Salmon olfaction: Odor detection and imprinting in *Oncorhynchus* spp.

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

Master of Science

University of Washington

2008

Program Authorized to Offer Degree:
School of Aquatic and Fishery Sciences

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Abstract

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Salmon display a remarkable ability to undergo long migrations from freshwater rearing grounds to sea and then return to their natal streams to spawn. Olfaction plays a major role in the success of these homeward migrations. Juveniles rear in freshwater and imprint the scent of their natal stream before migrating to sea. Salmon return to the natal stream as adults using olfaction to detect their natal water. People have been aware of this homing behavior for many years, but we still lack a fundamental understanding of the chemical cues available within homestream waters for salmon to learn and discriminate. In order to better understand the specific chemical components that salmon imprint, we first need an assay to assess when salmon can discriminate between two odors.

There are critical windows when salmon are capable of imprinting, but some hatchery practices or land use practices (habitat destruction, dams, pollution, etc.) may impede proper imprinting by juveniles, leading to excessive straying. The release of hatchery or captively-reared salmon into the wild at inappropriate life stages, or after insufficient periods of exposure, may result in elevated levels of straying. Captive broodstock programs have been established to preserve the genetic resources of some threatened and endangered salmon populations. Specifically, a captive broodstock program was established in 1991 for the ESA-listed sockeye (*Oncorhynchus nerka*) salmon in Redfish Lake (RFL), Idaho, to conserve the population. The program reintroduces juveniles at several different stages (eyed eggs, fry, and smolts) directly into the lake so they will imprint before migrating out to sea. However, there has been no research on the effectiveness of these different release strategies for imprinting.

The two primary goals of this research were to: 1) develop an assay that will detect changes in the ventilation rate of coho (*O. kisutch*) salmon fry following the introduction of an odorant using a non-invasive technique, and 2) determine the effectiveness of a captive broodstock program's release strategies for sockeye salmon. The assay development utilized a combination of existing techniques for conditioning in other fish species, however the coho were not successfully conditioned using our protocol. This sparks many questions regarding the ability of coho to be conditioned that must first be addressed before attempting to isolate specific imprinting and homing chemical constituents. For sockeye salmon, we observed a tendency to imprint by all exposure groups, confirming the effectiveness of current release strategies and supporting the hypothesis that sockeye are capable of imprinting earlier than the parr-smolt transformation.

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ACKNOWLEDGEMENTS

The past two years have been an incredible experience, and I have many people to thank for helping me along the way. Dave Baldwin, at the Northwest Fisheries Science Center (NWFSC), was a tremendous asset for program-writing and electrical expertise on the conditioning experiment. Kellii Schurb was an integral part of the sockeye salmon behavioral study, assisting with construction and testing, and Dave Rose provided day-to-day assistance with facility maintenance and fish care.

This research would not have been possible without generous support and funding from the NWFSC internal grant program and the Bonneville Power Administration.

The faculty, staff, and fellow graduate students at SAFS have been a valuable resource. Specifically, Brandon Chasco taught me R programming and Paul Clayton set up several databases for me; they were always available and willing to help.

It has been a pleasure to be a part of the Quinn lab, which has been a welcoming and supportive environment in which to work. Many thanks to Jonny Armstrong, Katy Doctor, Keith Denton, Neala Kendall, Joe Anderson, Josh Chamberlin, George Pess, and Todd Seamons.

I have had a wonderful thesis committee that has supported my work and offered invaluable guidance along the way. It has been a privilege to work with Tom Quinn, who has been an amazing mentor and teacher throughout my academic career. Andy Dittman has had an integral role in developing and implementing my research topics, and his availability and patience have been exceptional. Joe Sisneros was always willing to offer his time and expertise for experimental design and data analysis.

Finally, to all of my friends and family for their support and encouragement: my mom and dad, Megan, Nick, Kelly, Lisa, Chris, Peter, Caroline, Adam, Jaime and many more. You have all been amazing and I cannot thank you enough for everything you do.

General Introduction

Olfaction is an important sensory system, essential for processing biologically relevant information throughout the life history of many fishes. Olfaction plays a role in detection of pheromones associated with breeding, kin recognition, conspecific attraction, and predator avoidance (Berejikian et al. 1999; Johnsson et al. 2001; Stacey 2003). Many fish use the olfactory sense to find or attract suitable mates (McLennan 2004; Plenderleith et al. 2005; Rafferty and Boughman 2006; Johnson et al. 2006), female sea lampreys (*Petromyzon marinus*) have such a strong attraction to water scented with spermiating males that this response may facilitate trapping female lamprey (Johnson et al. 2006). The olfactory sense often controls homing behavior for many species through detection of home site-specific cues. Black rockfish (*Sebastes inermis*) only move tens of meters from their home territory and use olfaction to return to their home habitat (Mitamura et al. 2005), which demonstrates homing is important even on a small scale.

Olfaction plays a role in homing by many species, but it has been studied a great deal in salmonid fishes and especially Atlantic salmon, *Salmo salar*, and Pacific salmon, *Oncorhynchus* spp., (Hasler and Scholz 1983). Indeed, homing is a defining characteristic of the salmon life history. Most Atlantic and Pacific salmon are anadromous; they are spawned in freshwater, embryos hatch but remain buried in the gravel while they complete development, then emerge from the gravel as fry. Depending on the species, juveniles typically spend anywhere from a few days to 1-2 years feeding in freshwater, then migrate to the ocean to feed, and finally return to their natal freshwater site to spawn. Different species vary in their life history patterns and preferred habitats. For example, coho salmon (*O. kisutch*) spend one or two years rearing in their natal stream, sometimes moving to off-channel habitats for feeding and refuge (Peterson 1982), whereas sockeye salmon (*O. nerka*) fry emerge from the gravel and immediately migrate (typically downstream) to a nursery lake to feed for 1-2 years (Quinn and Dittman 1990; Burgner 1991). When a juvenile is ready to migrate to sea, it undergoes the parr-smolt transformation (smolting). This involves a series of

physiological changes, which prepare the fish for ocean conditions (i.e., silvering, sleeker body form, increased sodium-potassium gill ATPase activity for osmoregulation in salt water; Hoar 1976; Folmar and Dickhoff 1980; Dickhoff and Sullivan 1987). After smolting, juveniles migrate to sea to feed for one to four years where they acquire >99% of their adult weight. When they are ready to spawn, salmon begin the long migration home to their natal river. Maturing salmon may travel 2,000-4,000 km from the ocean feeding grounds to their natal freshwater streams to spawn (Quinn 2005). While the mechanisms for orientation at sea are still unknown (Quinn and Dittman 1990), the extraordinary accuracy by which salmon return to natal streams once in freshwater has long been attributed to recognition of olfactory cues (Hasler and Wisby 1951; Scholz et al. 1976; Quinn and Dittman 1990). The homeward migration by adults is often prolonged and arduous, with many obstacles and opportunities to stray. Successful homing is important because it ensures spawning will occur in suitable habitat, which increases survival of offspring (Quinn 2005).

Olfactory cues directing the homing migration must be learned during their early freshwater residence (Hasler and Wisby 1951). Juveniles living in streams and lakes imprint the scent of their natal stream before migrating to sea. Hasler and Wisby (1951) hypothesized that streams must have a unique chemical composition, which persists year after year, and that salmon are able to discriminate between their natal stream and other stream waters. They also suggested that the parr-smolt transformation is a critical learning period of for imprinting (Hasler and Scholz 1983). Several studies, primarily using coho salmon, have been conducted using artificial odorants to examine the imprinting process. Coho salmon exposed to phenyl ethyl alcohol (PEA) or morpholine as smolts and released returned to a tributary scented with the imprinting odor (Scholz et al. 1976; Hasler et al. 1978). Nevitt et al. (1994) determined that maturing coho salmon, exposed to PEA during the parr-smolt transformation, exhibited an increased preference for PEA-scented water in a two-choice arena during the period when fish would be homing. To assess the possibility of additional learning periods in coho salmon, individuals were exposed to PEA as alevins, parr or smolts (Dittman et al. 1996). Only

the smolt exposure group demonstrated a preference for the PEA-scented water in behavioral tests as adults, revealing the parr-smolt transformation to be an important period for imprinting in hatchery coho salmon. Almost the entire body of imprinting work has focused on coho salmon, which are commonly produced in hatcheries. However, it is also important to recognize and determine critical learning periods in other species. For example, learning must occur at earlier periods for sockeye salmon because they typically migrate into a lake as juveniles a year or two before smolting, but home back to their natal stream. In the wild, successful homing involves olfactory learning, at a specific juvenile stage, and discrimination, as an adult, of chemical cues unique to the natal stream water. Despite continued examination of salmon homing using artificial odorants, the specific chemicals involved in olfactory imprinting in the wild are still unknown.

Objectives

This thesis consists of two chapters, each of which addresses one aspect of salmon olfaction. The first chapter reports efforts to develop a classical conditioning assay for juvenile coho salmon, which focuses on the fish's ability to detect and discriminate different stream water sources. Ultimately, we believe this new technique will facilitate determination of the chemicals involved in imprinting and homing. The second chapter is an assessment of imprinting by testing adult sockeye salmon for responses to odors to which they were exposed at different early life history stages. These experiments are designed to further our understanding of the role of olfaction in salmon life history.

Chapter One: Development of a bioassay for natural odor detection and discrimination in salmonids (*Oncorhynchus* spp.)

Introduction

Fishes have an acute sense of smell, and olfaction is important in virtually every aspect of their lives (e.g., feeding, reproduction, parent-offspring interactions, aggregation, migration, and predator avoidance: Hara 1992). With regard to migration and homing, Hasler and Wisby (1951) used a conditioning paradigm to demonstrate that bluntnose minnows (*Hyborhynchus notatus*) used olfaction to discriminate between water from two different creeks. This experiment led to a series of experiments demonstrating that salmon homing is an olfactory-mediated process (reviewed by Hasler and Scholz 1983). The unique characteristics of water from a specific, local source are used by homing salmon to identify their natal stream, as revealed by numerous field experiments (Jensen and Duncan 1971; Johnsen and Hasler 1980; Hansen and Jonsson 1994). Decades have passed since this early work yet we still do not know the chemical components involved in imprinting and homing. Existing methodologies are not sufficient to examine the complex olfactory characteristics of water chemistry.

A classic behavioral assay used to study salmon olfaction is a two-choice Y-maze but using this approach to assess homing in adult salmon is logistically difficult. Rearing fish to maturity is expensive, labor intensive and time consuming. Physiological tests, such as the electro-olfactogram, provide information regarding odor detection, but lack the ability to test odor discrimination. This method is also extremely invasive and ultimately results in death of the fish. Both methods have the power to determine whether a fish is able to detect a particular odor, but neither provides the ability to test fine-scale water source discrimination. The proposed research was designed to address these shortcomings by developing a more reliable and comprehensive assay for odor detection and discrimination in salmonids.

The model for development of an odor detection and discrimination assay with salmonids was the classical conditioning paradigm introduced by (Pavlov 1927) for learning and memory experiments with dogs. Previous studies have used this classical conditioning model to assess different behaviors in various fish species. Predator avoidance behaviors are often assessed by pairing an alarm substance (i.e., skin extract of a conspecific) with a predator odor (Berejikian et al. 1999; Scholz et al. 2000; Korpi and Wisenden 2001). Conditioning has also been used to determine whether fish can detect particular chemical cues in their environment (Valentincic et al. 2000; Leduc et al. 2007) or the effect of toxins on the olfactory system (Scholz et al. 2000). Several studies have paired light with either food reward or electric shock to assess learning in juvenile rainbow trout, *Oncorhynchus mykiss*, (Parkyn et al. 2003), medaka, *Oryzias latipes*, (Eisenberg et al. 2003), and goldfish, *Carassius auratus*, (Yoshida et al. 2004). Hasler and Wisby (1951) conditioned bluntnose minnows (*Hyborhynchus notatus*) by pairing two stream water sources to either a positive (food) or negative (electric shock) reinforcement. The two stream waters were then introduced simultaneously at each end of an aquarium and the fish's position in the tank was monitored prior to, and during, stream water introduction. Successful conditioning and discrimination was indicated when the fish swam toward the positive stream water and away from the negative, but it took over a month of training and only assessed behavioral responses, but they were not able to assess physiological responses simultaneously.

We sought to develop a new experimental method incorporating behavioral monitoring with the ability to record quantifiable, physiological response data. Several studies have used heart rate or ventilation rate as a proxy for stress (Cairns et al. 1982; Laitinen et al. 1996; Johnsson et al. 2001; Hawkins et al. 2004; Brown et al. 2005; Sundstrom et al. 2005) or toxin effects (Gerhardt 1998; Gerhardt et al. 1998; Belanger et al. 2006) on various fish species. A few studies have used physiological monitoring of heart rate or ventilation rate to assess conditioned responses (Hawkins and Johnstone 1978; Morin et al. 1987; Morin et al. 1989; Vogel and Bleckmann 2001), and these

studies required the fish be anesthetized or have electrodes surgically implanted for testing.

The main objective of this project was to design and evaluate a conditioning and testing protocol that allows monitoring of ventilation rate in free-swimming salmon using a known odorant. The development of this assay combined methods from classic behavioral tests and modern physiological tests to gain a deeper understanding of salmon olfaction. Ultimately, our goal was to develop a new technique that would permit us to do further experiments to identify the natural water sources learned by coho salmon.

Methods

The experiment took place at the University of Washington (UW) Research Station at Big Beef Creek (BBC), WA. This site was chosen for access to water from a well that is of constant temperature (10°C $\pm$ 1°) and pH (7.2). These values are very suitable for rearing salmon and the lack of variation is also important for olfaction tests. Exposure to water with a low pH measurement (<6.0) has been linked to impaired olfaction in salmon (Moore 1994). In addition, BBC has very few electrical devices that would interfere with the ventilation signal and so provided an electrically "quiet" environment for the tests.

Fish and maintenance

The experiment used 400 coho salmon parr for conditioning and testing. Eyed eggs were acquired from the George Adams Hatchery, on the Skokomish River in Hood Canal, WA. They were transported to and reared at the BBC Hatchery in a 15-L trough supplied with well water under a constant 12light:12dark photoperiod. Flow was set to encourage upstream orientation, but allow for refuge under a cover on the upstream portion of the tank. Fish were fed *ad lib* several times throughout the day for the first month and gradually shifted to fewer feedings later in the day. While testing was in progress, they were fed only once at the end of each day. Experiments began when fish reached at least 40 mm in fork length. All testing took place over a 1-month period in late spring following emergence. The nature of the recording equipment used to monitor ventilation rate necessitated a compressed time frame to ensure that the fish being tested

were of approximately equal size. Some of the parameters for the protocol had to be re-established because of a size difference between the fish used in a pilot study and those used for testing (on average, 51 mm and 1.5 g, compared with 85 mm and 8.0 g, respectively).

Tank design

Conditioning and testing were carried out in four experimental tanks (50 x 20 x 10 cm) housed in the same room and supplied with well water. Experimental tanks were constructed of white Plexiglas (Fig. 1.1) and water was supplied to each tank via Tygon tubing with the flows set daily at approximately 2 L/min. Each tank had a 17.5-cm long opaque tube (3-cm inner diameter) with removable stainless steel mesh electrodes at the upstream and downstream ends. Individual fish were placed inside the tube for testing and the electrodes secured in place. This design provided the most uniform and finite odor exposure, compared to several different flow regimes that were evaluated. A blind was constructed between the tanks and the observer to prevent any behavioral response to the observer's movements. Experiments were run during the day under the same photoperiod as the rearing conditions.

Protocol Development

Conditioning Trials. — Many of the variables for this study had to be determined before beginning any conditioning trials. To determine the necessary acclimation period for the fish's ventilation rate to settle down and plateau, 10 fish were placed in the experimental tank and left undisturbed while their ventilation rate was recorded for 80 min. On average, a resting rate of approximately 1.6 Hz was achieved by at least 45 min, so the acclimation period was conservatively set at 45 min. The magnitude of voltage was based on previous studies (Morin et al. 1989; Eisenberg 2003), but was also verified for this setup. Fish were placed in clear PVC tubes identical to the opaque test tubes and monitored with video cameras to determine the appropriate voltage. The shock started at 0.5 V and was incrementally increased by 0.5 V until the fish's response switched from voluntary (i.e., turning around or tail flick) to involuntary (i.e., a C-start response). A 2.5 V AC shock appeared to be strong enough for the fish to react

but was not harmful. Also, the timing of odor and shock delivery was determined to ensure that the fish experienced the odor and the negative stimulus simultaneously. Dye tests were conducted in each tank so that the odor reached the front wall of the fish enclosure 2 sec after it was turned on and it reached the back of the enclosure 9 sec after being turned on.

For each conditioning run, a 5 month old coho salmon parr was placed into a test tank and allowed to acclimate for 45 min, at which point a 10^{-6} M concentration of phenyl-ethyl alcohol (PEA) or L-Arginine was metered into the tank by a peristaltic pump such that the odor fully mixed with the inflowing water. Previous imprinting studies have used PEA because it does not cause an innate behavioral response in salmon (Hasler and Scholz 1983; Dittman et al. 1996), but acts as a behavioral attractant following imprinting, and L-Arginine has been identified as a potent odorant for fish (Dittman et al. 1996; Shoji et al. 2003). During the conditioning runs, a two-second, 2.5 V, square wave shock was delivered 9 seconds after the odor delivery. As mentioned before, the fish was enclosed in the test tube by stainless steel mesh walls, which serve a dual purpose as electrodes for: (1) delivering the electrical stimulus, and (2) recording the electrical signal of the fish's opercular movements. Delivery of the unconditioned stimulus (shock) was manually controlled using a LabView program, Pulse Generator. The duration between conditioning runs was set at 2 min, based on the protocol described in Morin et al. (1989). Groups of 20 fish were trained individually with 5, 10, 20, or 30 runs to determine the minimum number of conditioning runs necessary to elicit a conditioned response. For each group of 20 fish conditioned with odor-shock associations, 20 control fish were tested with equivalent number of runs but with only the odor exposure and no shock. The controls were needed to demonstrate that the fish had no innate behavioral or ventilatory response to PEA or L-Arginine.

Test run. — After each fish had been conditioned with the prescribed number of odor/shock associations, it was tested for conditioned responses. The test was a two-second pulse of PEA (or L-Arginine); ventilation rate and behavior were monitored before, during and after exposure. The expected result was a branchial suppression

response (BSR), a decrease in ventilation rate following the stress of electric shock (Xu 1997). The percent change in ventilation rate was calculated as follows: [100 x (1-pre/post)], where pre is the rate (opercular movements/sec) for the 10 sec immediately prior to odor exposure and post is the rate for the 10 sec beginning 9 sec after the odor delivery (i.e., when the fish would first detect the odor). The time periods chosen correspond to the fish's resting ventilation rate (pre) and the time when the shock would have been delivered (post). Fish were only conditioned and tested once to prevent any effect of learning or memory extinction that may have confounded conditioned responses. After testing, fish were euthanized with a lethal dose of tricaine methanesulfonate (MS-222).

Electro-olfactogram

The success of this assay depends on the fish's ability to detect the odor used for training. Therefore, electro-olfactogram (EOG) testing was conducted following trials to verify that these coho salmon were able to detect PEA and L-Arginine. EOGs are a way to measure olfactory sensitivity to a particular odorant by summing the receptor-generator potentials (Sorensen et al. 1995; Cole and Stacey 2006). Methods were modeled after Baldwin and Scholz (2005) (Figure 1.2). A fish was anesthetized in a 50 mg/L MS-222 solution and injected with a 3 mg/ml gallamine triethiodide solution to paralyze the muscles before careful removal of the nares covering. The fish was then placed on the EOG apparatus and acclimated for approximately 30 min with artificial freshwater (AFW) flowing across its gills and the olfactory epithelium (Baldwin and Scholz 2005). Chilled background water was continuously perfused across the olfactory epithelium at all times while the fish was on the apparatus except during the odor pulses. After acclimation, the electrodes were positioned and AFW applied to the olfactory epithelium until the baseline stabilized. Each fish was exposed to an ordered series of odor pulses approximately 2 min apart: AFW (control), 10^{-5} M L-Serine, 10^{-6} M PEA, 10^{-5} M PEA, 10^{-6} M L-Arginine, 10^{-5} M L-Arginine, and 10^{-5} M L-Serine. Responses were first measured for a pulse of L-Serine to determine proper electrode placement. Once the electrodes were positioned, odor pulses were delivered and the magnitude of

response (mV) recorded for each odor. EOGs were conducted on four fish from the coho salmon population used in the conditioning study.

Data acquisition and analysis

A computer monitored ventilation rate of four fish simultaneously and the raw data were backed up daily to an external hard drive. The electrical signal from the branchial muscles of the fish was detected by the electrodes, amplified, and routed to the computer. A LabView 7.0 program, Acquire, (written by David Baldwin, NOAA-Fisheries, 2006) recorded the amplitude and frequency of branchial activity for the 30 sec immediately prior to odor exposure, during exposure, and 90 sec post-exposure. Previous studies determined that the electrical signal of fish ventilation is approximately 0.5-4 Hz (Laitinen et al. 1996; Gerhardt 1998; Hawkins et al. 2004), so a bandpass filter was set for 0.5-5 Hz.

Because the exact timing of response to the shock in conditioning runs was unknown, the time period of expected ventilatory response had to be identified. This was done in two different ways. First, instantaneous frequency was plotted for individual fish for the 10 sec before and 20 sec following the odor delivery. Second, the time window of greatest change in instantaneous frequency (i.e., greatest slope) was calculated for several different time bin lengths. The significance of change in ventilatory rate following odor exposure was evaluated by a paired t-test of the pre- and post-ventilation rates.

Results

The electro-olfactogram results confirmed that this coho salmon population was capable of detecting both PEA and L-Arginine at the concentrations used in the conditioning study (Fig. 1.3). The responses show a strong negative potential for both odors, indicating this population had receptors that were activated by the test odors and the fish were not anosmic. Although EOGs were conducted on only four fish from the test population, the results were unequivocal evidence that the test odors were detectable by these fish.

We assessed the conditioned response by determining the instantaneous frequency before, during and after odor exposure in the test run. This analysis revealed no consistent ventilatory response to the odor, indicating no apparent conditioned response (Fig. 1.4). We also examined the time of greatest change in instantaneous frequency for many different time bin lengths using a sliding window approach. The slope was calculated as: $[frequency_i\text{-}frequency_{(i-n)}] / [peaktime_i\text{-}peaktime_{(i-n)}]$ for n in 1-10 time steps. By plotting the time of greatest change in ventilation for all fish, we expected to see a pattern of decreased ventilation rate following odor exposure. However, we observed no consistent patterns with any response window examined.

Because there did not appear to be a consistent time period or duration of response, we chose to examine a 10 sec window corresponding to when a fish would have experienced the shock and odor simultaneously for the "post" average ventilation rate and compare this to the 10 sec immediately prior to the odor delivery. The selection of these windows was based on previous literature values (Morin et al. 1989). We observed no difference in the average percent change in frequency from pre- to post- odor exposure for either control or treated fish (Figs. 1.5 and 1.6). We hypothesized that there would be no change in ventilation frequency for control fish, but that treatment fish would decrease their ventilation frequency in response to odor presentation, and that the response would increase in magnitude with more conditioning runs.

The pre- and post- ventilation rates were evaluated as a paired sample for individual fish. Paired t-tests, conducted for each group, treatment and test odor (Table 1.1), revealed no significant change in ventilation with control or treatment fish for any of the four groups with PEA or L-Arginine. This indicated the absence of a conditioned response for any treatment level, regardless of the test odor used.

Discussion

The goal of this experiment was to develop a new, robust assay for assessing odor detection and discrimination in salmonids. Using a combination of techniques, we sought to design a new protocol that would allow measurement of ventilation rates for free-swimming fish and use this to quantify the conditioned response of coho salmon trained

with an odor and negative stimulus (shock) association. However, collecting data from free-swimming fish was challenging. Whenever a fish swam erratically, the ventilation signal was lost in the "noise" of the larger electrical signal generated by the fish's tail muscles. To reduce their mobility, fish were then placed in a PVC tube for testing. The result was a much cleaner ventilation signal, but the responses could no longer be validated with video recording.

After reviewing data from all treatment groups for both odors, there did not appear to be a conditioned response using this protocol (Figs. 1.5 and 1.6). The most likely reason for the lack of conditioning is that the timing of the odor/shock presentation was not precise enough to cause any associative learning with the odor. Because the fish enclosures were 17.8 cm long, a fish could be exposed to the odor for a full 7 sec before receiving any shock. Alternatively, a fish at the downstream end of the tube might only experience the odor for 1 sec before being shocked. It is also possible that there were too few conditioning runs, but several studies have shown conditioned responses in fish after 1-20 conditioning trials (Berejikian et al. 1999; Valentincic et al. 2000; Korpi and Wisenden 2001). Several studies (Morin et al. 1987; Eisenberg et al. 2003) performed training in blocks of 5 or 10 trials with periods of 1 hour to several days between trainings, so possibly the rapid training and testing sequence dampened a conditioned response because there was not enough time to recover.

A pilot study was conducted on a few fish at the Northwest Fisheries Science Center (NWFSC) hatchery in the spring of 2006 to determine some of the initial parameters before conducting the study in 2007. Preliminary results indicated that some fish had a reduction in ventilation rate when presented with the odor after conditioning. However, there were a number of differences between these fish and those tested in the actual experiment. The fish were from different populations, held in different water, and tested in an early version of the apparatus. Perhaps most significant was the difference in size; fish used in the preliminary study were approximately 51 mm and 1.5 g, whereas the test fish were approximately 85 mm and 8.0 g. Most of the test fish appeared to be undergoing the parr-smolt transformation in their first year of life. This transition

normally takes place in the second year of life in these populations (Weitkamp et al. 1995) but can be induced early if growth is rapid (Brannon et al. 1982), as was the case with the experimental fish. This physiological change may have affected their ability to be conditioned to an odor.

The original test apparatus was a 20 x 20 cm square enclosure set inside the Plexiglas tank with a flow straightener approximately 5 cm upstream of the enclosure. The purpose of the flow straightener was to ensure laminar flow of the odor-bearing water through the fish enclosure. However, after running 20 fish through the conditioning and testing protocol, none of the fish showed any signs of a conditioned response. In an effort to improve the design, a number of technique variations used in similar studies were attempted. The first change was an improvement of the flow regime. Because the square enclosure was so large, the fish were swimming and this made the ventilation signal difficult to identify. Also, visually counting opercular beats from video recordings of constantly moving fish was difficult and not entirely accurate. Another problem with the square enclosure involved the flow and the time it took for the odor to clear from the enclosure. A clear acrylic tube replaced the square cage design, which significantly decreased the duration of odor presence in the test tube compared with the previous setup. However, with the clear tube the fish's ventilation rate did not settle to a resting level, so an opaque PVC tube was used for the final design. The other unknown was whether the electric shock was an effective unconditioned stimulus for these fish. We decided against using a food reward as a positive unconditioned stimulus, fearing that the food odor might confound the conditioned response to the odor of interest. Food reward has been used in previous work successfully, but it did not appear to elicit any behavioral or ventilation change when presented to these fish. Another commonly used unconditioned stimulus is homogenized skin extract (alarm substance), which elicits a fear response similar to a stress response in fishes (Berejikian et al. 1999; Scholz et al. 2000; Korpi and Wisenden 2001), and pilot trials were conducted for this approach. Alarm substance was made from conspecifics to form a 1.0 mg/ml solution, which was injected in volumes of 0.66 ml, 1 ml or 2 ml. The solution was injected into the test tanks

for several fish and the ventilation rates monitored for 5 min following injection, but no rate change was observed. Lastly, to ensure that lack of conditioning was not specific for PEA, the experiment was repeated using another known odorant for salmon, L-Arginine. As mentioned above, we determined that the coho were able to smell both PEA and L-Arginine.

After trying several variations, I ultimately settled on the protocol described above, but there were inherent problems. Perhaps the largest problem with this experimental design was the inability to record a ventilation signal immediately following delivery of the shock. The electrodes were used both to acquire the electrical signal and to deliver the shock, but the ventilation signal needed to be amplified 10,000x to be detected by the computer program. Therefore, in order to keep the computer from receiving a 25,000V shock, the amplifier was switched off during the shock. Unfortunately, it took approximately 40 seconds for the input signal to stabilize again after being switched back on, which meant that there is no ventilation signal data to show the expected decrease in ventilation rate. We were unable to overcome this serious problem, but it needs to be addressed in the future if this approach is to be successful. Another problem with the protocol was that because the test tube was opaque, it was impossible to know the fish's position in the tube to deliver the shock based on position or verify its response to the shock to ensure the proper strength.

Despite the various efforts to produce a conditioned response in coho salmon, this assay design did not perform as expected. However, the knowledge gained throughout this process has created some suggestions for further protocol development. Most importantly, the ventilation response to the unconditioned stimulus needs to be established. In this case, equipment was a problem for obtaining ventilation rate immediately following the shock. One option would be to further explore alarm substance or even a strobe light (Sager et al. 2000) for the unconditioned stimulus, as these would allow continuous data recordings. Another improvement would be investing in high-speed video equipment that would record in low-lighting conditions. This might allow the fish to be tested in a clear tube where video recordings could be used as a

backup for counting opercular beats and/or use behavioral changes as an indicator of conditioned responses. A clear tube and video monitoring of the fish could also be used to test fish individually so that training is only conducted once the fish has reached its pre-defined resting rate. This would decrease the overall sample size but perhaps provide better quality conditioning for each fish. Lastly, shortening the duration of odor exposure, whether by decreasing the length of the test tube or increasing the flow rate into the tank, may provide a more accurate association with the shock and thereby increase the conditioned response.

Conclusions

The main goal of this study was to design an assay to study salmon olfaction and gain a better understanding of the ability of coho to discriminate between stream odors. The protocol was designed using a combination of classic techniques and new technology in an attempt to further our basic knowledge of salmon olfaction and its role in homing. Chemical signals are essential for olfactory-mediated homing in salmon. Hasler and Wisby (1951) found evidence that vegetation and soil contributed distinct odors to natal streams, which they postulated to be the odors salmon were able to learn, detect and recognize to guide their homeward migration. However, little progress has been made since that early research to isolate and identify the odors used in homing. While the results of our research were not entirely conclusive, there were many valuable insights gained throughout the process. Hopefully the methods outlined above can provide the framework to further explore olfactory discrimination and isolating the specific chemical components in natal waters which salmon are able to imprint and detect.

Table 1.1. Paired t test results for the comparison of pre- and post- ventilation rates for individual fish. Values reported are the mean of the differences and the p-value for a 2-tailed paired t-test.

		PEA		
Runs	**Control**	**P-value**	**Treatment**	**P-value**
5	-0.04	0.6876	0.04	0.6137
10	0.06	0.1898	0.02	0.7737
20	-0.05	0.7117	-0.06	0.4163
30	0.03	0.716	0.03	0.5776
		L-Arginine		
Runs	**Control**	**P-value**	**Treatment**	**P-value**
5	0.21	0.0003	-0.04	0.390
10	0.05	0.591	-0.0001	0.999
20	0.06	0.342	-0.03	0.693
30	0.03	0.6362	-0.04	0.722

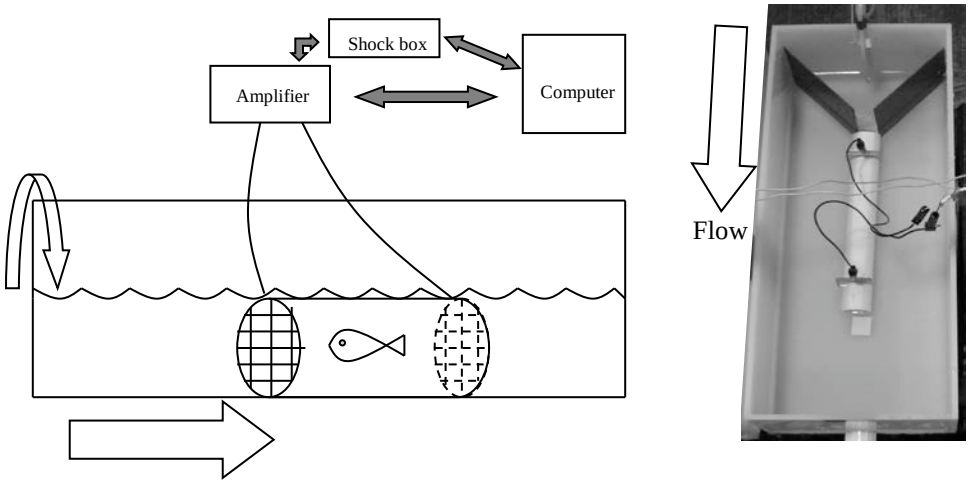


Figure 1.1. a) Schematic lateral view of conditioning/test tank and b) an overhead photo of tank with PVC enclosure tube. Arrows denote direction of water flow. The fish was placed in the PVC tube for the duration of the experiment. The grids at both ends of the tube are the recording/shock delivery electrodes.

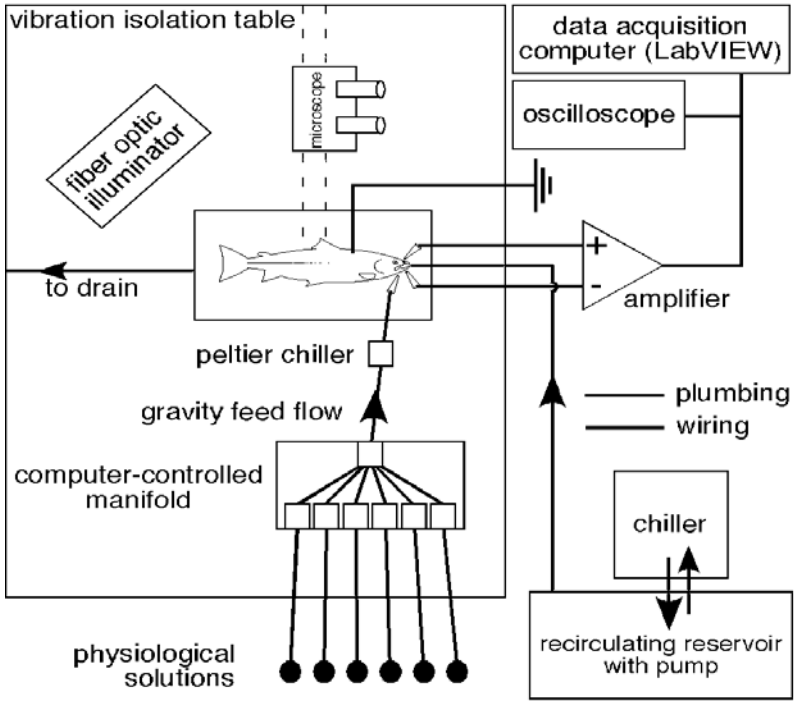
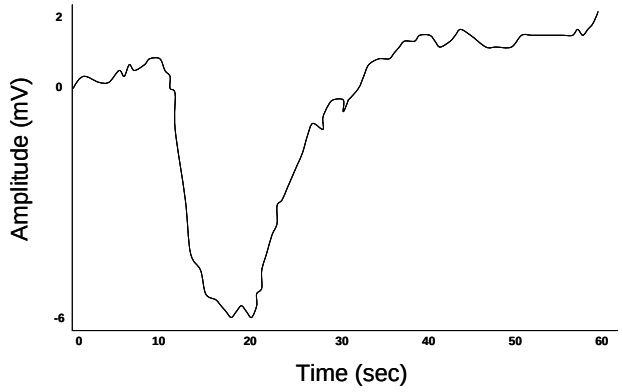


Figure 1.2. Setup for the electro-olfactogram. Graphic taken from Baldwin and Scholz (2005).

a



b

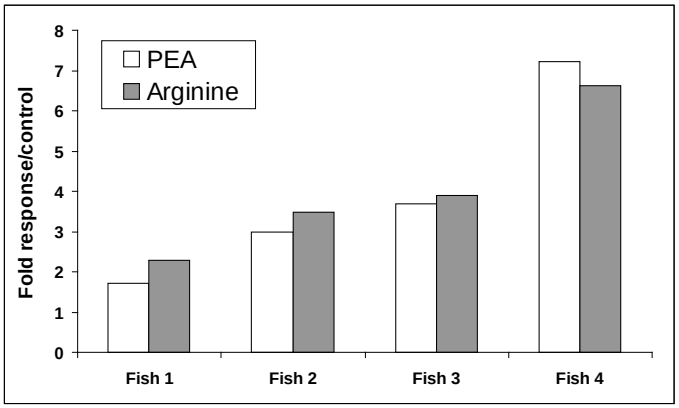


Figure 1.3. Electro-olfactogram results: a) representative EOG response (mV) of one fish to 10^{-6} M PEA, b) fold responses (mV) over control to 10^{-6} M PEA and 10^{-6} M L-Arginine for 4 representative individuals from the coho population reared at BBC.

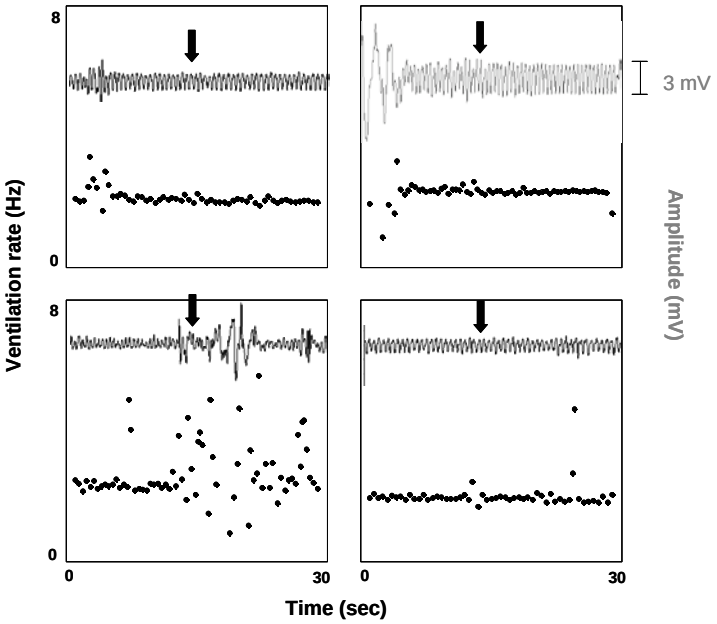


Figure 1.4. Representative individual frequency plots for fish conditioned to PEA with 30 paired odor/shock runs. The data is from a continuous recording 14 sec prior to the odor exposure, to the 16 sec following odor exposure. These instantaneous frequency plots were used to determine the response window when the fish's ventilation rate changes due to a conditioned response. Arrows denote the time when PEA reached the upstream electrode of the fish enclosure.

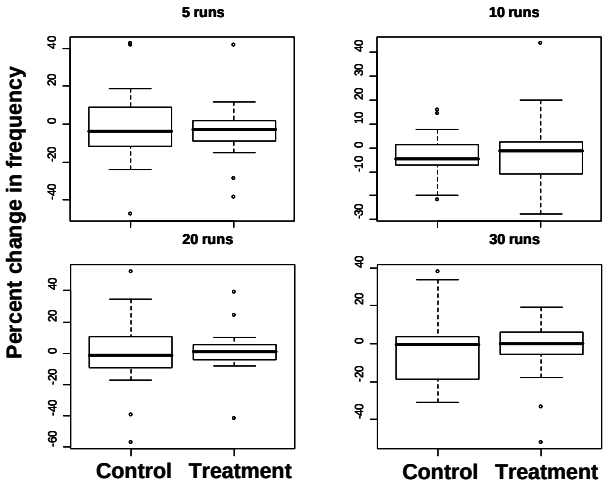


Figure 1.5. Average percent change in ventilation rate from pre to post exposure to PEA. Negative values represent a decrease in ventilation rate, positive values indicate an increase. The box plots show the median as a dark horizontal bar, with the box encompassing the interquartile range and the whiskers are the maximum and minimum values, with no significant difference between control and treatment in any of the conditioning groups. Data are the 20 control and 20 treatment fish from each of the 4 different conditioning levels.

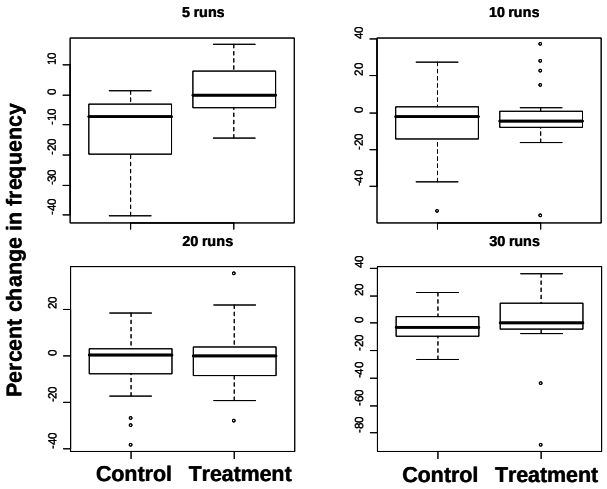


Figure 1.6. Average percent change in ventilation rate from pre to post exposure to L-Arginine. There is no significant difference between control and treatment in any of the conditioning groups. Data are the 20 control and 20 treatment fish from each of the 4 different conditioning levels.

Chapter Two: Effective captive broodstock release strategies for successful olfactory imprinting in sockeye salmon

Introduction

Over the last several centuries, the world's human population has drastically increased. Because of this expansion, a large number of species have either become extinct, or are currently at risk of extinction, due to over-harvest and habitat destruction. Conservation biology is the application of science to the preservation and conservation of plant and animal species and natural resources. While the field of conservation biology began in the 19th century, it has gained momentum in the United States since Congress passed the Endangered Species Act (ESA) in 1973. The ESA was designed to protect the species by prohibiting any "take" or destruction/modification of the listed species' habitat. Harcourt and Ehrenfeld (1992) defined conservation biology "…by its goal – to halt or repair the undeniable, massive damage that is being done to ecosystems, species and the relationships of humans to the environment". While many of the endangered and threatened species listings could be attributed to habitat degradation, poor communication between land managers and biologists sometimes led to management for crisis control rather than preventative planning to minimize species' loss (Diamond and May 1985). Consequently, in some cases the species reached such critically low levels of abundance that the slow pace of habitat restoration might not be adequate to prevent extinction. Therefore, as a tool for the conservation and eventual recovery of endangered populations, programs have been initiated to capture individuals from wild populations and breed them in captivity. The ultimate goal of these programs is to reintroduce and establish self-sustaining populations in the wild. One such example is the California condor, *Gymnogyps californianus*, which sparked a heated debate over whether the appropriate conservation approach was to remain hands-off or take action by capturing all remaining individuals for a captive breeding program (Alagona 2004). This highly publicized and expensive program was successful, with the ongoing release of captively bred individuals into the wild. Other captive breeding programs have been initiated for a

wide range of species across the globe, including primates (Britt et al. 2003) and other mammals such as marmots and wolves (Cassimir et al. 2007; Hedrick and Fredrickson 2008), birds (Fulai et al. 1995), reptiles (Brito et al. 1999) and fishes (Philippart 1995).

Captive broodstock programs have been established to preserve the genetic resources of some threatened and endangered Pacific salmon populations (genus *Oncorhynchus*). One example is the sockeye salmon, *O. nerka*, in the Stanley Basin of Idaho in the Snake River system. Snake River sockeye salmon were listed as endangered by NOAA Fisheries in 1991 and a program began that year with the intention of maintaining an anadromous population through captive broodstock propagation. Salmon are anadromous fishes that are spawned in freshwater, rear in lakes and streams as juveniles, migrate to sea for 1-5 years, and then return (home) to their natal stream to spawn. Prior to leaving freshwater, juvenile salmon learn (imprint) certain chemical compounds in the water that will direct their migration back to the spawning grounds as adults (Hasler and Scholz 1983). Low levels of straying (spawning in a non-natal stream) occur in wild populations (Quinn 1993) and are important for maintaining gene flow between populations and reducing the chance of inbreeding (Hendry et al. 2004). However, excessive straying of captive broodstock fish prevents conservation of that population's genetic line, and may also weaken the genetic integrity of the recipient population (Quinn 1993). Due to limited knowledge of the physiological and developmental processes involved in imprinting, the Stanley Basin Sockeye Technical Oversight Committee (SBSTOC) and the Idaho Department of Fish and Game (IDFG) initiated a "spread-the-risk" strategy to avoid any negative results from employing only one release strategy. Adults are taken into a hatchery, spawned, and the offspring reared for varying amounts of time before being released into Redfish Lake to imprint before they migrate to sea. Currently, the IDFG and SBSTOC are using a number of release strategies to reintroduce Snake River sockeye salmon into the wild: egg boxes are planted along spawning beaches in RFL and fry volitionally migrate out, fry are reared in net pens in the limnetic zone the fall before smolting to rear for approximately 1 month before releasing, and smolts are released at the outlet of the lake in the spring (Kline and

Heindel 1999). These release strategies reflect the dilemma that although juveniles tend to be safer in a hatchery than they would be in the natural environment (hence pressure to retain them longer), their behavior is compromised by the artificial rearing and so post-release survival may decline with the duration of rearing. In addition to other effects on behavior, rearing can affect imprinting and salmon that do not experience their natal water during appropriate juvenile stages are more likely to stray rather than home as adults. Therefore, managers need to balance higher survival against successful imprinting when deciding appropriate release strategies.

In this study, we examined the timing and duration of exposure necessary for successful olfactory imprinting in juvenile sockeye salmon. The imprinting periods chosen for our experiment parallel the "spread-the-risk" releases of fish in Stanley Basin captive broodstock program. Juveniles were exposed to an amino acid odorant at different developmental stages for varying periods of time, and behavioral assays were conducted on maturing adults to determine the appropriate hatchery release strategies (i.e., life stage and duration of exposure) necessary to ensure proper imprinting by olfactory recognition in migrating adult sockeye salmon.

Methods

Study site and populations

Experiments to determine critical imprinting periods of sockeye salmon were initiated on two different populations in the fall of 2004. The first population was Redfish Lake Sockeye salmon obtained from the National Marine Fisheries Service (NMFS) Redfish Lake Sockeye Salmon Captive Broodstock program at Burley Creek, Washington. Given their endangered status, only a limited number of these fish were available. The second population was the F1 offspring of captively-reared Okanogan River sockeye salmon obtained from the Cassimer Bar Salmon Hatchery. This population was used as a surrogate in these studies because like Redfish Lake sockeye salmon, they are a Columbia River population that spawns after a very long upstream migration from the ocean with similar timing. The Okanogan River fish are more relatively abundant and therefore more experimental treatments were possible. Eyed

embryos from both populations were transported to the Big Beef Creek, Washington field station (BBC) in December 2004. The Okanogan River population was divided into six experimental groups, based on the odor to which they were exposed and the timing of exposure (Fig. 2.1): (i) control – odorant naïve; (ii) alevin to smolt L-Arginine exposure (January 2005 to May 2006); (iii) fall to smolt L-Arginine exposure (October 2005 to May 2006); (iv) smolt L-Arginine exposure (May 2006); (v) pre-smolt only L-Arginine exposure (February to March 2006); (vi) L-smolt – smolt exposure to L-Leucine (May 2006). These experimental groups emulate the various "spread-the-risk" release strategies employed by the Redfish Lake sockeye salmon captive rearing program. Due to a limited number of available embryos, the Redfish Lake population was divided into the (i) control – odorant naïve group and (iv) smolt L-Arginine exposure (May 2006) group.

All treatment groups were maintained separately, and then marked with distinctive fin clips by treatment on 15 June 2006, after the parr-smolt transformation had occurred. This is the process by which salmon physiologically prepare to enter saltwater. Gill Na+K+ ATPase production increases in smolting fish to allow the gills to regulate ions in saltwater. Therefore, to ensure that all treatment groups were experiencing this process simultaneously, gill tissue samples were taken every 6 weeks from a subset of each group. The Na+K+ ATPase levels confirmed all groups had completed the parr-smolt transformation by June 15, 2006. L-Arginine was used as the primary imprinting odor because it has been identified as potent odorant for fish (Hara 1992), and salmon can use the compound as an imprinting odorant (Havey and Dittman unpublished). L-Leucine was used as a control odor because it does not activate the L-Arginine receptor (Speca et al. 1999). Odors for the different treatment groups were metered into each rearing tank by a peristaltic pump to achieve a final concentration of 100 nM. Fish were reared in BBC well water (constant $10° \pm 1°$ C) for three years, at which time a majority of the fish reached sexual maturity.

Behavioral testing

Behavioral responses of maturing sockeye salmon to imprinting odors were tested in three two-choice mazes at the BBC facility. Each maze consists of two arms (3.05 x 0.5 x 0.5 m tanks) flowing in to the main are of the tank (3.05 x 1.22 x 1.22 m). A plastic mesh divider separated the main tank from the arms to limit access to each arm before the start of a trial (Fig. 2.2). To provide novel background water (i.e., different from rearing water) for testing, water was pumped from a side channel of BBC into each arm and the flow maintained at approximately 15 L/min/arm. Flows into each arm were checked each day, as was the flow rate of the peristaltic pump delivering odor to each arm. Odors (10^{-3} M solutions of L-Arginine and L-Leucine) were metered into the arms of the maze using a peristaltic pump to deliver a final concentration of 100 nM/arm. Fish were acclimated for 1-2 days in holding tanks supplied with channel water to account for differing temperatures between rearing (well) water and testing (channel) water.

To start a trial, a mature fish was moved from the holding tank into the downstream section of the main area of the tank and allowed to acclimate for 30 min. The experimental group to which the fish belonged was unknown to the observer during the trial. A removable mesh screen was placed across the main raceway at the upstream end to prevent the fish from swimming in the arms before the start of a trial. During the last 5 min of the acclimation period and for the remainder of the trial, one odorant (L-Arginine or L-Leucine) was pumped into each arm of the y-maze to ensure that the odors reached the main tank by the start of the trial. After the acclimation period, the screen was lifted and the fish swam freely for 15 min. Each trial was recorded using a digital video recorder and viewed later. At the end of each trial, the peristaltic pump delivering the odor mixture was turned off, the fish removed, and its fin clip recorded to determine the experimental group. Each maze was flushed with channel water for 5-10 min between trials to remove residual odors. The mazes were drained and scrubbed each day, and the odor delivery tubes switched between arms daily to eliminate any inherent arm preference. There were six possible combinations of y-maze (3) and odor arm (2) testing scenarios (e.g., Maze 1 with L-Arginine in arm A and L-Leucine in arm B, and vice

versa). Approximately equal numbers of fish from each treatment group were tested in all y-maze combinations to remove any inherent y-maze or arm bias. The sexual maturity of each fish was assessed at the end of the trial and only data from maturing fish were retained for analysis.

Data analysis

Videos were reviewed without prior knowledge of treatment group to prevent any biased observations. For all analyses, the odor arm refers to the arm scented with the odor to which the fish was imprinted as a juvenile. Responses were only compared within population or within treatment group.

The data were examined in several different ways to ascertain whether the fish's choice of arm and water source differed from random movement in the tanks. We first examined the first arm the fish entered during the trial, the last arm the fish entered, and the arm in which it spent the majority ($\geq$50%) of its time. These data were compiled by treatment and population and analyzed by a Z-test. In addition, the total amount of time spent in the imprinted odor arm, and the average time per entry were log-transformed. Both populations (except the L-Leucine smolt exposed group) were averaged across treatment groups and compared to the appropriate control using two-sample, one-tailed t-tests with unequal variances. Differences in proportion of time spent in the odor arm were compared between experimental groups and the control group using a two-sample one-tailed t-test after the proportions were normalized using an arcsine square root transformation. The frequency of entries into each arm was compared within each treatment group using a paired t-test. In all cases, responses were tested at the P=0.05 significance level. Fish that did not enter either arm during a trial are referred to as "no choice" fish and were removed from further analysis.

Results

Of the 342 maturing fish from both populations that were tested, 126 made a "choice" by swimming into one or both arms during the trial (Fig. 2.3). The response variable that most strongly indicated attraction to the odorant was the average time fish

spent in the odor arm per entry (Fig. 2.4). On average, fish from all treatment groups spent more time per entry in the odor arm than control fish. The pre-smolt exposure group spent significantly greater time per entry in the Arginine arm than control fish (P=0.015) and the smolt exposure group from the Okanogan population showed a nearly significant difference in time spent per entry (P=0.076). This pattern was also seen with the Okanogan egg-smolt and RFL smolt groups, which spent more time per entry in the Arginine arm than control fish, but the differences were not significant (P=0.33 and 0.187, respectively). For all treatment groups, there was a tendency for more total time spent in the odor arm than the control group except the L-Leucine smolt exposure (Fig. 2.5), though in no comparison did the experimental fish differ significantly from the controls (ANOVA, F=0.512, d.f. 4 and 88, P=0.727). No significant differences in the percent of total time spent in the L-Arginine arm averaged across treatment group were observed in any treatment group relative to controls (Fig. 2.6).

The frequency of entries into control and odor arms is another way to assess choice in y-maze studies (e.g., Yambe et al. 1999; Yambe and Yamazaki 2000) but there were no significant differences found between the treatment groups and controls (Table 1). The first and last arm choices for each fish have also been used as an indicator of arm preference in a y-maze study (Yambe and Yamazaki 2001), and these data were summed across treatment group and the Z value given for within treatment differences in arm choice (Table 2). Fish only making one choice during a trial were included in the first arm analysis above, but were excluded from the last arm choice analysis (Table 3). Lastly, the number of fish that spent more than 50% of their total choice time in the odor arm was summed across treatment groups. Counts were compared within treatments by a Z-test (Table 4). None of the treatments showed a significant difference in any of these types of data: first arm, last arm or majority of time spent in either arm.

Discussion

Based on our results, several of the release strategies currently being used (planting eyed eggs, fall and smolt releases into the lake) appear to be adequate for successful homing of sockeye in Redfish Lake. Also, our findings indicated that sockeye

salmon were capable of olfactory imprinting at multiple life stages and over varying exposure durations. The pattern is best illustrated with the average time fish spent during each entry into the odor arm (Fig. 2.4). Fish exposed to L-Arginine for any duration as juveniles (i.e., all treatment groups and both populations) spent longer, on average, in the odor arm per entry than the control arm. A similar but weaker pattern was also seen in the overall time spent in each of the odors (Fig. 2.5).

An important aspect of this study was to examine similarities and differences in olfactory learning between a critically endangered population and a potential surrogate population, based on life history and migration strategies. One of the applications of this work was to show that we can apply the results from behavioral studies to aid in the conservation efforts for an endangered species that may not be accessible for lab experiments. There was no evidence of between-population differences (i.e., Okanogan and Redfish Lake stocks) in olfactory learning. However, given the generally weak responses, we cannot conclude from our data that Okanogan sockeye are an appropriate surrogate population for further research on RFL sockeye. Qualitative observations throughout rearing indicate the populations inherently behave different in the hatchery, and small sample sizes made it difficult to directly compare behavior responses between the populations.

Our results indicated that sockeye salmon imprinting is a complex process and we still have a great deal to learn. An important aspect of this experiment to emphasize is that these populations were reared entirely in a hatchery environment. Fish were maintained in a relatively stable environment with a constant flow, temperature, and water source, which may reduce their ability or tendency to imprint relative to wild fish, as suggested by Dittman and Quinn (1996). Wild fish undergo migrations from freshwater to sea with a multitude of changing environmental factors, whereas hatchery fish are generally reared under stable conditions. These changing environmental factors may play a large role in elevating thyroxine hormone levels, which have been linked to olfactory imprinting (Dickhoff et al. 1982; Nishioka et al. 1985). Thyroxine may be involved in a positive feedback loop where increased levels induce migration (i.e.,

sockeye migrating downstream to a nursery lake following emergence, etc.) and these migrations through different environmental stimuli lead to an increase in thyroxine levels (Dittman and Quinn 1996). Therefore, hatchery fish that are unable to migrate may only experience a critical learning period associated with the developmental increase in thyroxine, associated with the parr-smolt transformation, whereas wild fish may experience several peaks in thyroxine with changing temperature, flow, water sources, etc. Dittman et al. (1996) saw no evidence for imprinting by coho salmon exposed to the same water throughout their lives compared with coho exposed as smolts and released to allow migration. This suggests exposure to a water source during the parr-smolt transformation may be necessary but not sufficient for imprinting; the act of migration may play a major role in olfactory imprinting as well. As previously mentioned, sockeye salmon undergo an early migration to a nursery lake in addition to the migration following the parr-smolt transformation. Our sockeye salmon were held in freshwater throughout their lives and not allowed to undergo any migration, which may have affected the degree to which these fish were able to imprint at the different exposure windows. While we saw trends of attraction to the imprinted odor, perhaps the responses would have been stronger if the fish were able to migrate as fry and smolts.

A reduction in sample size resulted from an unanticipated problem with the maturation rate of the Okanogan population. Fish from both populations were reared under similar conditions and were weighed periodically. Food rations were adjusted to maintain similar growth rates to ensure that all fish reached the parr-smolt transformation together. We assumed that both populations would mature at age 3, but only 75 % of the Okanogan population matured in the fall of 2007. This, combined with the fact that not all fish made a “choice” in the experiments, decreased our expected sample sizes to a range of 11-24 “choice” fish (i.e., fish that entered one or both arms) per group for all Okanogan treatment groups.

An interesting phenomenon arose with respect to population differences in behavior. As mentioned earlier, only 22 of the 138 (~16%) Redfish Lake fish made a choice during the trials. The small percentage of RFL fish that made a choice compared

with Okanogan fish (~51%) may indicate behavioral differences between the populations. Qualitative observations noted during rearing indicated RFL fish were quite skittish compared with Okanogan fish. Throughout rearing, the RFL fish were very tentative when feeding; often the person feeding them could only dispense a few pellets at a time to coax them to the surface, and they schooled more readily than did the Okanogan fish. Behavioral differences were noted throughout their lives and thus the differences between the two groups in the adult behavioral testing may reflect fundamental aspects of their behavior. The video coverage area did not include the entire main tank so it was not possible to quantify any alternative behavior as a metric for successful imprinting or other aspects of behavior. In future work, video footage could encompass the entire testing area to allow "no choice" fish to be analyzed for movement patterns as an index of motivation.

Rearing and maintaining salmon through adulthood is extremely time-consuming and costly. Because it is such a large investment, it is desirable to collect as much data as possible by having several treatment groups. However, the result can come at the cost of sample size. The trends shown with these fish support the hypothesis that sockeye are capable of olfactory learning during periods prior to and including the parr-smolt transformation, but small sample sizes limited our ability to determine statistical significance. A power analysis using data from a similar previous study determined that sample sizes of 75 fish/treatment making a choice would be necessary to detect significant differences in behavioral responses (A. Dittman, pers. Comm., August 2007).

As stated above, preference of the imprinted odor arm is apparent with the total time, but much stronger when shown as time per entry. This may be attributed to the number of entries being factored in to the time per entry analysis, and imprinted fish had a tendency to remain in the odor arm longer for a given entry than the control arm. In the wild, exploratory behavior is characteristic of homing salmon (Griffith et al. 1999; Keefer et al. 2006; Keefer et al. 2008). Johnsen and Hasler (1980) proposed a model for salmon homing behavior, which describes how salmon enter rivers along the shoreline and swimming upstream (positive rheotaxis) if it detects the appropriate natal stream

olfactory cues. However, if the fish does not detect its home stream water, it will swim back downstream (negative rheotaxis) and continue along the shoreline. Our findings seem to support prior observations of exploratory behavior in wild fish during the homing migration. Thus it was not surprising that the fish moved back and forth rather than immediately entering the odor arm and remaining there for the rest of the trial.

Behavioral studies to examine imprinting and homing in salmon often do not reveal the same strong patterns seen in wild populations (e.g., Dittman and Quinn 1996; Courtenay et al. 1997). Testing fish in an artificial setting can be extremely difficult. While many factors are "controlled", there are clearly a number of factors influencing homing behavior in streams that are not included in these artificial arenas. Two-choice y-mazes are a useful tool to show strong preferences or aversions to particular odorants, but may be limited in their ability to reveal weak patterns in attraction or aversion. However, this kind of approach can be very informative for populations that cannot be studied in their natural setting or those with small numbers (i.e., Redfish Lake sockeye). Results are never as clear-cut or strong as we see in naturally migrating fish, but a conservative interpretation of behavioral studies can deepen our general understanding of imprinting and homing.

Conclusions

In summary, our findings supported the hypothesis that the experimental sockeye salmon were capable of olfactory imprinting at multiple windows, but there is still more to learn about the imprinting and homing processes in wild salmon. Significant results from this study were: 1. Sockeye imprinting occurred at developmental windows before and during the parr-smolt transformation; 2. Several release strategies employed by the RFL Captive Broodstock Program appear to be viable strategies to achieve successful homing back to Stanley Basin, but the smolt release may be the most successful when including egg-smolt survival. Overall, the trends shown in this study suggest that sockeye salmon have a complex imprinting strategy and more research is necessary to address this complexity.

Table 2.1. Average of entries per fish for each arm (mean ± s.e.m.). A paired t-test was used to compare within each treatment and the P values listed.

Treatment	*Number of Entries*			
	Arginine ±s.e.m.	**Leucine ±s.e.m.**	**No Choice**	**P-value**
Control	6±1.11	6.05±1.16	16	0.603
L-smolt	9.75±3.37	5.8±1.85	14	0.117
Egg-smolt	3.33±0.70	4.26±1.17	20	0.401
Fall-smolt	5.77±1.3	5.23±1.57	19	0.612
Presmolt	4.78±1.41	4.91±1.78	20	0.223
Smolt	5.5±1.48	5.17±1.76	11	0.101
RFL control	1.5±0.29	1.5±0.5	60	0.442
RFL smolt	1.67±0.37	2.7±0.5	56	0.132

Table 2.2. Number of fish that chose the Arginine or Leucine arm first. A Z-test was used to determine if the numbers that chose each arm first within each treatment group were significantly different.

Treatment	*Number of Fish*			
	Arginine	**Leucine**	**No Choice**	**P-value**
Control	14	10	16	0.816
L-smolt	6	5	14	0.302
Egg-smolt	11	12	20	-0.209
Fall-smolt	11	6	19	1.213
Presmolt	5	8	20	-0.832
Smolt	10	6	11	1.000
RFL control	4	4	60	0.000
RFL smolt	5	9	56	-1.069

Table 2.3. Number of fish that entered either the Arginine or Leucine arm last during a trial. Fish that only made one entry during the whole trial were not included in this analysis, but are listed separately. A Z-test was used to determine if the number of fish that entered each arm last within each treatment group were significantly different.

Treatment	*Number of Fish*				
	Arginine	**Leucine**	**One entry**	**No Choice**	**P-value**
Control	7	12	5	16	-1.147
L-smolt	4	3	4	14	0.378
Egg-smolt	8	8	7	20	0.000
Fall-smolt	6	5	6	19	0.302
Presmolt	6	2	5	20	1.414
Smolt	7	5	4	11	0.577
RFL control	2	1	5	60	0.577
RFL smolt	3	5	6	56	0.707

Table 2.4. Number of fish that spent more than 50% of their time in Arginine or Leucine arm. A Z-test was used to determine the number of fish that spent more time in each arm within each treatment group were significantly different.

Treatment	***Number of Fish***			
	Arginine	**Leucine**	**No Choice**	**P-value**
Control	13	11	16	0.408
L-smolt	6	5	14	0.302
Egg-smolt	12	11	20	0.209
Fall-smolt	9	8	19	0.243
Presmolt	5	8	20	-0.832
Smolt	10	6	11	1.000
RFL control	3	5	60	-0.707
RFL smolt	5	9	56	-1.069

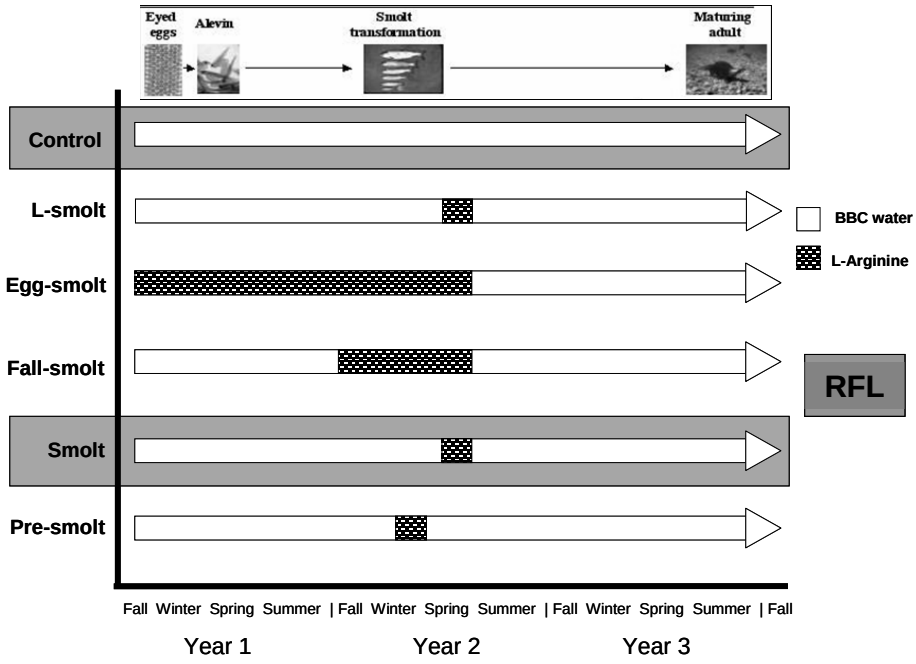


Figure 2.1. Description of different treatment groups for Okanogan (all 6 groups) and Redfish Lake (Control and Smolt) juveniles. The exposure periods encompass several developmental stages, and vary in duration.

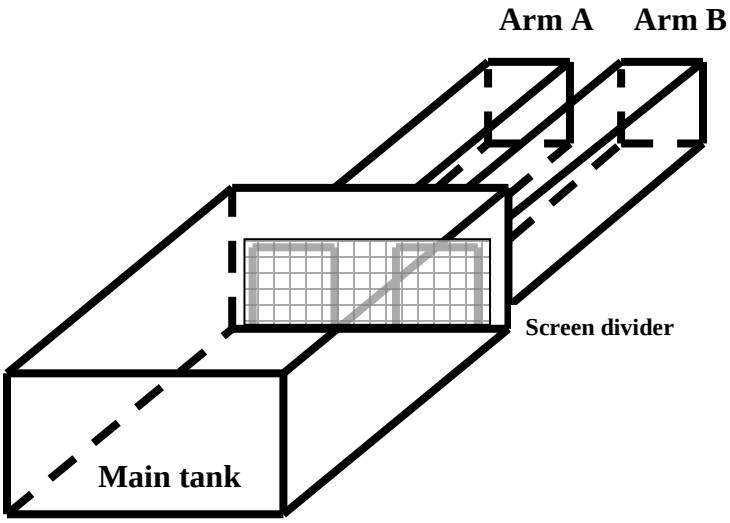


Figure 2.2. Schematic of a Y-maze for testing adult sockeye salmon. The two arms flow directly into the main tank and the mesh divider prevents upstream access to the arms until the trial begins. A camera was placed directly above the junction of the arms and main channel to monitor fish movement in and out of each arm.

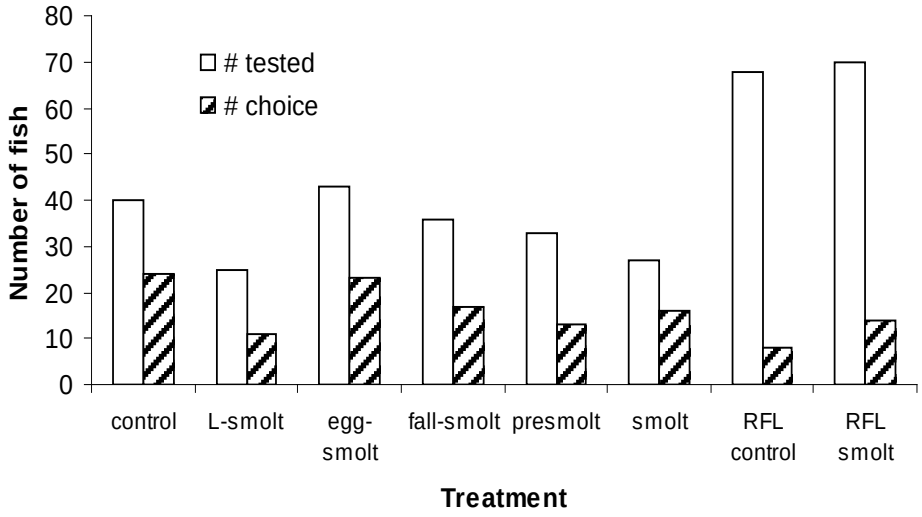


Figure 2.3. The number of fish from each treatment group tested in the Y-maze and the number of those making a choice by swimming into one or both arms during the trial.

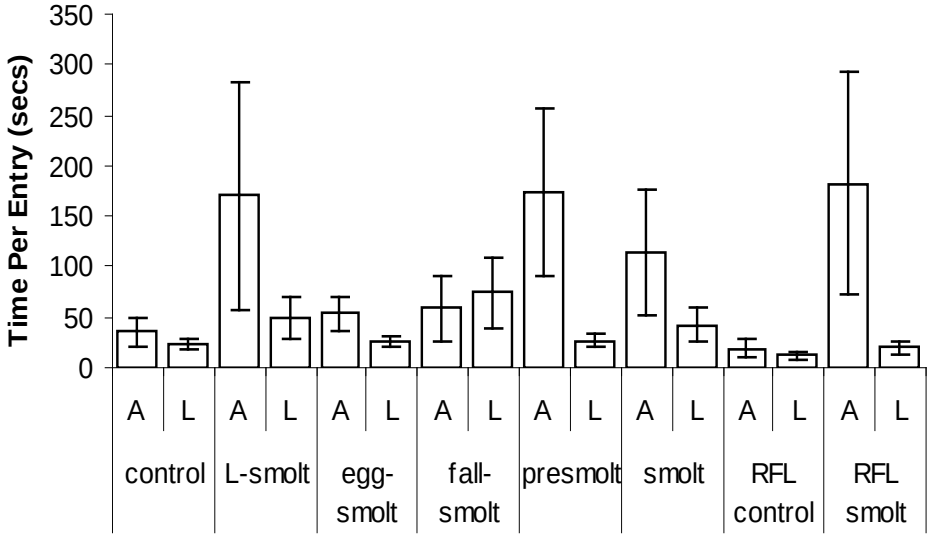


Figure 2.4. Average time per entry, in seconds, for each fish. Values for both the L-Arginine (A) and L-Leucine (L) arms are given for each group as the mean time per entry $\pm$ S.E.M.

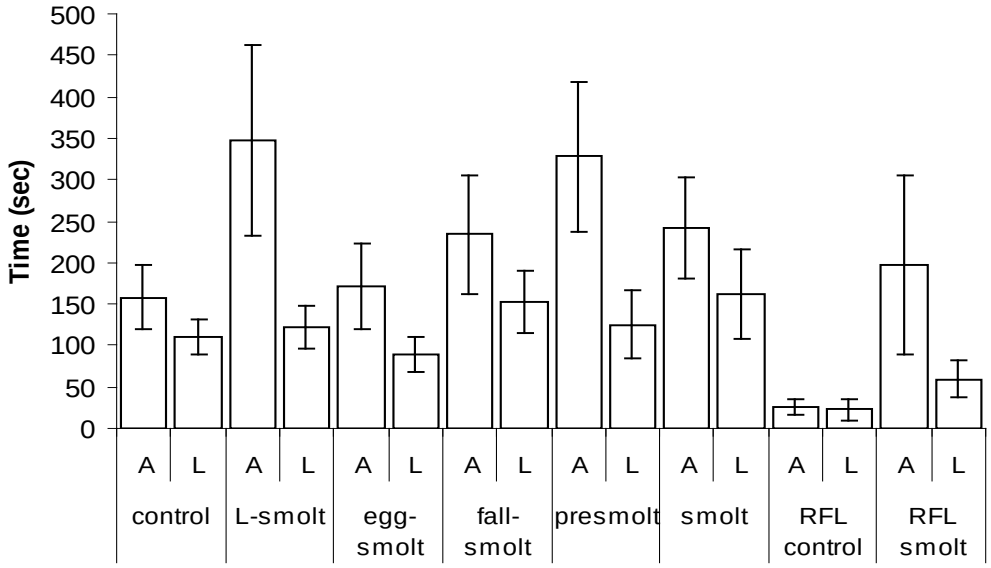


Figure 2.5. Average time spent in each odor arm averaged across treatment groups. Values for both the L-Arginine (A) and L-Leucine (L) arms are given for each group. Bars represent the mean in seconds ± the S.E.M.

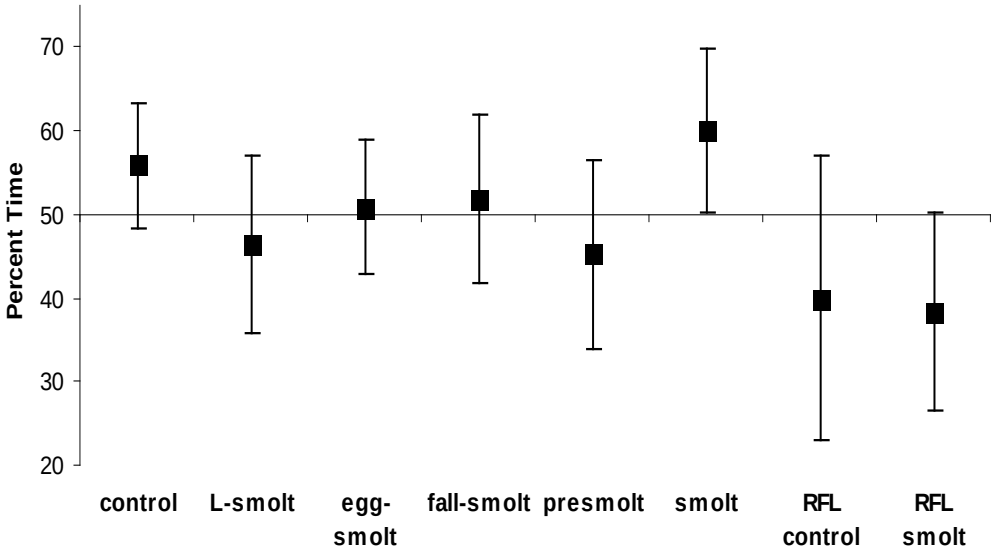


Figure 2.6. Percent of time spent in Arginine arm out of total time spent in both arms. Data are the mean of each treatment group ± S.E.M.

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