the water and allowed to fill. When the sampling bottle was removed, a sterile 10mL syringe was used to extract water from the Nalgene sampling bottle, a 0.45 µm PTFE syringe filter was attached to the syringe, and water was filtered into a sterile 15 mL centrifuge tube. The centrifuge tube containing the filtered sample water was placed on wet ice in a cooler for 3 hours and then stored in a -20°C freezer (Appendix 2). Frozen samples were shipped on dry ice to the Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan for subsequent DFAA analysis.

II. UHPLC and Statistical Analysis

Water samples for DFAA analysis were derivatized within 8 hours of thawing. Deriviatization followed the protocol outlined by Lemanski and Chen (Appendix 3). Samples were analyzed for DFAAs using ultra-high performance liquid chromatography (LaChrom Elite UHPLC, Hitachi High-Tech Corp., Tokyo, Japan) via fluorescence detection (Lachrom Ultra L-2485U, Hitachi High-Tech Corp., Tokyo, Japan). EZ-Chorme Elite software was used to analyze fluorescence charts and determine types and quantities of DFAAs within samples (Yamamoto *et al*. 2013). DFAAs in derivatized water samples were quantified by analyzing florescence charts produced by a UHPLC. Machine calibration was performed with known concentration samples. Three technical replicate injections were performed for each subsample to account for machine detection variability (total $N = 54$) and these replicates were averaged to provide a single concentration of each DFAAs in each subsample. Averaged subsample DFAA data were used for statistical analyses (DFAA measurements by site shown in figures 3.5-3.8). Means and standard deviations of each analyte in each subsample can be found in Appendix 4. Two analytes, cysteine and methionine, are known to have high detection variability and low detection precision with UHPLC and thus were not included in this analysis.

To determine whether hatchery water samples differed significantly from Fall Creek water samples, NMDS plots were created to visualize clustering of data and mixed effect analysis of variance (MEANOVA; DFAA ~ Location + (1|Site)) tests were performed for concentrations and percent compositions of each DFAA to determine whether any DFAAs differed significantly between hatchery water and river water. Degrees of freedom for MEANOVA calculations were calculated by using a

Satterthwaite approximation (Satterthwaite 1946). All analyses were performed with R-statistical software (R Core Team 2013) using the Vegan (Okansen *et al.* 2013), lme4 (Bates *et al.* 2014) and lmerTest (Kuznetsova *et al.* 2014) packages. By using these two analyses, I determined if there were any significant differences between locations (i.e. river vs. hatchery) based on the types and quantities of DFAAs.

Results

NMDS plots showed high clustering with low stress (Figure 3.2 and 3.3; stress = 0.1947 and 0.1941 respectively). High clustering of DFAA and subsample points near the center of the plots suggest that DFAAs are highly similar within and among sites. MEANOVA tests provided similar results, and showed no significant differences between hatchery and river water DFAA concentrations or percent composition (Table 3.1 and 3.2; critical value = 0.05). While it has been hypothesized that overall concentrations of DFAA within samples is not as important ecologically as the molar percentages of each DFAA within a sample, neither concentration nor molar percentage were different between sites. Therefore, I was unable to reject my null hypothesis and found no evidence to suggest that the DFAA concentrations and percent compositions differed between river water and water that had passed through the hatchery.

Discussion

When hatchery-reared salmonids are released into rivers to migrate to the ocean and return as adults to target return locations, it is assumed that these fish have imprinted on the chemical nature of the water at their target return location. However, straying might occur whenever the chemical nature of water from the hatchery and spawning

habitat for naturally reproducing salmon are similar. The goal of my study was to improve our understanding of causes of straying by hatchery-reared salmonids by determining if the DFAA profiles of river water and hatchery water (which utilizes river water for its operations) are significantly altered by hatchery equipment or processes.

Several studies have shown that DFAAs appear to be important to salmon for homing and home stream identification (Shoji *et al.* 2003; Ueda 2011; Yamamoto & Ueda 2007; Yamamoto *et al*. 2010, 2013). While this study presents evidence that could help explain some observed straying in hatchery-reared salmon, there is still critical information that needs to be gathered to fully understand how the similarity in river water and hatchery water might relate to straying. For example, it would be useful to quantify and compare DFAA concentrations at the watershed and/or drainage level. Different rivers within a watershed may have very different DFAA profiles (E. Chen, personal communication). Using the same methods and analyses as described here, Chen demonstrated that the DFAA profiles from three different rivers within a single drainage were distinctly different (Figure 3.4; Table 3.3). Given the magnitude in differences between data comparing three rivers to my data, it seems reasonable to suggest that differences in DFAAs between river water and hatchery water are potentially negligible for salmon to properly differentiate between two water sources based solely on DFAA.

As discussed before, differences between hatchery water and river water may be observed, but it is more important to consider whether the observed differences are relevant to salmon navigating back to their natal spawning grounds or target return locations. The minimum amount of change in a DFAA profile that is needed for salmon to distinguish between two water sources, and to what extent a change in the DFAA

profile of a water source is needed to elicit a behavioral response has yet to be explored. However, a large body of information is available on salmon olfactory recognition and sensitivity to varying concentrations of several odorants including DFAA, and how these odorants might relate to homing (Døving *et al.* 1974, 1980; Hara 1973; Hara *et al.* 1984; Hara 1994; Olsen 1989; Shoji *et al.* 2000; Shoji *et al.* 2003; Selset *et al.* 1980; Stabell 1987; Ueda 2011; Yamamoto and Ueda 2007, 2009; Yamamoto *et al*. 2010, 2013). Examining the level of difference needed in the DFAA profile of water(s) to elicit a behavioral response in salmon would provide a critical piece of information for understanding the DFAA homing hypothesis in much greater detail.

The major conclusion of my study is that the DFAA profiles of hatchery and river water at the OHRC did not differ significantly from one another. My findings suggest a hypothesis: that hatchery-reared salmon may stray from target return locations simply because DFAA profiles between target return locations and spawning areas for natural populations are highly similar. My study offers a baseline for a plethora of testable new research hypotheses that should be investigated, such as whether hatchery managers could successfully imprint juvenile salmon to hatchery water that has a slightly altered DFAA profile, and whether fish imprinted to hatchery water with a slightly altered DFAA profile as juveniles would more accurately identify their target return locations as spawning adults. Studies of this nature could provide hatchery managers with novel tools that could reduce stray rates in hatchery-reared salmon, and thereby limit negative interactions between hatchery and naturally spawning salmon.

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Figures and Tables

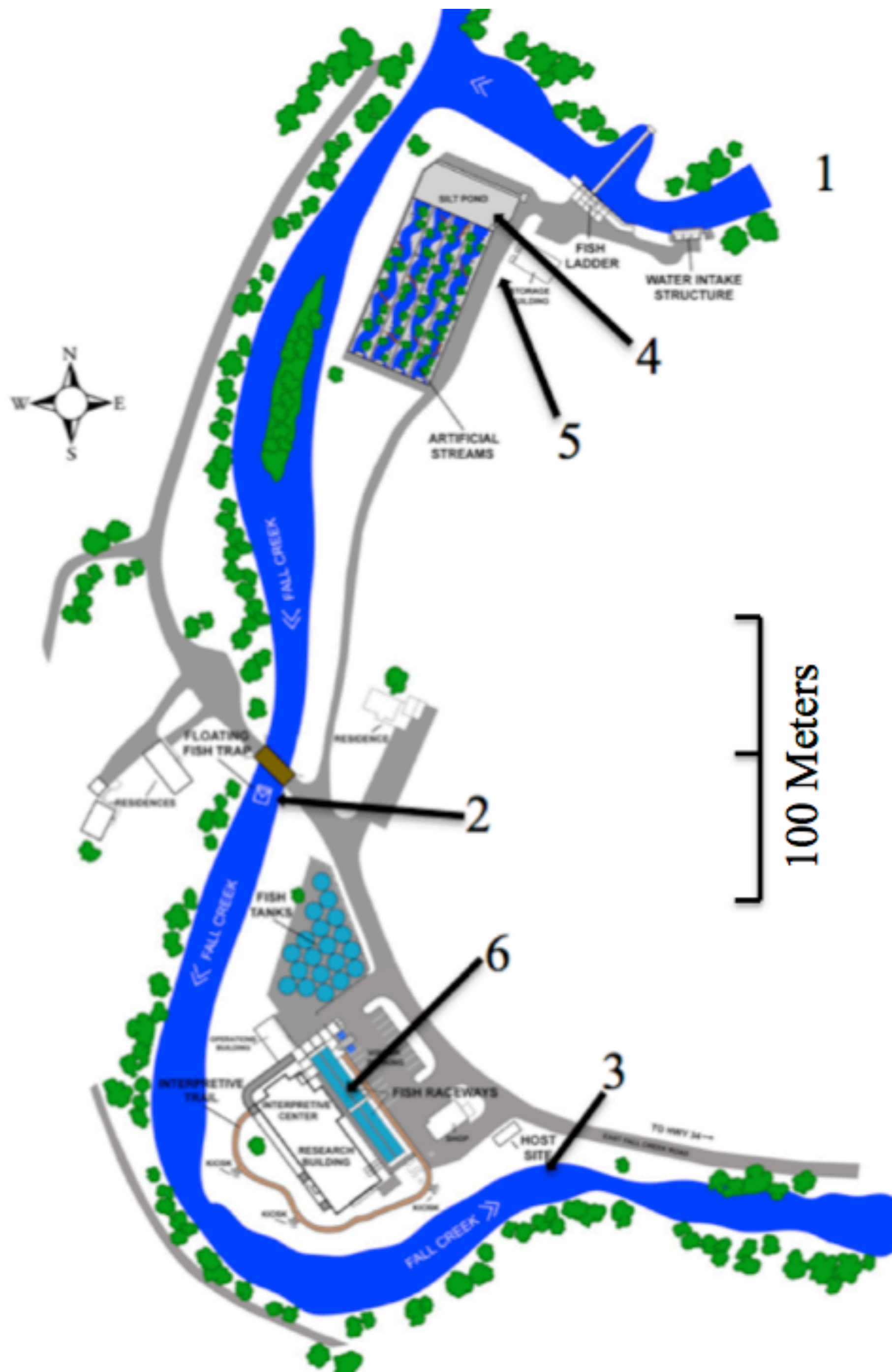

Figure 3.1. Map of sampling sites. Sampling sites 1-3 located within Fall Creek, Alsea, OR. Sites 4-6 located within the Oregon Hatchery Research Center, Alsea, OR (44° 25.283'N, 123° 33.893'W). Utility shed at site 5 drum filter location unmarked on figure.

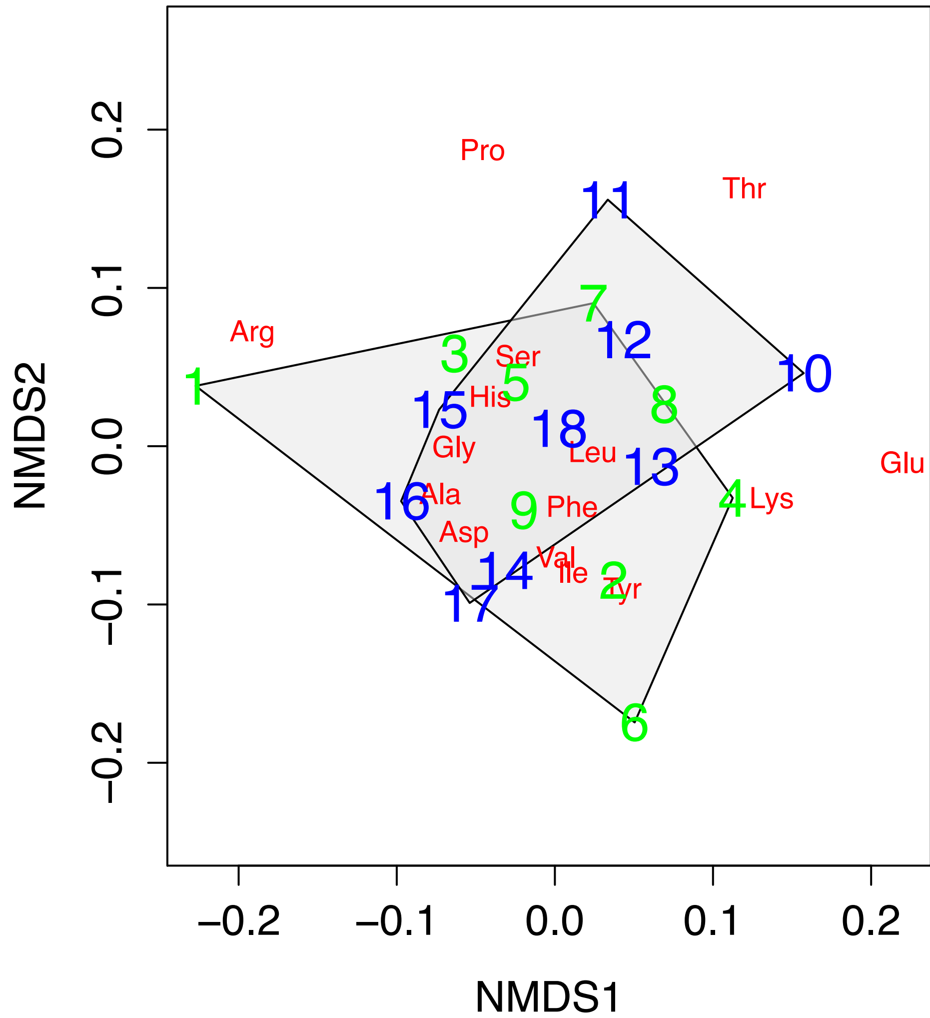

Figure 3.2. NMDS plot of individual DFAA and subsamples based on percent composition data. Subsamples 1-9 (green) represent river water samples; subsamples 10-18 (blue) represent hatchery water samples (stress = 0.1947).

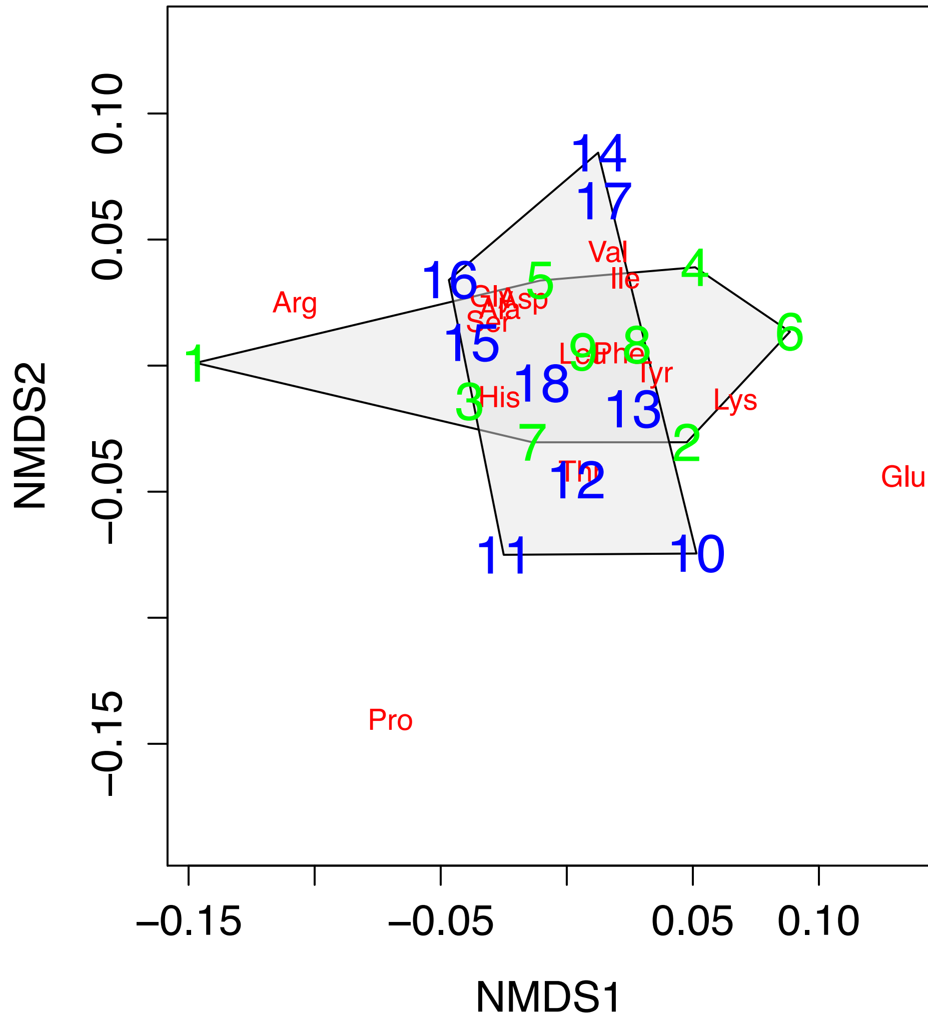

Figure 3.3. NMDS plot of individual DFAA and subsamples based on molar concentration data. Subsamples 1-9 (green) represent river water samples; subsamples 10-18 (blue) represent hatchery water samples (stress = 0.1947).

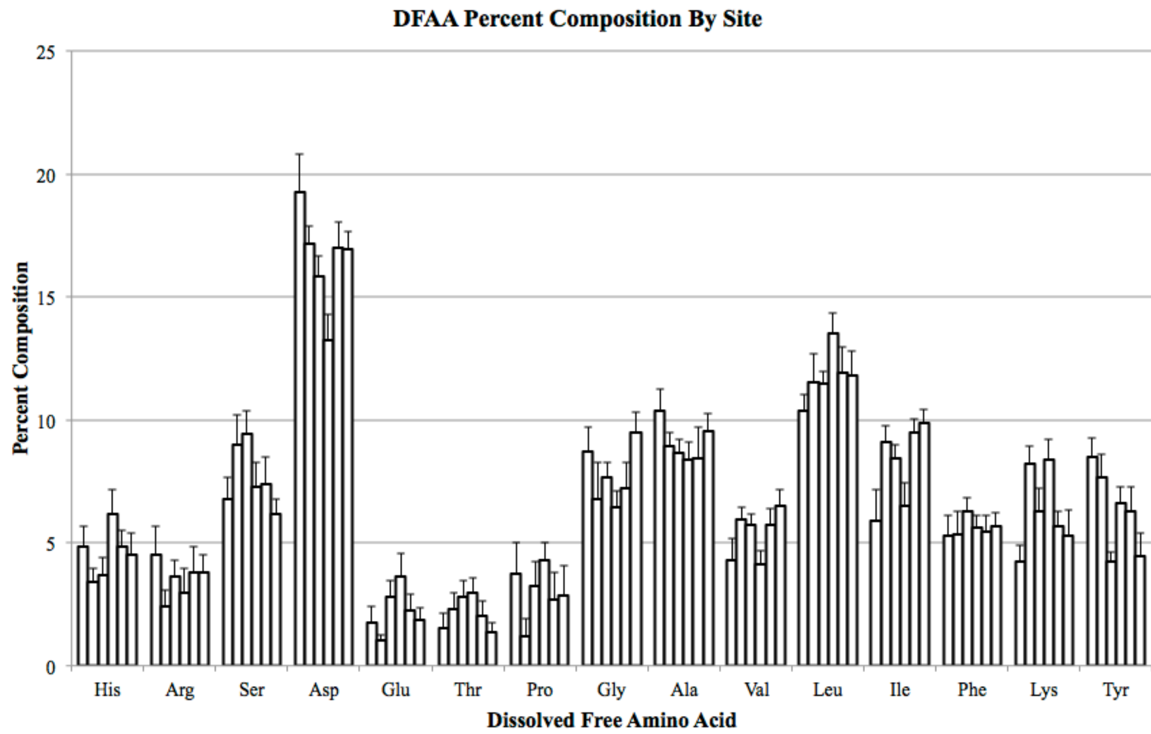

Figure 3.4. Bar chart of mean DFAA percent composition by site (bars represent sites left to right in numerical order). Error bars represent +1 standard error.

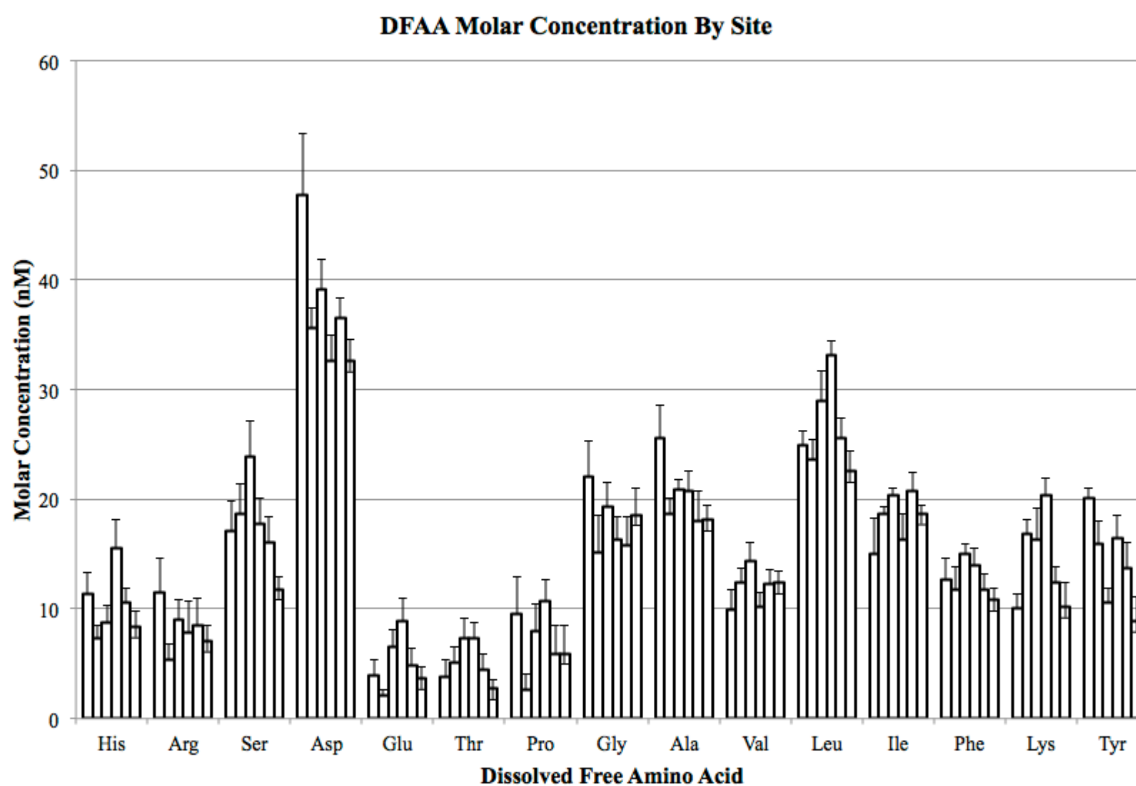

Figure 3.5. Bar chart of mean DFAA molar concentration profile by site (bars represent sites left to right in numerical order). Error bars represent +1 standard error.

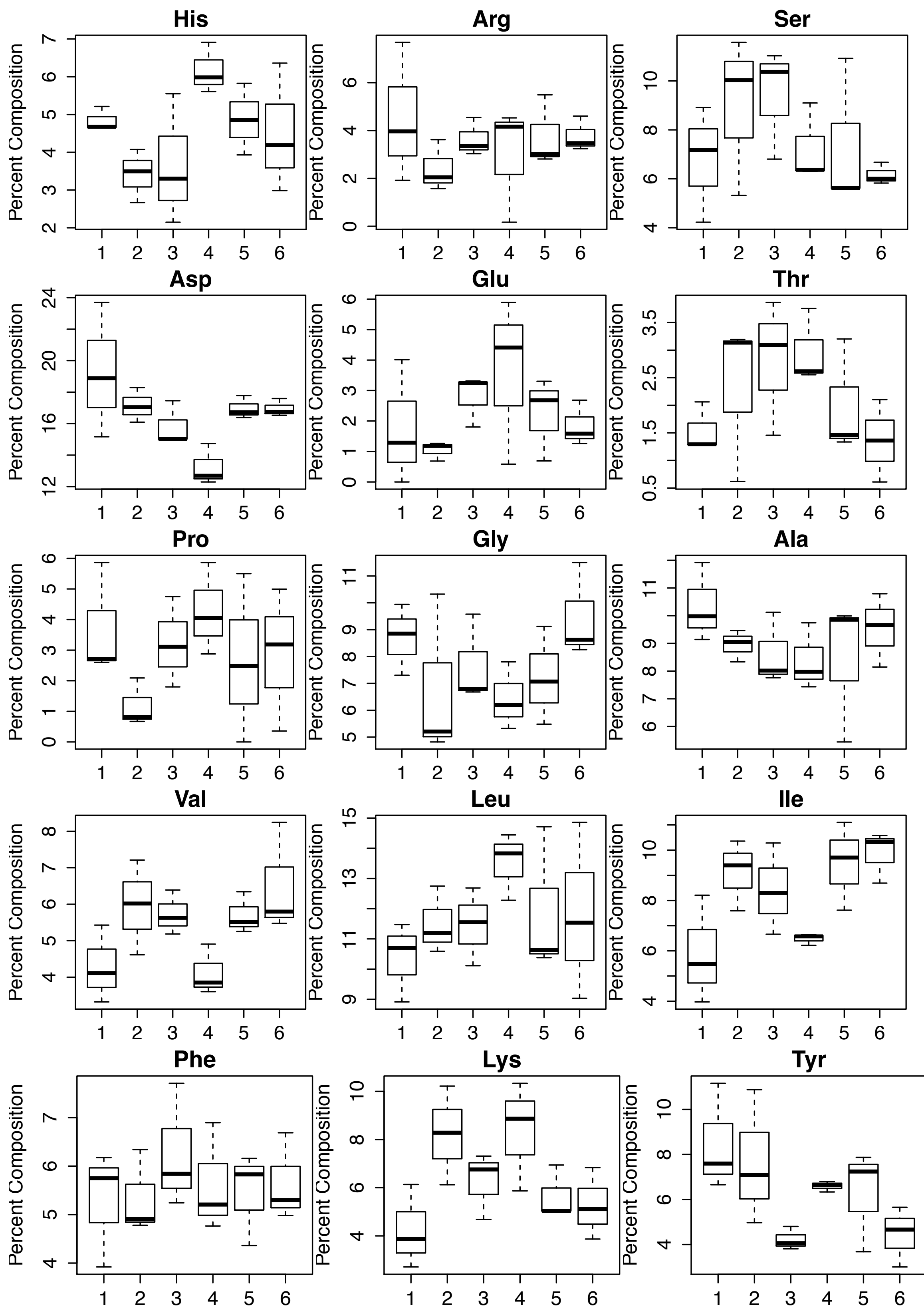

Figure 3.6. Boxplots of mean percent composition for 15 DFAA by site (sites1-6).

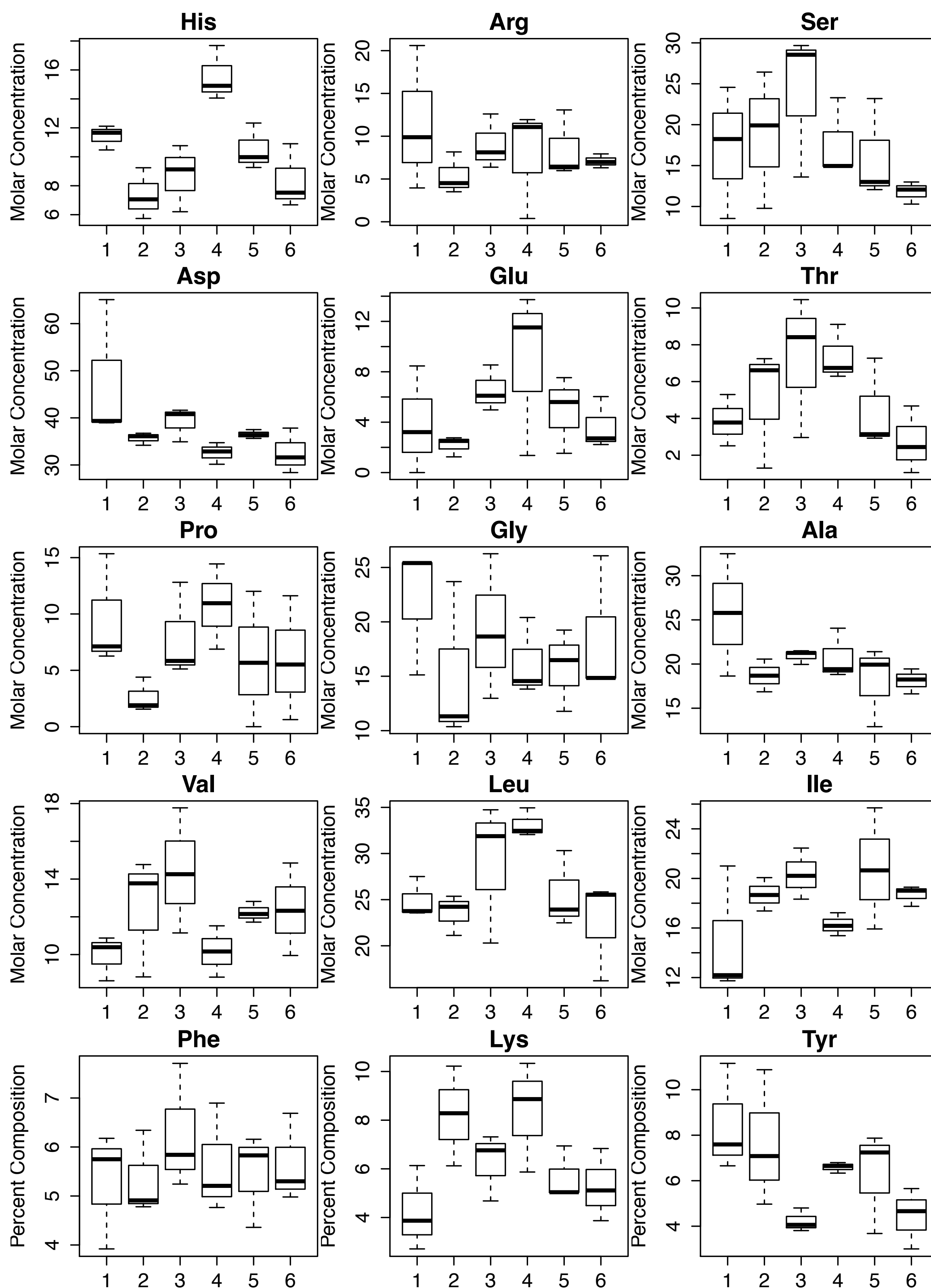

Figure 3.7 Boxplots of mean molar concentration of 15 DFAA by site (sites 1-6).

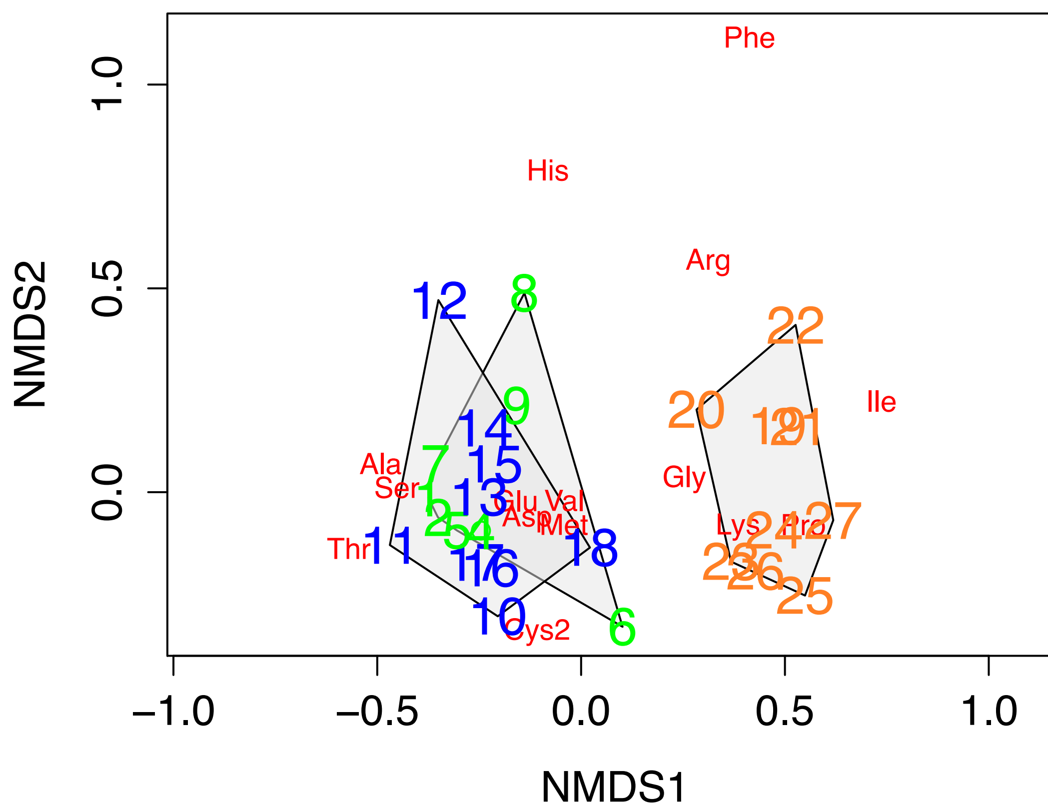

Figure 3.8. NMDS plot of individual DFAA and subsamples based on molar composition data from major salmon migratory rivers in Hokkaido, Japan. Subsamples 1-9 (green; Ishikari River, Japan) represent water samples from river 1; subsamples 10-18 (blue; Toyohira River, Japan) represent water samples from river 2, subsamples 19-27 (orange; Chitose River, Japan) represent water samples from river 3 (stress = 0.1609) (E.Chen, personal communication)

Table 3.1. Table of mixed effect analysis of variance (MEANOVA) results comparing DFAA percent composition profiles by location. No significant differences were observed (critical value = 0.05).

DFAA	Estimate	Std. Error	t value	Pr(>|t|)
His	1.208	0.669	1.806	0.145
Arg	-0.026	0.799	-0.033	0.974
Ser	-1.444	1.065	-1.356	0.194
Asp	-1.686	1.591	-1.06	0.349
Glu	0.699	0.743	0.94	0.361
Thr	-0.111	0.597	-0.185	0.862
Pro	0.544	0.925	0.589	0.588
Gly	-0.013	1.061	-0.012	0.991
Ala	-0.527	0.702	-0.751	0.464
Val	0.12	0.873	0.137	0.897
Leu	1.303	0.822	1.585	0.132
Ile	0.8	1.451	0.552	0.610
Phe	-0.054	0.465	-0.116	0.909
Lys	0.2	1.501	0.133	0.901
Tyr	-1.013	1.462	-0.693	0.526

Table 3.2. Table of mixed effect analysis of variance (MEANOVA) results comparing DFAA molar concentration profiles by location. No significant differences were observed (critical value = 0.05).

DFAA	Estimate	Std. Error	t value	Pr(>|t|)
His	2.326	2.441	0.953	0.395
Arg	-0.845	2.204	-0.383	0.707
Ser	-4.725	3.099	-1.525	0.147
Asp	-6.96	3.836	-1.814	0.144
Glu	1.603	2.022	0.793	0.472
Thr	-0.546	1.688	-0.323	0.763
Pro	0.818	2.65	0.309	0.773
Gly	-1.916	2.619	-0.731	0.475
Ala	-2.763	2.232	-1.238	0.283
Val	-0.679	1.468	-0.463	0.668
Leu	1.256	3.551	0.354	0.741
Ile	0.565	2.048	0.276	0.796
Phe	-0.949	1.383	-0.686	0.530
Lys	-0.094	3.779	-0.025	0.981
Tyr	-2.493	3.532	-0.706	0.519

Table 3.3. Table of mixed effect analysis of variance (MEANOVA) results comparing unpublished DFAA percent composition profiles by river. Five significant differences were observed (* denotes significant difference; critical value = 0.05).

DFAA	Estimate	Std. Error	t value	Pr(>|t|)
His	-0.453	0.975	-0.465	0.656
Arg	0.536	0.742	0.723	0.493
Ser	-6.758	3.246	-2.082	0.076
Asp	-3.938	1.163	-3.385	0.012*
Glu	-1.649	0.59	-2.793	0.027*
Thr	-1.738	0.556	-3.127	0.017*
Pro	5.539	1.489	3.72	0.007*
Gly	1.141	0.741	1.539	0.136
Ala	-4.153	2.107	-1.971	0.089
Val	-0.618	0.358	-1.726	0.128
Met	-1.013	1.495	-0.678	0.520
Ile	0.077	0.063	1.227	0.231
Cys2	-1.366	0.615	-2.222	0.036*
Phe	-0.004	0.011	-0.344	0.734
Lys	2.327	1.073	2.169	0.067

CHAPTER 4: GENERAL DISCUSSION

Hatcheries have been utilized for many decades in the Pacific Northwest but may pose numerous threats to naturally spawning populations of salmon (Waples 1999; Flagg *et al.* 2000; Naish *et al.* 2008; Rand *et al.* 2012). Effects on naturally spawning populations of salmon by hatchery-reared salmon can have many ecological impacts as well as detrimental genetic effects (Berejikian *et al.* 1996; Flagg *et al.* 2000; Weber and Fausch 2003; Lee and Berejikian 2008; Araki *et al.* 2007a, 2007b, 2008). My thesis addressed two unanswered questions about how certain hatchery practices and operations might affect odor recognition and behavioral responses that could influence straying as adults, with the intent to contribute to the improvement of hatchery operations and practices designed to reduce the impacts of hatchery-reared salmon on natural populations.

Odor Exposure During Incubation

While olfactory-mediated conspecific recognition has been shown to be widespread among salmonids and can facilitate certain types of behavior, its precise role in homing by salmonids has yet to be determined. Common rearing can affect the ability to distinguish between kin and populations (Brown and Brown 1992: Courtenay *et al.* 1997; Reviewed in Griffiths 2003), but the importance of exposure timing to unfamiliar conspecific odors had not been fully investigated. Specifically, I examined the effect of exposure to unfamiliar conspecific odors during incubation on population recognition to provide information about effects from hatchery space utilization during incubation as well as potential risks from using certain water sources during incubation. Dittman *et al.* (2015) provided evidence that newly-emerged Chinook salmon preferred their incubation water source over well water or water from another river when subjected to y-maze

testing. Dittman *et al.* (2015) also provided insight into odor imprinting and learning at the earliest stages of life in salmonids.

By considering the principle of odor recognition in newly-emerged salmon, but as it applies to population specific odors, I showed that exposure to these odors during incubation had no significant effect on water preference. My results suggest that steelhead do not respond to population specific odors during the embryonic interval of life. Newly emerged steelhead may be able to recognize population specific odors but these odors may not be relevant at this early life stage (i.e. the odors may not elicit any particular behavioral responses as observed in studies of older fish). My results also suggest that common incubation could be implemented in a hatchery system to conserve incubation stack space without risking potential impacts associated with unintended odor learning. To fully understand whether common incubation affects population recognition and homing behavior, further studies will need to be performed to determine whether common incubation produces long-lasting behavioral effects that do not manifest until later in life.

Dissolved Free Amino Acids in Hatchery Water

A great deal is known about the ability of salmonids to identify their natal stream by olfaction (Quinn 2005). While there have been some attempts to identify the compound(s) that salmon use for home stream identification, dissolved free amino acids have been proposed as likely candidate odorants (reviewed in Ueda and Tsukamoto 2014). Many studies have shown that salmon can and do utilize DFAAs as home stream identification odorants (Yamamoto & Ueda 2007, 2009; Yamamoto *et al.* 2010, 2013). Most of those studies were conducted in Japan, where the concern around straying of

hatchery salmon (and the potential impacts of straying) is quite different than the Pacific Northwest due to management strategies that provide salmon for commercial harvest, and seldom for conservation and/or ecological purposes. Due to this difference in regional salmon management practices, the question of whether hatchery-reared salmonids are capable of distinguishing between the DFAA profiles of target return locations and spawning areas for naturally reproducing salmon has yet to be addressed.

By combining knowledge gained from my Japanese colleagues and an understanding of hatchery management in the Pacific Northwest, I provided useful insight for hatchery managers about how similarity between the DFAA composition in hatchery system water and river water might be a cause of straying of hatchery fish, or low return rates of hatchery fish to targeted return locations such as fishways. My results suggest that the OHRC's equipment and operations do not alter the DFAA profile of Fall Creek water in any significant manner. High similarity between hatchery water and river water is a potential explanation for some straying by hatchery-reared salmonids. Moreover, I propose that in situations where the DFAA profiles of river and hatchery water are highly similar, homing fidelity might be improved by imprinting hatchery salmon to water with slightly altered DFAA profiles. Such an approach should be considered by agencies trying to minimize interactions between hatchery and natural populations.

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APPENDICES

APPENDIX 1: Y-MAZE MATERIAL LIST AND DESIGN

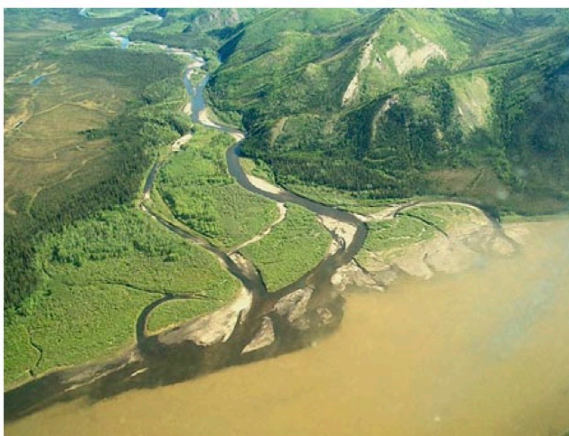
Table 1. Material list displaying materials and quantities of materials need to construct four juvenile y-mazes for behavioral testing. Note: acrylic sheets will need to be cut to size with plastic saw blade.

Name	Material	Quantity Needed	Supplier/Brand	Part # (if available)
White Acrylic Sheet	Colored Cast Acrylic (48”x36”x1/4”)	2	McMaster Carr	8505K96
Head Tank	Rectangular Polyethylene Batch Can 15-Gallon 26-5/8" Long 14-5/8" Wide 13-1/4" High	2	McMaster Carr	1255K65
Hosing	Tygon 2001 Surgical Grade Tubing 3/8” I.D. 1/2” O.D.	Minimum 8’	Tygon	N/A
Acrylic Glue	Sci-Grip #3 (Acrylics)	1-Pint (16oz)	Weld-On/IPS Corporation	N/A
Caulking	920FS High Performance Marine Grade Sealant	10.1 FL.OZ	Bostik Marine	N/A
Saw Blade	10”x80-Tooth Non-Ferrous/Plastic Saw Blade	1	Diablo	D1080N
Water Pump	Submersible Water Pump 115 Volts A.C. 60 Hz 24 Watts 0.3 Amps	1	Aquatic Ecosytems Inc.	E160713
Gates	2mm Perf-Plate	(Dependent upon design)		
PVC Piping	Schedule 40 PVC Piping	(Dependent upon design)		

APPENDIX 2: DFAA COLLECTION PROTOCOL

DFAA collection techniques

Dissolved free amino acid sample collection techniques



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worth of samples. Historical water samples are irreplaceable, so do no take any risks. Liquid nitrogen is prone to spillage when driving over unmaintained rural roads, so ensure that the canister is properly sealed and secured in the vehicle. Once back in the lab, transfer the water samples into a reliable freezer capable of reaching temperatures of at least -20°C (Yang et al. 2009, van Pinxteren et al. 2012)

Step 5: Sample preparation

Fresh samples should be immediately derivatized and processed. Always consult your HPLC technician for proper filtration and handling of fresh samples prior to analysis. If outsourcing your samples, ensure whether or not filtering is required by the contractor.

Frozen samples should be slowly defrosted in a reliable refrigerator to 2°C. The samples should be promptly derivatized and processed after defrosting. If outsourcing your samples, ensure whether or not the contractor prefers frozen or fresh samples.

In any case, create sampling blanks that quantify contamination throughout the entire collection process (See Step 4: Bottling technique). The sampling blank should also be analyzed or outsourced and be used as a baseline in determining actual DFAA concentrations in the river water.

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collecting from a riverbank, a clean reusable cup, bottle, or container attached to the end of a painters-pole can be used to reach into or closer to the main flow channel. A laboratory-grade plastic bottle could serve as a simple and reliable sampling container. If collecting from a bridge, tie the container to the end of a rope so that it can be lowered into the main flow channel. The current can be stronger than it appears and detach your sampling container, so bring spare parts and tools.

CAUTION
Exercise extra caution when working with ropes around waterways. When lowering and elevating the sampling container from a bridge, ensure that your limbs are free from entanglement, which could potentially drag you into the river if you lose control of the sampling container under high flows.

Step 3: Collection method

Sampling containers should be rinsed with sample water 3 to 5 times to reduce contamination. At least three subsamples should be taken at each site to account for within-site variability. The sampler should obtain water at a depth that is practically feasible, which is usually 2-6 inches below surface level, but again, consistency is key. Attempting to dip the sampling container deeper may not be advantageous, as the water collected may not necessarily reflect deep water.

Step 4: Bottling technique

The bottling technique for water sample varies for short-term (up to 8 hours) and long-term (over 8 hours) storage. When done properly, short-term storage does not require freezing (Mopper and Lindroth 1982). The sampler should choose which technique is feasible given location and access to high performance liquid chromatography (HPLC) analytical equipment. Consistency, however, is paramount, and a sampler should strive to stick with the same bottling technique within each waterway.

Even with clean equipment and careful handling, contamination is always present and should be quantified for all techniques. Sampling blanks should be used to account for the contamination from the sampling equipment and vials. Sampling blanks should follow the same rinsing and sampling procedure as the regular samples, but using ultraclean DFAA free water (MilliQ, 18 megaohm, or HPLC-grade).

Step 4a: Bottling technique for short-term storage

If the water sample can be analyzed by HPLC within 8 hours after collection, freezing is not necessary. Throughout the sampling and analysis of DFAAs, an effective way to reduce contamination is by pyrolyzing all glassware at 500°C for 4

hours in a muffle furnace; this is especially advantageous if the sample can be analyzed promptly as it negates the need for freezing, which is often detrimental to glassware. If pyrolyzed glassware is not available, silanized vials can be used as an alternative. Silanization inhibits materials from adhering to the vial surface, thus minimizing adsorption effects and wall-induced hydrolyses. Many laboratory-grade glass vials can be purchased pre-silanized. 10 to 20-mL capacity vials are sufficient for water sampling. Volume should not be an issue as only 20-40 µL of each sample is required for HPLC analysis.

After river water is collected with the sampling container, pour the sample water from the sampling container onto the ground while maintaining a steady flow. After ¼ to ½ of the container is emptied, place the pyrolyzed vial under the steady flow until sufficient quantity is vialed. This method reduces the presence of foreign objects that may have adhered to the edges of the sampling container while the water was being collected. The sample should then be stored at 2°C in a dark environment. An ice-filled plastic cooler with a cover could act as a good storage compartment. Cold and damp vials are extremely difficult to label, so each vial and cap should be properly pre-labeled before sampling to prevent confusion. Always bring plenty of spare vials.

Step 4b: Bottling technique for long-term storage

For long-term storage exceeding 8 hours, the water sample should be frozen to halt microbial and photosynthetic activity that could alter DFAA compositions. However, as glassware is vulnerable to breakage when frozen, 10 to 20-mL plastic vials with screw caps are the preferable option when freezing is necessary. Prior to freezing and vialing, the samples should be gently filtered through 0.22 or 0.45 µm filters (Fuhrman and Bell 1985). Syringe filtering is a simple and inexpensive procedure that can be easily performed in the field. Polytetrafluoroethylene (PTFE) syringe filters, which are available through many laboratory equipment retailers, are certified for HPLC analysis due to its inherent resistance against aggressive chemicals and solvents. 10-mL capacity syringes are sufficient for extracting ample sample volume, and both the syringe and filter should be disposed of after each extraction and never reused. Filtering samples carries two significant advantages: prevention of DFAA release as a result of chemical and mechanical procedures and preservation of HPLC column life.

After river water is collected with the sampling container, use a syringe to extract water directly from the container. Attach the filter to the syringe tip and gently filter the water into a plastic vial for storage. Ensure that the syringe tip and filter remain free from any contact with foreign objects prior to usage. To avoid cracking, the vials should be no more than ⅔ full. The vials should then be flash-frozen, which is best accomplished in the field with either a plastic or styrofoam cooler filled with dry ice or a stainless steel insulated canister filled with liquid nitrogen. When collecting samples, it is generally good practice to bring two liquid nitrogen canisters or two dry ice coolers as breakage of one could result in the loss of a day's

worth of samples. Historical water samples are irreplaceable, so do no take any risks. Liquid nitrogen is prone to spillage when driving over unmaintained rural roads, so ensure that the canister is properly sealed and secured in the vehicle. Once back in the lab, transfer the water samples into a reliable freezer capable of reaching temperatures of at least -20°C (Yang et al. 2009, van Pinxteren et al. 2012)

Step 5: Sample preparation

Fresh samples should be immediately derivatized and processed. Always consult your HPLC technician for proper filtration and handling of fresh samples prior to analysis. If outsourcing your samples, ensure whether or not filtering is required by the contractor.

Frozen samples should be slowly defrosted in a reliable refrigerator to 2°C. The samples should be promptly derivatized and processed after defrosting. If outsourcing your samples, ensure whether or not the contractor prefers frozen or fresh samples.

In any case, create sampling blanks that quantify contamination throughout the entire collection process (See Step 4: Bottling technique). The sampling blank should also be analyzed or outsourced and be used as a baseline in determining actual DFAA concentrations in the river water.

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APPENDIX 3: DFAA DERIVITIZATION STANDARD OPERATING PROCEDURE

Dissolved Free Amino Acid Derivatization for Analysis by LaChrom Elite UHPLC (Hitachi High-Tech Corp., Tokyo, Japan) via LaChrom Ultra L-2485U Fluorescence Detection
(Hitachi High-Tech Corp., Tokyo, Japan).

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Introduction:

This standard operating procedure (SOP) is designed to be a step-by-step guide to derivitizing water samples for dissolved free amino acids (DFAA) via ultra high-performance liquid chromatography (UHPLC and HPLC may be used interchangeably). Although this standard operating procedure is written in a general sense, many of the equipment, methods, and materials are specific to the ***Hitachi High-Tech UHPLC system***, which utilizes a ***fluorescence detector, binary gradient pump, autosampler, rapid-resolution column***, ***column oven***, and ***EZ-Chrome Elite software***. If attempting to use any equipment, methods, or materials other than those listed above and throughout this SOP, please consult the manufacturer on the use of proper equipment, methods, and materials before conducting any analysis. Failure to confirm such things could result in major machine damage and/or very costly repairs.

Procedure:

1.) Derivatizing Dissolved Free Amino Acids (DFAA) in Samples

1.a) Sample Preparation

When derivatizing samples collected for DFAA analysis, follow sample collection procedures outlined in standard of procedure (SOP) for DFAA water sample collection by Ernest Chen and Marcus Welker. Samples that have been collected and will be derivatized within 8 hours of collection should be kept at 2 °C in a dark environment in between collection and deriviatization. Samples that will not be derivatized within 8 hours of collection should be frozen to -20 °C immediately and kept frozen until deriviatization of samples is possible. When derivatizing frozen samples, move frozen samples from freezer to refrigerator set at 2 °C and allow to thaw until no ice crystals are visible in collection containers. Once samples have thawed, reassure that no large ice crystals remain in the samples.

1.b) Deriviatization Methods

When derivatizing samples it is important to minimize light exposure and contamination for extended periods of time with vials uncovered. Attempt to minimize the amount of time that a cap is removed from either collection containers or HPLC grade amber vials (HPLC grade amber vials should be pre-pyrolyzed at 500 °C for 4 hours). Following this practice will ensure that samples remain unaltered and lack contamination. Multiple samples can be prepared at once, but avoid trying to do more than 7 at a time when running triplicate injections and 20 at a time when running single injections. The sequence injection limits will vary depending on system health and environmental conditions, but it is safer to err on the side of caution in reducing sequence sizes. The following steps should be completed for each HPLC sample vial in the order which they are listed and, when necessary, within the certain time limits.

1- Allow samples to thaw to 2 °C and lack ice crystals (if frozen)

2- Place 20 μL of sample into ***HPLC amber vial***(s)
3- Add 160 μL of ***buffer solution***
4- Add 20 μL of ***reaction reagent***
5- Mix via ***touch mixer*** for 10 seconds
6- Submerse vial(s) in 60 °C water bath for 60 seconds to complete reaction
7- Remove from water bath and add 800 μL of ***reaction neutralizer*** to stop reaction.
8- Mix via ***touch mixer*** for 10 seconds

1.c) Storage of Derivatized Samples

Samples that have been derivatized can be stored for long periods (> 1 year) of time if stored in a refrigerator with no exposure to light.

APPENDIX 4: DFAA SITE AND SUBSAMPLE DATA

Site 1

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	12.11	3.75	10.47	2.46	11.66	10.31
Arg	20.60	6.72	3.96	2.96	9.88	8.89
Ser	24.56	6.39	8.54	4.33	18.24	4.49
Asp	65.09	14.16	39.35	9.62	38.95	11.50
Glu	0.00	0.00	8.46	2.74	3.21	3.52
Thr	3.78	6.54	2.51	4.35	5.30	3.82
Pro	7.12	1.64	6.27	10.85	15.34	13.94
Gly	25.46	16.34	15.12	3.76	25.40	3.69
Ala	32.48	5.06	18.65	5.30	25.79	10.18
Val	10.39	6.76	10.88	3.76	8.61	7.49
Leu	23.76	1.90	23.56	2.37	27.50	5.54
Ile	11.74	11.54	12.19	11.64	21.00	5.28
Phe	9.91	6.69	12.23	7.35	15.85	2.34
Lys	7.57	5.85	12.59	0.38	9.99	3.83
Tyr	18.11	2.37	22.73	2.09	19.44	2.80

Site 2

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	7.06	1.10	9.24	1.83	5.74	5.79
Arg	3.51	4.50	8.16	3.91	4.52	4.68
Ser	19.91	6.48	26.43	2.52	9.77	3.79
Asp	34.18	2.88	36.73	4.60	36.09	8.77
Glu	2.51	0.21	2.76	2.24	1.25	1.10
Thr	6.61	4.82	7.24	4.16	1.30	2.25
Pro	1.55	2.69	1.90	2.96	4.39	7.61
Gly	10.37	9.72	23.70	8.12	11.32	9.86
Ala	16.86	3.43	20.56	3.34	18.68	5.91
Val	14.77	3.61	13.77	3.25	8.82	1.73
Leu	25.37	2.14	24.23	8.48	21.13	6.26
Ile	18.66	0.57	17.37	2.86	20.06	1.52
Phe	11.13	9.80	10.92	4.07	13.05	7.05
Lys	16.74	4.10	14.01	3.78	19.84	0.95
Tyr	14.68	4.53	11.27	3.04	21.80	6.22

Site 3

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	9.13	2.46	6.20	8.05	10.77	0.96
Arg	12.60	6.76	8.11	4.58	6.38	3.78
Ser	28.55	8.93	29.67	6.22	13.61	3.52
Asp	41.62	9.52	40.79	7.51	34.92	9.20
Glu	4.98	2.69	8.54	7.64	6.10	3.14
Thr	10.45	6.07	8.41	5.44	2.96	3.15
Pro	12.81	8.06	5.12	8.87	5.83	5.77
Gly	18.65	0.68	26.26	5.91	12.98	2.44
Ala	21.26	3.11	21.50	2.78	19.96	2.72
Val	17.77	5.75	14.26	5.63	11.15	1.69
Leu	31.88	3.01	34.74	7.23	20.30	6.20
Ile	18.33	0.53	22.45	0.99	20.21	2.26
Phe	16.08	1.74	13.95	3.68	15.16	2.87
Lys	20.34	7.29	18.74	8.80	9.89	8.81
Tyr	11.22	1.50	13.02	3.41	7.55	4.12

Site 4

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	14.06	6.29	17.69	12.33	14.91	6.70
Arg	0.39	0.67	11.94	12.15	11.08	4.71
Ser	14.90	3.52	14.95	8.72	23.29	6.17
Asp	34.73	9.24	30.15	8.26	32.87	5.95
Glu	13.73	5.47	1.36	2.35	11.52	1.66
Thr	6.29	6.17	9.11	4.16	6.74	2.43
Pro	6.88	5.30	14.44	3.68	10.95	7.20
Gly	14.56	0.97	13.82	9.19	20.40	5.92
Ala	18.82	4.61	24.06	5.83	19.42	5.87
Val	11.52	6.33	8.81	3.04	10.16	2.50
Leu	32.46	2.50	34.95	6.42	32.05	3.28
Ile	15.38	3.20	16.17	14.03	17.23	0.82
Phe	12.29	2.44	11.54	1.67	18.23	5.69
Lys	24.21	3.75	21.53	3.01	15.30	2.34
Tyr	14.81	4.17	17.47	8.99	17.15	6.32

Site 5

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	9.26	6.00	9.98	0.57	12.33	5.10
Arg	13.07	11.33	5.98	5.29	6.44	5.28
Ser	13.00	1.15	12.06	9.02	23.19	2.63
Asp	37.50	9.34	36.37	5.63	35.65	3.18
Glu	7.53	4.95	5.60	4.85	1.53	2.65
Thr	7.27	3.49	2.92	3.43	3.14	5.44
Pro	5.67	4.92	0.00	0.00	12.01	10.00
Gly	16.48	10.95	19.24	6.48	11.78	5.11
Ala	12.91	12.20	19.93	3.85	21.40	4.68
Val	12.15	3.87	12.82	5.18	11.72	4.53
Leu	23.93	1.01	30.31	4.04	22.49	7.50
Ile	25.69	3.68	15.92	3.85	20.65	0.75
Phe	14.03	6.40	8.81	1.72	12.48	3.14
Lys	16.10	3.13	10.57	5.03	10.62	3.24
Tyr	16.65	4.61	16.82	8.96	7.81	1.47

Site 6

	Subsample 1		Subsample 2		Subsample 3	
DFAA	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
His	10.90	4.48	7.52	6.59	6.68	1.22
Arg	7.93	3.54	6.31	4.69	6.98	6.27
Ser	10.29	3.66	12.07	4.07	12.99	2.94
Asp	28.39	6.02	31.61	2.24	37.83	6.07
Glu	2.72	2.00	2.23	3.03	6.03	4.00
Thr	1.06	1.65	2.44	2.41	4.67	2.61
Pro	5.52	8.93	0.63	1.08	11.62	8.01
Gly	14.84	4.09	14.82	3.96	26.07	7.52
Ala	16.63	3.32	19.45	4.03	18.25	5.42
Val	9.95	3.12	14.85	3.87	12.32	1.63
Leu	25.53	3.59	16.21	2.44	25.83	3.39
Ile	17.75	1.57	19.01	1.49	19.29	4.04
Phe	8.57	2.13	12.03	4.40	11.77	2.05
Lys	6.64	2.54	12.32	6.82	11.59	9.46
Tyr	5.13	4.66	8.41	4.02	13.12	9.56