Exploring native oyster shell microchemistry to characterize population connectivity in Puget Sound, WA

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Abstract

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Olympia oysters (*Ostrea lurida),* a species of concern in Washington State, have failed to fully recover after both overexploitation and environmental degradation. State agencies, tribal nations, and environmental groups in Washington have made it a priority to restore *O. lurida* because they are the only native oyster on the west coast of North America and provide key habitat and ecosystem services to the Puget Sound. Despite this interest, our understanding of *O. lurida* larval dispersal patterns remains limited. The early life stage of Olympia oysters play a key role in their restoration because it is the only time when they can disperse to other populations. While being brooded, Olympia oyster larvae incorporate trace elements present in estuarine waters into their

shell, creating a chemical “signature” of their natal site before release and dispersal providing a way to track their larval connectivity patterns. However, collecting brooded larvae from a species undergoing restoration efforts poses procedural challenges because it traditionally requires sacrificing significant numbers of adults to find enough reproductive females. The first objective of this study, therefore, was to develop a non-lethal sampling approach to collect brooded larvae. Exposure to magnesium sulfate ($MgSO_4$) was assessed in lab trials as an anesthetization method to allow for the non-lethal collection of brooded Olympia oyster larvae. In the field, over 14,000 oysters were then anesthetized to assess reproductive status and validate the procedure. The anesthetization method of 45 min air exposure followed by 45 min submersion in 100 g/L $MgSO_4$ was found to have a success rate > 80% in the field. This anesthesia method for *O. lurida* allows for non-lethal sampling of assessing reproductive status and collection of brooding larvae, as well as for obtaining tissues for genetic analysis, biopsy for diseases. The second objective of this work was to determine the viability of elemental fingerprinting for tracking larval dispersal patterns of *O. lurida*. With the use of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), the provenance signatures of larvae and recruits can be compared, and potentially matched, to signatures of source populations. Unique elemental fingerprints of *O. lurida* larval shells were found in three regions in Puget Sound. This provides an approach to quantifying larval dispersal that can be used to determine the extent to which these *O. lurida* populations self-seed, seed other sites, and exchange larvae with other populations.

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DEDICATION

To my wonderful loving mother, Angela Beth Hintz, who is no longer with us, but continues to inspire me each and every day.

Chapter 1 INTRODUCTION

Early commercial fisheries of Olympia oyster (*Ostrea lurida)* – Washington's only native oyster – overharvested the species to near extinction. Populations were reduced to low numbers by a combination of overharvesting, water pollution, loss of habitat, and the introduction of nonnative predators (Blake 2012). Despite improved water quality and reduced harvest, self-sustaining populations have not reestablished throughout Puget Sound and *O. lurida* is now listed as a species of concern in Washington State. (Trimble 2009).

Restoration efforts have been ongoing for over fifteen years, but their efficacy remains equivocal. Active management practices to restore the native oyster involve habitat enhancement and stock enhancement (outplanting of oyster seed). Restoration efforts have successfully increased the spatial extent and abundance of Olympia oysters in few bays in Puget Sound (Blake and Bradbury 2012). However, the distribution of 12 million seed at nearly 80 locations and several habitat enhancements have failed to reestablish Olympia oyster populations in Puget Sound as of the last evaluation in 2012 (Blake and Bradbury 2012). Washington Department of Fish and Wildlife's plan for rebuilding Olympia oysters recommends focusing efforts on 19 priority sites (Blake and Bradbury 2012). Efforts have improved but it is vital to understand the level of connectivity between the sparse subpopulations of Olympia oysters in order to restore a self-sustaining network of populations. One reasons the restoration efforts have had equivocal success could be due to low levels of connectivity because without adequate recruitment of new individuals populations cannot sustain themselves.

Ensuring population connectivity, the exchange of individuals among geographically separated subpopulations, is imperative to successful management of marine species. Primarily

sedentary species, such as bivalves, disperse to neighboring subpopulations during their only mobile phase, the larval phase. The level of connectivity within a population will inform whether targeted stock enhancement to improve demographics of existing populations or establishing several small populations will establish a self-persistent metapopulation (Puckett and Eggleston 2016). Recruitment of new individuals is a critical factor governing adult population densities (Wasson et al. 2016). Establishing connectivity is critical for maintaining the genetic health and persistence of a population. Populations persist when connectivity in combination with demographics guarantees each adult is replaced by at least one recruit that survives to reproduction (Puckett and Eggleston 2016). Population connectivity dynamics will inform the restoration approach to establishing a self-sustaining network of populations and ensure the genetic health of the population.

Larval dispersal dynamics are complex and difficult to assess. Many microscopic larvae are released into vast bodies of water and the dispersal of these larvae is highly dependent on water flow. Additionally, high larval mortality limits dispersal and connectivity of populations (Puckett 2014). Although water flow dominates larval dispersal, factors influencing the dispersal and the dynamics cannot be simplified down to oceanographic conditions and pelagic larval duration (Cowen & Sponuagle 2009). Larval behavior, such as vertical swimming speed and direction, can strongly influence larval dispersal patterns. For many years larvae were thought to be passive particles at the whim of the currents because larval swimming speed is 1-3 orders of magnitude slower than the speed of the currents (Young 1995), however research has shown larval swimming behavior via vertical migration significantly influences dispersal and increases retention (Peteiro and Shanks 2015). Several biological processes and their interactions drive dispersal and connectivity patterns (Puckett 2014). The complexity of factors and their

interactions lead to high difficulty in directly observing larval dispersal patterns and predicting dispersal patterns with biophysical modeling.

Population connectivity is often studied with genetic analysis. It is much easier to obtain the genetic structure of a marine population to assess connectivity than it is to obtain direct observations of dispersal distance (Shanks 2009). The genetic connectivity of native oyster populations in Puget Sound has been characterized using microsatellites (Stick 2012). Population genetics determines population connectivity at time scales of multiple generations (Kool et al. 2013). However, the level of exchange of individuals ecologically relevant is several orders of magnitude higher than the exchange required for genetic homogeneity among subpopulations (Cowen & Sponuagle 2009). Restoration is occurring on shorter time scales than genetic analysis and we aim to understand ecological relevant dispersal. The exchange of few individuals from outside populations can homogenize genetic signatures, and the import of a few individuals is less ecologically relevant than many individuals or a substantial self-seeding population (Cowen & Sponuagle 2009).

Geochemical signatures retained in the hard parts of certain species provide another method for measuring larval dispersal and population connectivity in many marine species, and might be particularly useful for *O. lurida*. These signatures are based on stable isotopes and / or elemental ratios to identify spatial variation in potential source locations of larvae (Cowen & Sponuagle 2009). Calcified structures record the chemical conditions during formation creating natural tags that allow for the tracking of pelagic larvae for many fish and invertebrates. Trace elemental fingerprinting provides a means to track individual recruits back to their bay of origin and quantify larval dispersal at ecologically relevant time scales important for restoration of *O. lurida*. Larval shells track environmental change by incorporating elements into their calcified

structures (i.e. shells) based on availability in the water, temperature, and salinity (Zacherl et al. 2009) providing tags to track larval movements (Carson 2010).

The goal of this research was to determine if trace elemental fingerprinting could be applied as a method to track larval dispersal patterns of *O. lurida* in Puget Sound, WA. Thorrold et al. (2002) identified Washington as potentially an ideal location to generate natural geochemical signatures due to the high annual rainfall and extreme variation in land use. Elemental concentrations in shell show strong ontogenetic shift during the transition from larval to settler/adult shell and therefore the chemistry of juvenile or adult shells cannot be used as a proxy for the elemental signature incorporated into the larval shell (Zacherl et al. 2009). Thus, to determine the elemental signature incorporated into larval shells and establish a map of potential source locations it is essential to sample larvae that have development in the natal site. Luckily, *O. lurida* larvae develop in the adults' mantel cavity for 7-12 days (Baker 1995) and brooded larvae can be sampled and collected from potential known sources. However, collecting brooded larvae from a species undergoing restoration efforts poses procedural challenges because it required sacrificing significant adults to find reproductive females and collect brooded larvae (Zacherl et al. 2009). Therefore, the first objective of this research was to develop a non-lethal sampling approach to collect brooded larvae and to use the non-lethal sampling method to monitor adult reproduction and collect brooded larvae. The second objective was to use the collected brooded larvae to determine the viability of elemental fingerprinting for tracking larval dispersal patterns of *O. lurida*.

Chapter 2 A NON-LETHAL ANESTHESIA PROTOCOL FOR ACCESSING THE MANTLE CAVITY OF OLYMPIA OYSTERS IN THE LAB OR FIELD

ABSTRACT

An effective method of anesthesia for Olympia oysters (*Ostrea lurida)* would allow for non-lethal sampling of tissues for genetic analysis, biopsy for diseases, assessing reproductive status, and collection of brooding larvae. The use of magnesium sulfate ($MgSO_4$) as an anesthetization method for Olympia oysters was assessed in lab trials and field use. Three replicate groups of 10 oysters were exposed to $MgSO_4$ at three concentrations (0, 75, 85, and 100 g/L) in the laboratory to investigate the optimal concentration for anesthetization. Laboratory trials determined that 45 minutes of treatment with 100 g/L $MgSO_4$ was the most effective. In the field, over 14,000 oysters were exposed to $MgSO_4$ as an anesthetic to assess reproductive status and validate the procedure. In field trials the anesthetization method of 45 min air exposure followed by 45 min submersion in 100 g/L $MgSO_4$ was found to have a success rate > 80%. No influence of sampling date, location, or reproductive status on anesthetization was detected. Shell height was negatively correlated with anaesthetization success rate, with small oysters more likely to open their shell in response to $MgSO_4$.

2.1 INTRODUCTION

In most bivalves, it is challenging to determine the health and reproductive stage in a non-lethal manner. Bivalves tightly close their two valves inhibiting inspection and sampling without removing the shell and sacrificing the individual. Non-invasive techniques have been developed including the use of magnetic resonance imaging (MRI) which allows for

visualization of soft tissue. Pouvreau et al. (2006) successfully used MRI to identify internal structures and assess gonadal evolution in *Crassostrea gigas*. These techniques are expensive, hard to implement in the field, and do not allow for the collection of samples.

Anesthesia has been used to collect tissue samples with oysters, *Pinctada margaritifera* (Acosta-Salmon & Southgate 2004), and tissue biopsies of freshwater mussels, *Actinonaias ligamentina* and *Quadrula quadrula* (Berg et al. 1995) with limited mortality. Anesthetization also allows for repeated sampling of the same individual and reproductive monitoring. Methods exist for anesthetizing various mollusks including conchs (Acosta-Salmón & Davis 2006) mussels (Lellis et al. 2000), scallops (Heasman et al. 1995) and oysters (Alipia et al. 2014, Suquet et al. 2009, Suquet et al. 2010, Butt et al. 2008, Culloty & Mulcahy 1992, Puchnick-legat et al. 2015), but species have varied responses to different anesthetics.

It would be beneficial to have an anesthesia method for the Olympia oyster, *Ostrea lurida* (Carpenter, 1864). The Olympia oyster is the only oyster native to the west coast of North America. Olympia oysters are an economically, culturally, and ecologically important species in the Puget Sound region of northwest Washington State, USA. These native oysters have been part of the diet of local Native American tribes for thousands of years (Blake 2003), were harvested in large quantities by European settlers between the late 1800s and early 1900s, (Blake & zu Ermgassen 2015, White et al. 2009), and continue to be an important species for both restoration and commercial aquaculture (Trimble et al. 2009). Although the wild fishery in Puget Sound ended in the early 1900s, farmed Olympia oysters continue to command high prices in a niche market (Trimble et al. 2009). Ecologically, *O. lurida* are ecosystem engineers that create biogenic habitat (Blake & Bradbury 2012) that increases biodiversity (Pritchard et al. 2015), and provide benthic-pelagic coupling by filtering local waters (Ruesink et al. 2005). Native oyster

reefs were depleted and degraded by 1920 in Washington State (White et al. 2009); the decline was attributed to overharvest from fisheries, decreases in water quality, and habitat loss (Kirby 2004, Pritchard et al. 2015). Over the last twenty years, multiple projects have aimed at restoring *O. lurida* populations (Peter-Contesse & Peabody 2005, Dinnel et al. 2009, Blake & Bradbury 2012).

It is important to have long term monitoring of these restoration projects and to evaluate their restoration success. Adult female *O. lurida* brood their fertilized embryos for 7-14 days, during this brooding period reproductive status and stage of females can be determined by visually examining the brood chamber. Nonlethal sampling of reproductive activity and tissue collection would improve both project efficacy and the ability to study this species of oysters. The ability to anesthetize *O. lurida* would allow for non-lethal examination of the internal structures, examination and removal of brooded larvae, tissue sampling, and experimental procedures on live bivalves without harming the individual. An optimal anesthetization method would be simple and quick for field applications, allow the animal to recover quickly to reduce stress, and have minimal to no mortality.

The goal of this study was to develop a cost effective, efficient anesthetization method that can be applied in the lab, hatchery, or field to assess the reproductive status of *O. lurida*. The use of magnesium salts has been shown as an effective anesthetic in numerous species of oysters (*Crassostrea gigas* (Suquet et al. 2009), *Ostrea edulis* (Suquet et al. 2010, Culloty & Mulcahy 1992), *Ostrea chilensis* (Alipia et al. 2014), *Saccostrea glomerate* (Butt et al. 2008), *Pinctada maxima* (Mamangkey et al. 2009)), and scallops (*Pecten fumatus* (Heasman et al. 1994)). In the current study, a non-destructive method of anesthetization was developed for relaxing *O. lurida* using magnesium sulfate ($MgSO_4$), sold commercially as common Epsom salts.

Laboratory trials were carried out to examine dose, pretreatment, and post-treatment mortality, followed by field trials to assess effectiveness as measured by the proportion of oysters successfully anesthetized.

2.2 METHODS

2.2.1 *Laboratory Trials*

Wild adult Olympia oysters from south Puget Sound (mean shell height 42.5 ± 2.6 mm, 95% CI) were held in ambient seawater at the Puget Sound Restoration Fund shellfish hatchery in Port Gamble, WA prior to experiments. Oysters were submerged in concentrations of 0, 75, 85, and 100 g/L of $MgSO_4$ (SaltWorks, China, Ultra Epsom Premium Epsom Salt, unscented) to characterize optimal anesthetic conditions. Preliminary trials revealed that concentrations ≤ 50 g/L and ≥ 125 g/L were unsuccessful in anesthetizing high proportions of *O. lurida* in less than 2 hours (data not shown). Oysters were randomly distributed among three replicate treatments (n=10 per treatment), with coded labeling to remove observer bias at each concentration plus controls. Treatments were carried out in clear 5.7-liter plastic bins with 50:50 freshwater/seawater to maintain approximate salinity of seawater. The $MgSO_4$ was initially dissolved in freshwater before combining with equal parts seawater. Oysters were monitored at 15 minute intervals for over 2 hours for response to the $MgSO_4$ anesthetic. Oysters were considered successfully anesthetized when valves opened (gaped), did not close in response to light tapping, and remained open after removal from anesthetic bath for at least 10 seconds. The goal was to create an anesthetization method that was quick and easily applied during low tides in the field, therefore immersion times >1 hour were not viable options. Survival of oysters in all treatment and control groups was monitored for 1 month after the trial. The magnesium salt

magnesium chloride ($MgCl_2$) was not included in the trials; although effective with *Ostrea chilensis* (Alipia et al. 2014) and *O. edulis* (Culloty & Mulcahy 1992, Suquet 2010), $MgCl_2$ did not quickly anesthetize *O. lurida* in preliminary trials (data not shown).

2.2.2 *Field Application*

Anaesthetization of *O. lurida* using $MgSO_4$ was tested in the field for sampling the reproductive status of oysters. This method was applied to 14,262 *O. lurida* from 11 locations throughout Puget Sound, Washington from June - August 2015. After collection, oysters were exposed to (ambient temperature) air for 45 minutes, which was shown to increase the proportion of oysters that successfully responded to the anesthetic in preliminary studies (data not shown). After air exposure, oysters were transferred to an insulated bath containing 100 g/L $MgSO_4$ 50/50 seawater/freshwater solution for 45 minutes. The $MgSO_4$ was dissolved in freshwater in the lab and was subsequently combined with seawater collected on site. The ambient air temperature ranged from 12.8-21.7 °C at sampling events and the $MgSO_4$/seawater anesthetic ranged from 17-25 °C, reflecting the seawater temperature at the sampling locations. Shell height (defined as the distance from the umbo to the opposing valve margin), response to anesthetization (open or closed), and reproductive status (brooding or not, based on visual inspection) were recorded for each oyster.

To determine if the anesthetization method introduced a bias in reproductive data collection, reproductive rates from anesthetization to lethal methods of assessing reproductive status were compared. During June 2016 in Fidalgo Bay, WA (48.477810°N, -122.574217°W) oysters were collected from fourteen haphazardly placed 1/16 m^2 quadrats in aggregations of *O. lurida*. Seven of the quadrats were anesthetized and the other seven were lethally examined for reproductive status.

2.2.3 *Data analysis and statistics*

Differences in the success rates of anesthetization across treatments in lab trials were determined by transforming proportional data using the arcsine-square root transformation (Zar 2010) followed by a two-way analyses of variance (ANOVA) and Tukey post-hoc tests. Linear regression analysis weighted by sample size was used to evaluate the success of anesthesia as a response to oyster shell height. To determine if there were statistically significant differences in the success rate of anesthetization spatially, one-way analyses of variance (ANOVA) was performed to compare the 11 different populations sampled. Similarly, to determine if there was a temporal effect, 15 sampling events were pooled from the two locations sampled most frequently, Fidalgo Bay and Mud Bay, and a one-way analyses of variance (ANOVA) was performed on anesthetization success. One-way analyses of variance (ANOVA) was also performed on the arcsine-square root transformation proportion of reproductive oysters found in each quadrat to compare the two methods of assessing reproductive status. Average anesthetization success is reported with 95% confidence intervals.

2.3 RESULTS

2.3.1 *Laboratory Trial*

Magnesium sulfate was found to be a successful anesthetic for *O. lurida* in lab trials. After submersion for 45 minutes, the 100 g/L $MgSO_4$ treatment successfully anesthetized the highest proportion (26.7% ± 6.5%, 95% CI) of the oysters compared to controls (3.5% ± 6.5%) and the 75 g/L treatment (3.5 ± 6.5 %) (Fig. 1, Table 1). After 60 min of treatment, 100 g/L was still the most successful treatment with 36.7% (± 6.5%) of the oysters anesthetized (Fig. 1, Table

1). Both time (ANOVA, $F_{(4,40)} = 22.185$, $P < 0.0001$) and concentration of $MgSO_4$ (ANOVA, , $F_{(3,40)} = 11.099$, $P < 0.0001$) were shown to be significant factors in the success of the anesthetic. The interaction of time and concentration was significant (ANOVA, $F_{(12,40)} = 3.198$, $P = 0.0028$) indicating some of the earliest response were from oysters exposed to lower concentrations. The general trend was greater anesthesia success with less time at higher concentrations of $MgSO_4$. The treatment concentration of 100 g/L $MgSO_4$ was most successful as an anesthetic (Tukey post hoc HSD test, $P < 0.001$, Table 1). The proportion of oysters successfully anesthetized in each treatment continued to increase over the 2 hours of treatment reducing the differences in response among $MgSO_4$ concentrations. No post-treatment mortality was detected in oysters over one month of observations.

2.3.2 *Field Application*

The anesthesia method applied in the field had a high overall success rate, with 11,595 of the 14,262 oysters (81.3%) successfully anesthetized after treatment. The success of 43 sampling events ranged from 42.4 - 97.9%; averaging 82.0 ± 0.1% anesthetization success among sampling events. No spatial effect was detected among the 11 locations (ANOVA, $F_{(10,38)}=1.862$, $P=0.0948$) (Fig 2); likewise no temporal effect was detected among 15 pooled sampling events between June and August ($F_{(2,14)}= 2.0892$, $P = 0.1665$). No effect of reproductive status (brooding or not brooding) on efficacy of the anesthetic was observed ($F_{(1,13)}= 0.7953$, $P = 0.3901$). As shell height increased, the success of anesthesia decreased significantly from 98 to 76% at 15 and 55 mm shell heights, respectively ($F_{(1,52)}= 229.1$, $P < 0.001$, $R^2 = 0.8114$, 95% CI [0.7261, 0.8967]) (Fig 3).

2.4 DISCUSSION

This study developed and field-tested a non-lethal method for accessing and sampling the mantle cavity of *O. lurida*. A combination of 45 min air exposure with subsequent immersion in 100 g/L $MgSO_4$ in a 50:50 mix of fresh and seawater proved to be an effective method for the anesthetization of *O. lurida.* This method induced rapid anesthesia, no detected mortality, and was easily applied in the field during the course of a low tide. Although there were varied responses in field applications, an average of 82.0% of the individual oysters exposed were successfully anaesthetized and at least 40% were anaesthetized in every trial. The addition of a 45 minute desiccation before submersion in the $MgSO_4$ anesthetic (45 min) increased the anesthesia success from 27% in the laboratory trials to 82.0 % in the field application. There were no temporal, spatial, or reproductive status effects on anesthetization effectiveness detected in field trials.

Larger oysters were slightly less likely to be anesthetized (Fig 3), possibly due to their larger adductor muscles. Magnesium sulfate relaxes the adductor muscle of the oyster. The two valves are connected via the adductor muscle that contracts to close the shell; when the adductor muscle is relaxed the ligament is the acting force opening the two valves. Oysters can keep their adductor muscle contracted and their valves closed tightly for long periods of time allowing them to live intertidally. It is likely that the larger individuals with larger adductor muscles require more exposure to $MgSO_4$, either by an increase in concentration or exposure time to relax the adductor muscle and induce gaping. It is also possible that larger individuals are less exposed to the anesthetic because they do not require the intake of new seawater as often as smaller individuals.

Magnesium sulfate has been shown as a successful anesthetic in a variety of other mollusks but is often not the recommended method of anesthesia. The European flat oyster (*O. edulis*) was effectively anaesthetized by $MgSO_4$ with a 74% success rate but the large concentrations (300 g/L) and time necessary (24 hr) were cited as disadvantages of using $MgSO_4$ as a method (Table 2; Culloty & Mulcahy 1992). Magnesium sulfate was shown to induce high mortality (> 90%) post anaesthetization in scallops (*Pecten fumatus*) with a similar concentration of 80 g/L $MgSO_4$ (Heasman et al. 1995) and juvenile abalone (Sagara & Ninomiya 1970 as cited in Heasman et al. 1995; Table 2). In the current study $MgSO_4$ was not found to induce mortality post anaesthetization in laboratory trials on *O. lurida*. In addition, Heare (2015) applied this anaesthetization method at a slightly lower concentration of 75 g/L $MgSO_4$ to the same Olympia oysters repeatedly with no mortality reported associated with anesthetization.

This study developed an *O. lurida* anesthetic procedure that shows a high anesthetization success rate with minimum mortality. This non-lethal method of accessing the mantle cavity and brood chamber is a valuable tool with many potential applications. An anesthesia method allows for the non-lethal sampling of *O. lurida* tissue for genetic analysis, biopsies for disease, checking the stage of gametogenesis, and collection of brooding larvae. Each of these tools can be applied to answer scientific questions and remove the limitations of lethal sampling. The $MgSO_4$ anesthesia protocol reported here was recently used by Barber et al. (2016) to monitor reproduction of *O. lurida* restoration sites in northern Puget Sound. While applied to a species of conservation concern for this study, this technique is broadly applicable to bivalve research. The use of an anesthetic is widely applied in aquaculture for tissue biopsies and for monitoring gamete development and could be applied to the aquaculture of *O. lurida*. This non-lethal

sampling method allows for a large sample size of wild populations without harming active restoration efforts as well as repeated sampling of individuals.

2.5 FIGURES

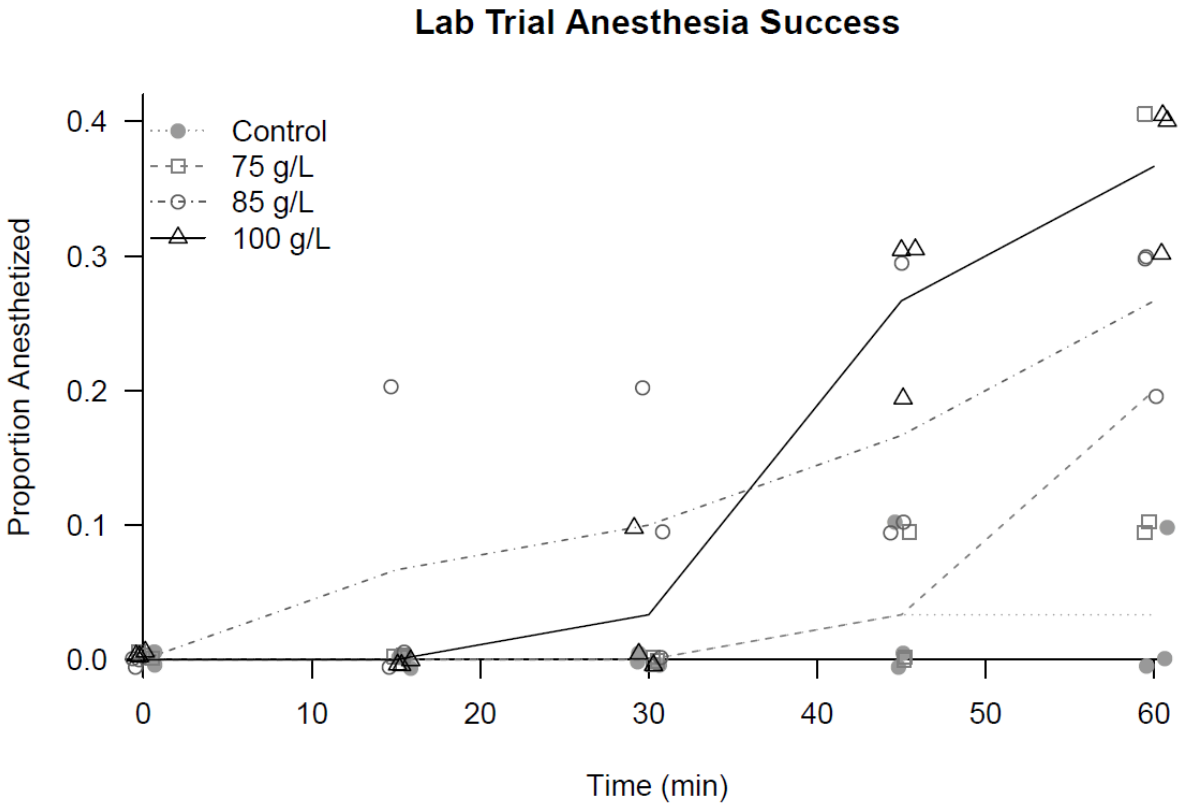

Figure 2.1 Anesthesia success of $MgSO_4$ in lab trials, three concentrations of $MgSO_4$ were assessed and compared to a control for their anesthesia success of *O. luria* and monitored over time. Replicates are represented by individual data points, the average of each treatment is represented by the lines. replicates were combined for overall proportion successfully anesthetized. Jitter was used to add a small amount of noise to the data points along both axis to improve visualization of overplotting.

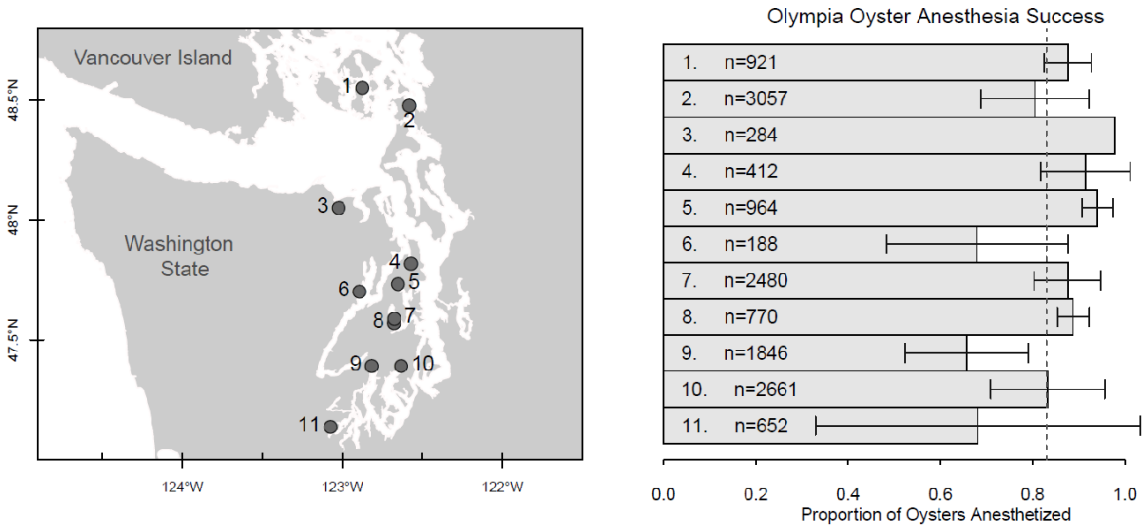

Figure 2.2 Olympia oyster anesthesia success at each population of oysters sampled in Puget Sound, WA. Left, a map of all populations sampled; right, average proportion of oysters successfully anesthetized at each population. Error bars represent 95% CI, and dashed line represents the average anesthesia success. Location of populations sampled 1) Lopez Island, 2) Fidalgo Bay, 3) Sequim Bay, 4) Port Gamble, 5) Liberty Bay, 6) Dosewallips, 7) Mud Bay, 8) Oyster Bay, 9) North Bay, 10) Burley Lagoon, 11) Little Skookum Inlet.

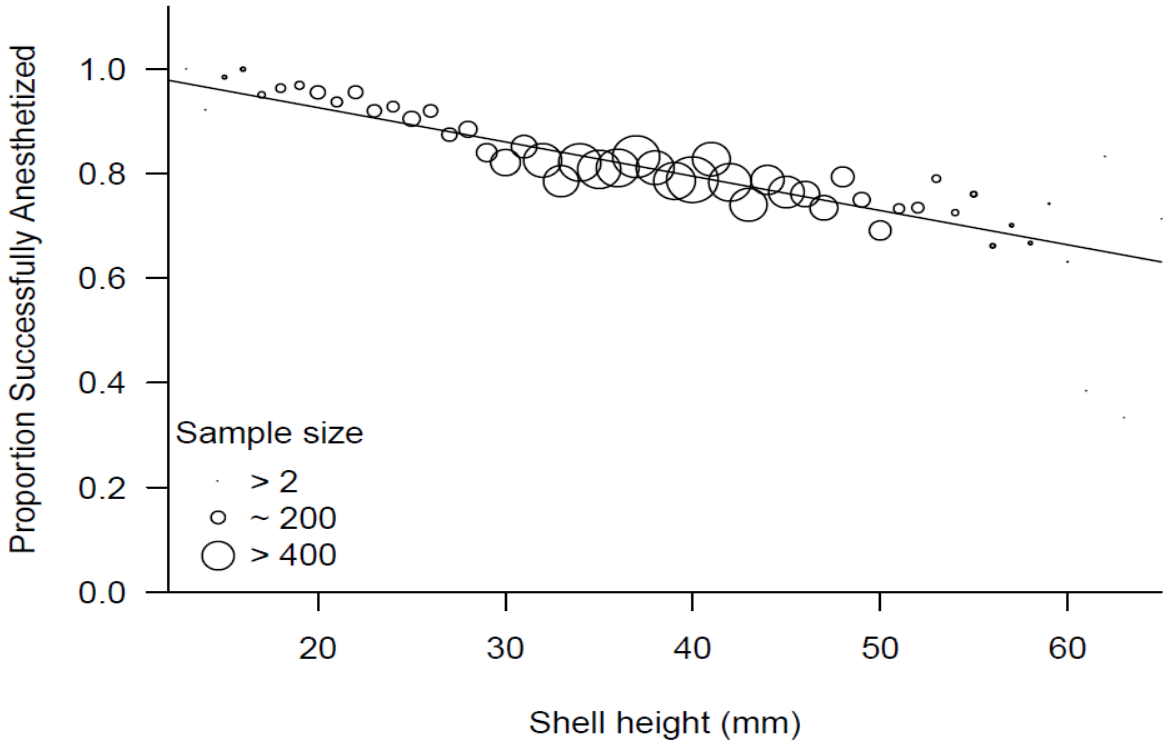

Figure 2.3 Proportion of oysters successfully anesthetized as a response to oyster shell height, each bubble represents the number of oysters sampled with that shell height. Fit with a linear regression weighted by the sample size ($y_i = 1.057 + -6.5571E^{-3}\ x_i$, $R^2 = 0.8114$, $F_{(1,52)} = 229.1$, $P < 0.001$).

2.6 TABLES

Table 2.1 Anesthesia success of three concentrations of $MgSO_4$ on *Ostrea lurida* over time in lab trials. Different letters by column represent significant differences among treatments by Tukey HSD test ($P < 0.05$)

Time (minutes)	$MgSO_4$ Concentration	Mean proportion anesthetized	
60	100	0.37	a
45	100	0.27	ab
30	100	0.03	c
15	100	0.00	c
0	100	0.00	c
60	85	0.27	ab
45	85	0.17	abc
30	85	0.10	bc
15	85	0.07	bc
0	85	0.00	c
60	75	0.20	abc
45	75	0.03	c
30	75	0.00	c
15	75	0.00	c
0	75	0.00	c
60	0	0.03	c
45	0	0.03	c
30	0	0.00	c
15	0	0.00	c
0	0	0.00	c

Table 2.2 Comparing anesthesia methods on mollusks using magnesium salts.

Species	Anesthetic	Concentration	Time	Success Rate	Source
Ostrea lurida	$MgSO_4$	100 g/L	45 min	82%	*Current study*
Ostrea edulis	$MgSO_4$	300 g/L	24 hrs	75%	Culloty & Mulcahy 1992

Ostrea edulis	$MgCl_2$	35 g/L	90 min	100%	Culloty & Mulcahy 1992
Ostrea edulis	$MgCl_2$	50 g/L	2-3 hrs	80 ± 20%	Suquet et al. 2010
Ostrea chilensis	$MgCl_2$	30-50 g/L	3 hrs	100%	Alipia et al. 2014
Crassostrea gigas	$MgCl_2$	50 g/L	16 hrs	100%	Suquet et al. 2009
Pecten fumatus	$MgCl_2$	30 g/L	~ 5 min		Heasman et al. 1994
Pecten fumatus	$MgSO_4$	80 g/L	60 min	high mortality (>90%)	Heasman et al. 1994
Saccostrea glomerata	$MgCl_2$	50 g/L	6 hrs	100%	Butt et al. 2008

Chapter 3 A PRECURSOR TO LARVAL TRACKING: REGIONAL DIFFERENCES IN ELEMENTAL FINGERPRINTS OF THE BIVALVE *OSTREA LURIDA* IN PUGET SOUND

ABSTRACT

Olympia oysters (*Ostrea lurida),* a species of concern in Washington State, have failed to fully recover after both overexploitation and environmental degradation. State agencies, tribal nations, and environmental groups in Washington have made it a priority to restore *O. lurida* because it is the only native oyster species on the west coast of North America and provides key habitat and ecosystem services to the estuarine waters. Despite this interest, understanding of *O. lurida* larval dispersal patterns remains limited. The early life stage of Olympia oysters play a key role in their restoration because it is the only stage where they can disperse to other populations. Brooded Olympia oyster larvae incorporate trace elements present in seawater into their shell, creating a chemical "signature" of their natal site before release and dispersal. With the use of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), the provenance signatures of larvae and recruits can be compared, and potentially matched, to signatures of source populations. It is paramount to determine the resolution of unique elemental signatures and create a "map" of signatures to track individual recruits to their natal origin. Unique elemental fingerprints of *O. lurida* larval shells were found to three regions in Puget Sound. This provides an approach to quantifying larval dispersal that can be used to determine the extent to which these *O. lurida* populations self-seed, seed other sites, and exchange larvae with other populations.

3.1 INTRODUCTION

Natural geochemical tags incorporated into calcified structures of marine organisms can provide a record of where an organism has been. Geochemical tags are generated by variations in environmental conditions (seawater chemistry, temperature, and salinity) and can be used to directly track newly settled recruits back to their location of origin (Thorrold et al. 2007). With the ability to track individual recruits population connectivity can be quantified on ecologically relevant time scales that are important for informing restoration. Indirect genetic approaches to evaluating population connectivity estimate connectivity over evolutionary time scales (Hedgecock et al. 2007) and the exchange of only a few individuals can maintain genetic homogeneity among subpopulations (Slatkin 1993); ecologically relevant larval exchange important for restoration is several orders of magnitude higher. One important question in restoration science is, to what extent does a restored or enhanced population self-seed, provide neighboring populations with recruits, and rely on recruitment from other populations? Trace elemental fingerprinting provides the means to answer these questions by determining the origin of individual recruits within a reproductive season.

Ensuring population connectivity, the exchange of individuals among geographically separated subpopulations, is imperative to successful management of marine species. For primarily sedentary species the planktonic larval phase is crucial to population connectivity. Planktonic larvae are capable of traveling vast distances and for many species the larval phase is the only opportunity to disperse to neighboring subpopulations. Metapopulation dynamics comprising the level of connectivity and significance of self-recruitment informs spatial management of many marine invertebrates (Puckett et al. 2014). Larval connectivity is an important factor to consider in conservation, restoration, and management of marine species that

can help determine size, configuration, and location of reserves and restoration efforts with the ultimate goal of establishing self-sustaining populations (Cowen and Sponaugle 2009; Puckett and Eggleston 2016; Kool et al. 2013; Levin 2006). Quantifying larval connectivity dynamics is complex and remains difficult to determine due to small larval size, high mortality, and large dispersal potential. For this reason, larval connectivity dynamics are often poorly understood despite their high importance.

One species that would benefit greatly from a better understanding of larval connectivity is the Olympia oyster, *Ostrea lurida*, (Carpenter, 1864). As the only native oyster in Washington State, they hold cultural, ecological, and economic significance in the region. Olympia oysters were reduced to near extinction over 100 years ago and less than 5% of their historical abundance remain in Puget Sound today (Blake and Bradbury 2012; White et al. 2009; Pritchard et al. 2015). Efforts to restore the native Olympia oyster to Puget Sound have been ongoing for over 15 years and have successfully increased their spatial extent and abundance, however self-sustaining populations have not re-established as of 2012 (Blake and Bradbury 2012). Restoration efforts would benefit from knowledge of larval dispersal patterns, dispersal distances, and the level of population connectivity in order to establish a network of self sustaining subpopulations.

Olympia oyster shells track environmental change by incorporating elements into their calcified structures based on availability in the water, temperature, and salinity (Zacherl et al. 2009) providing tags to track larval movements (Carson 2010). The goal of this study is to determine the spatial scale of chemical variability in shell microchemistry in Puget Sound and to evaluate whether elemental fingerprinting methods can be applied to quantify the larval dispersal of shelled mollusks such as *O. lurida* in Puget Sound. Elemental fingerprinting has been applied

to shelled mollusks to distinguish between estuaries along the coast (Becker et al. 2007; Carson 2010; Gomes et al. 2016; Sorte et al. 2013) and small scale within small estuaries (1-10 km, Norrie et al. 2016; Broadaway and Hannigan 2012), but has never been used to distinguish locations within a large estuarine system such as Puget Sound for invertebrates. Within Puget Sound, elemental signatures have been applied to multiple fish species to determine maternal origin of salmon offspring (Volk et al. 2000), distinguish between estuarine and ocean natal sources (Gao et al. 2005), identify nursery habitats (Chittaro et al. 2009), and improve the understanding of population structure (Chittaro et al. 2010) of fish utilizing otolith chemistry. Significant spatial variability in trace elements of otoliths allowed for successful classification among regions in Puget Sound (Chittaro et al. 2010) with fine scale resolution identifying regions with as little as 10 km separation (Chittaro et al. 2009).

Puget Sound is a large glacially carved estuarine system in the northwest corner of the continental United States. As one of the most productive nearshore bodies of water in the world, the Sound is home to a diverse and economically important ecosystem. This complex estuarine system is an extension of the Strait of Juan de Fuca with over 3,000 kilometers of intricate shoreline with frequent freshwater inputs surrounding numerous islands of all sizes (Kruckeberg 1991). Water flow within Puget Sound and among its five geographically defined sub-basins is dominated by the strong tidal influence (Moore et al. 2008) leading to a mean residence time of 57 days within these regions (Sutherland et al. 2011). The diversity of land use, geology, and freshwater inflow throughout the estuary potentially provide high variability of elemental signatures spatially (Thorrold et al. 2007).

In order for geochemical signatures to serve as a method to track recruitment back to a source population of mollusks, elemental signatures incorporated into larval shells must be unique on a relevant geospatial scale. Larvae are required to determine source elemental signatures and establish a map of source populations because neither juvenile or adult shells can be used as a proxy due to ontogenetic shifts in metal uptake (Zacherl et al. 2009). To determine if unique elemental signatures are incorporated into larval shells and at what spatial resolution unique elemental signatures can be detected, brooded larvae were collected from populations of *O. lurida* throughout Puget Sound, WA. Ultimately this will lead to establishing a trace elemental "map" of shell chemistry that can be used to determine source locations of recruits.

3.2 METHODS

3.2.1 *Field Sampling*

To characterize unique elemental signatures within the Puget Sound region, 13 sites with populations of *Ostrea lurida* were sampled for brooded larvae (Fig 1). Collection sites were chosen using existing information about adult oyster populations, prioritizing populations of substantial size while encompassing a large geographic range. Each site was visited repeatedly from June 15th to Aug 15th, 2015 to assess the reproductive status of individual *O. lurida* and maximize the number of brooded larvae collected. Two of the locations sampled, Fidalgo Bay and Mud Bay, were sampled biweekly throughout the summer to assess temporal variation of elemental signatures. Eleven other sites were sampled 1-4 times to maximize both the number of locations brooded larvae were collected from and the number of brooded larvae samples collected. Brooded larvae were successfully collected from 9 of the 13 locations in Puget Sound sampled (Fig 1, Table 1).

3.2.2 *Larval collection*

To limit trace element contamination of samples, all containers, equipment, and other materials coming in contact with larval samples in the field were acid-leached in 0.1 mol /L hydrochloric acid overnight, and sample storage vials were acid leached in 1.0 mol/L hydrochloric acid at subboiling temperatures for 8hrs, then rinsed five times with ultrapure water (resistivity > 18.2, Millipore).

Adult oysters were collected non-destructively on site and anesthetized using $MgSO_4$ (Hintz et al. 2017). Treated individuals opened their valves and were visually inspected for brooding larvae. Early-stage trochophore larvae identifiable by their light color were noted and discarded. Mid to Late D-stage veliger larvae that had formed their initial calcified shell, the prodissoconch, were collected. These late stage brooded larvae were carefully rinsed from the mantle cavity with filtered seawater (75 microns, collected at location). All larvae were rinsed in three different solutions each with decreased salinity, filtered sea water, 50:50, and ultrapure water to remove any remaining anesthetic solution in the sample before being stored in ultrapure water. Larval samples were stored in two 1.5-mL acid-leached and rinsed Eppendorf tubes per brood and frozen upon return to the laboratory. Late-stage brooded larval samples were found and collected from 99 oysters, spanning 9 different locations in Puget Sound (Table 2).

3.2.3 *Sample Prep for La-ICP-MS analysis*

All containers, equipment, and other materials coming into contact with larval samples in the lab were acid-leached in 1 mol /L hydrochloric acid for 1 week, and then rinsed five times with ultrapure water (resistivity > 18.2, Millipore).

Sample preparation methods were modified following Carson (2010) and Becker et al. (2007) to clean and prepare early stage bivalves for trace elemental fingerprinting. To clean the shells and remove organic matter, isolating the elemental signatures of the inorganic component of the shell, shells were cleaned with 15% hydrogen peroxide (H_2O_2, (UltraTrace, Fisher Scientific) buffered with 0.05 mol/L NaOH (Trace Metal Grade, Fisher Scientific). A few hundred brooded larval shells per parent oyster were carefully isolated and placed in 1 mL of buffered hydrogen peroxide cleaning solution overnight (12-18h) at room temperature. Shells were rinsed in ultrapure H_2O three times before being transferred into a petri dish and visually inspected. Only clean larval shells were transferred into the final rinsing vial and rinsed with ultrapure water two more times then carefully crushed to homogenize. The homogenized shell mixtures were dried on a clean glass slide before being transferred and mounted on 3M-brand Scotch double-sided tape affixed to glass petrographic slides. Larval samples were prepared and mounted in a random order and completed slides were then stored in a laminar flow hood until elemental analysis.

3.2.4 *LA-ICP-MS analysis*

Shells were analyzed using a New Wave Research UP 213-nm laser attached to an Agilent 7500 series ICP-MS. Each petrographic slide was placed into a ‘‘super cell’’ low-volume sample chamber with helium used as the carrier gas. The chamber also contained the U.S. Geological Survey microanalytical carbonate standard number three (MACS-3), a matrix matched standard. Sets of three ablation passes through the standards before, during, and after each slide were used to calibrate the ICP-MS and correct for instrument drift. Brooded larval shells from each parent oyster were mounted on double-sided tape affixed to glass petrographic slides as concentrated masses of shell mixtures to produce a composite of the elemental signature

of the sample reducing the variability between individual larvae within a sample. Ablation passes for each piece of double-side tape were used to monitor for contamination and none of the analyte elements were detectable in any analysis of the tape. Concentrations of 12 elements were quantified: ^{26}Mg, ^{27}Al, ^{31}P, ^{42}Ca, ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{67}Zn, ^{69}Ga, ^{88}Sr, ^{137}Ba, ^{208}Pb, ^{238}U. These elements were chosen because they are found in Puget Sound, WA waters or sediment or have previously been successful in elemental fingerprinting of bivalves (Carson 2010; Zacherl et al. 2009) and were detected in larval shell samples in preliminary trials (data not shown). Homogenized brooded larvae samples were ablated at a fluence of 2-3 joules per second and 10 Hz. One millimeter long line scans of homogenized shells were conducted using a 55 micron spot size and a raster rate of 30 micron per second. These relatively weak settings were used to minimize burn-through of the shell into the mounting tape determined by visual inspection. Elemental values for each ablation line were calculated from raw values using Glitter Software.

3.2.5 *Influence of non-lethal sampling method on elemental signatures*

To determine if the non-lethal sampling method of collecting brooding larvae by anesthetizing the adults in a $MgSO_4$ solution influenced the elemental signatures of larval shells we compared the elemental concentrations in shells collected using the non-lethal $MgSO_4$ anesthetic to the lethally collection method of shucking the adult. During June 2016 in Fidalgo Bay, WA (48.477810°N, -122.574217°W) we collected oysters from fourteen haphazardly placed 1/16 m2 quadrats in aggregations of *O. lurida*. All oysters collected were checked for reproductive status using either the $MgSO_4$ anesthetic or the lethal method of collection. Late stage brooded larval samples were collected from 9 different oysters; 4 of which were collected using the $MgSO_4$ anesthetic and 5 of which were lethally sampled. All brooded larvae samples were rinsed following the protocol used during collection in 2015. Elemental concentrations in

these brooded larvae shells were determined following the same LA-ICP-MS methods of preparation and analysis outlined above.

3.2.6 *Statistical analysis*

All elemental abundances in shells determined in LA-ICP-MS analysis were converted into molar elemental ratios to calcium (X:Ca) to correct for total amount of shell ablated. These data were screened and outliers were removed. The 10 replicate analysis of each brooded larvae sample were then averaged to create the best representation of the elemental signature for that brooded larvae sample, with the exception of the samples collected to determine the influence of non-lethal sampling method on elemental signatures. All statistical analysis were performed in R (R version 3.4.2, RStudio version 1.1.383).

3.2.7 *Discrimination and Classification analysis*

Larval samples from 99 individual Olympia oysters coming from 9 different locations of oysters in Puget Sound, WA were used to discriminate between geographically distinct regions using the elemental ratios. Two common approaches to discriminate and classify groups with elemental signatures are linear discriminant analysis (LDA) and random forest classification trees (RF). Both of these statistical approaches classify samples to predefined groups utilizing multiple variables.

LDA predicts group membership by building discriminant axis that are linear combinations of elemental ratios by minimizing the within group variance and maximizing the between group variance (Legendre and Legendre 2012). Assignment accuracy was determined by cross-validation leave one out (jackknife), where the model was created by leaving one

sample out and testing the classification success on the sample left out. This method assumes multivariate normality and equal co-variances which can often be meet by transforming the data.

RF is a machine learning classification approach based on classification trees which recursively split the data into binary groups, this nonparametric approach requires no distributional assumptions of the data set and is free of over fitting (Breiman 2001). Multiple classification trees are created with a random subset of the data using bootstrap sampling (the "in-bag" samples), then the samples not used in creating the classification algorithm (the "out-of-bag" (OOB) samples) are classified by assigning the class the majority of the trees selects (Breiman 2001). The OOB samples are used to measure the successful prediction of group membership and determine a classification error rate, therefore a follow-up cross-validation test to obtain an unbiased classification accuracy is unnecessary.

Just as method selection is vital, it is equally as important to select a combination of variables that maximize the classification to groups (Mercier et al. 2011). Both classification models (LDA & RF) were performed on all combinations of variables (11 variables, 1914 combinations) to determine which combination of variables produced the model with the highest assignment accuracy. Linear discriminant function analysis (LDA) was performed on log transformed (R, BIOSTATS, McGarigal 2014) elemental ratios to meet assumptions of normality and equal co-variances to assess our ability to distinguish between regions based on shell chemistry. LDA and RF were applied to group each individual site independently as well as the sites grouped together into geographically distinct regions; North Sound (NS), Sequim Bay (Seq), and Central Sound (CS) (Figure 1, Table 1).

LDA analysis classification success was determined by the overall cross-validated jackknifed assignment accuracy, and assignment accuracy to each group was determined following the same methods. RF analysis reports the prediction error on the out-of-bag (OOB) portion of the data and assignment accuracy was determined by 1- OOB error rate. The best performing model was determined by high overall assignment accuracy and similar assignment accuracy for each group (i.e. not a variable combination which was good at classifying one group much better than others) With unequal group sizes LDA jackknife may reclassify many samples accurately simply by chance (White and Ruttenberg 2007). To determine a more accurate estimate of classification success for the best performing variable combination for LDA to region a 90/10 random bootstrap sample was used. Randomly 90% of the data (84 samples) was subset to create the LDA classification model and the assignment accuracy was determined by the remaining 10% of the data (10 samples), the data was subsampled 5,000 times to determine the mean assignment accuracy and confidence intervals. Cohen's kappa statistic was also determined to interpret the classification of the LDA results indicating how well the discrimination is performing better than random chance (Titus et al. 1984). All elemental ratios used in the LDA classification model were compared between regions utilizing univariate one way analysis of variance (ANOVA, df=2) followed by post hoc Tukey's tests.

3.2.8 *Temporal Variation*

To examine the relative influence of temporal variation on the elemental ratios, non-parametric multivariate analysis of similarity (ANOSIM) procedures were conducted on Gower's similarity matrix and paired with Nonmetric Multidimensional Scaling (NMDS, two ordination axis and 100 random starts) on brooded larvae samples collected biweekly from two locations, Fidalgo Bay (North Sound) and Mud Bay (Central Sound). Samples collected from 6/15/2015 to

7/5/2015 were categorized as early season (n = 9 and 10 for Fidalgo Bay and Mud Bay, respectively) and samples collected from 7/6/2015 to 8/15/2015 were categorized as late season (n = 9 and 14 for Fidalgo Bay and Mud Bay, respectively). ANOSIM was conducted to test for significant differences between early and late season for each location sampled independently.

3.2.9 *Influence of non-lethal sampling method on signatures*

To evaluate the potential influence of our non-lethal sampling method for collecting brooded larvae using a $MgSO_4$ solution, Analysis of Group Similarities (ANOSIM) was conducted on Gower's similarity matrix and paired with Nonmetric Multidimensional Scaling (NMDS, two ordination axes and 100 random starts) on anesthetized and lethally sampled broods. The goodness of fit of the model was assessed by comparing the observed dissimilarity to the ordination distance. ANOSIM was conducted on individual LA-ICP-MS analysis (i.e. replicates from the same sample were not combined) to determine significant differences between brood samples (i.e. brooded larvae collected from the same adult) and in collection method between anesthetized and lethally sampled broods.

3.3 RESULTS

3.3.1 *Classification results*

Three regions were found to have unique elemental fingerprints in Puget Sound (Fig 2). At the site level, assignment accuracy was low (45-49%, Table 2), but oysters could be more accurately assigned into one of three broader regions with both classification methods. (Table 2, Fig 2). These three regions included 8 of the 9 sites where brooded larvae were collected. Brooded larvae collected from Little Skookum Inlet were not included in the regional

classification (Fig 2). Samples from Little Skookum Inlet were excluded because geographically they should be retained in their own region (South Sound) however with few (5) samples collected from this site and region the classification algorithms could not successfully classify samples from this site.

Classification success to region was similar with both RF and LDA, however each model performed best with different variables (Table 2). RF performed best with only 4 variables (Fe, Cu, Sr, Pb) where LDA reached the highest classification success with 8 variables (Mg, P, Mn, Fe, Ga, Sr, Pb, U), which included three of the variables used by RF (Table 2). Both classification models were best at classifying Central Sound (LDA 87%, RF 81%) followed by North Sound (LDA 75%, RF 72%) with similar success to both regions (Table 3). Classification accuracy was lowest when classifying Sequim Bay, but LDA performed better with 75% success over RF with only 50% success (Table 3). The overall classification success was greater with LDA analysis (81% compared to 74% with RF) with higher success among each grouping and outperforming RF in classification of Sequim Bay by 25% (Table 3). Therefore, LDA was determined to be a better model for classifying *O. lurida* brooded larval elemental signatures to region utilizing the following 8 elemental ratios: Mg, P, Mn, Fe, Ga, Sr, Pb, U (Figure 2).

Linear discriminant analysis successfully classified *O. lurida* brooded larvae into three regions with 79.0 $\pm$ 0.4 (bootstrap sampling with 5000 permutations, 95% CI) accuracy. The bootstrap assignment accuracy of 79.0% is less than the jackknife classification assignment accuracy of 81.9%, but provides a higher accuracy because the bootstrap sampling tests a larger proportion of the data repeatedly. The overall classification accuracy is 69.2% greater than expected by random chance (kappa = 0.6919). The first canonical function explained 73.52% of the total variance with positive loadings from Sr, Fe, and P and negative loaded by Zn, Mn, Pb,

Ba, and U (Fig 4). The remaining 26.5% of variance was explained by the second canonical function with positive loading of U and negative loading by all other elements (Fig 4). Central Sound and North Sound (85.7 $\pm$ 0.4 and 75.5 $\pm$ 0.7 assignment accuracy respectively, bootstrap sampling with 5000 permutations, 95% CI) were differentiated primarily by the first canonical function driven by significant differences in Fe (ANOVA, $F_{(2, 91)} = 14.03$, $P < 0.001$; Tukeys post hoc HSD test $P < 0.001$; Fig 4) and Sr (ANOVA, $F_{(2,91)} = 9.95$, $P < 0.001$; Tukeys post hoc HSD test $P < 0.001$; Fig 4) ratios from those regions (Fig 3 & Fig 4). Sequim Bay (66.2 $\pm$ 1.1 assignment accuracy, bootstrap sampling with 5000 permutations, 95% CI)) was distinguished from North and Central Sound with significant differences in Pb (ANOVA, $F_{(2, 91)} = 7.43$, $P = 0.001$; Tukeys post hoc HSD test, Seq-NS $P = 0.017$ and Seq-CS $P < 0.001$; Fig 4) and Mn (ANOVA, $F_{(2,91)} = 4.797$, $P = 0.011$; Tukeys post hoc HSD test, Seq-NS $P = 0.043$ and Seq-CS $P = 0.007$; Fig 4) elemental ratios seen as negative loadings on both canonical functions (Fig 4). Sequim Bay was more similar to North Sound than Central Sound, only differing significantly in Pb and Mn, where Sequim and Central Sound differed significantly in three additional elements (Zn, Sr, and Fe, Figure 3). Both P and U were important in discriminating between regions in the LDA even though there were no significant differences between the regions when examined independently (ANOVA, $F_{(2, 91)} = 2.14$, $P = 0.124$ and $F_{(2, 91)} = 2.08$, $P = 0.131$, respectively, Figure 3).

3.3.2 *Temporal variation*

The NMDS analysis of elemental ratios of brooded larvae collected from two locations, Fidalgo Bay and Mud Bay, that were sampled repeated throughout the season produced convergence with low stress indicating high confidence in sample scores within the ordination

plot (Fig 5). No within season temporal differences in elemental signatures were detected between early (6/15/2015-7/5/2017) and late (7/5/2015-8/15/2015) season at Fidalgo Bay and Mud Bay (ANOSIM, Fidalgo Bay: P = 0.128, Mud Bay: P = 0.572). This indicates there was no influence of temporal variation in the elemental ratios throughout the summer of 2015 and both locations produced stable elemental signatures.

3.3.3 *Influence of non-lethal sampling method on signatures*

The NMDS analysis of brooded larval collected under the two sampling methods, $MgSO_4$ anesthetic and lethal collection, produced convergence with low stress indicating high confidence in sample scores within the ordination plot (Fig 6). Visual assessment of the ordination plots suggests collection method to be a factor influencing sample scores. The results of ANOSIM show the two collection methods were significantly different, but with a low R statistic (0.1126) indicating the two methods are not very different (Table 4). Individual replicate analysis of shells collected from the same parent oyster showed significant grouping as well, showing more similarity within groups by sample indicated by a large R statistic (ANOSIM, Table 4). This indicates highest correlation from brooded larvae samples was from those collected from the same parent oyster.

3.4 DISCUSSION

The results of this study indicate unique element signatures are incorporated into *Ostrea lurida* natal shells in Puget Sound, WA and can be used to track larval dispersal patterns within this large estuarine system. Unique elemental signatures were detected to three distinct regions and were not site specific. Regional elemental signatures are typical of elemental fingerprinting studies of bivalves (Becker et al. 2005; Carson 2010; Sorte et al. 2013; Gomes et al. 2016).

Assignment accuracy of elemental signatures was low (<50%) to individual site, requiring the regional grouping of sites to increase the assignment accuracy.

These regional signatures can be used to track large scale larval dispersal patterns over relatively short temporal scales. Both statistical approaches (LDA and RF) to classifying brooded larvae samples to their region of origin performed well, with LDA outperforming RF with an 82% successful classification rate. Classification success varied between regions, however assignment accuracy increased with increased sample size. Central Sound had the highest assignment accuracy (87.8%) and the largest sample size (n=49). The other two regions, North Sound and Sequim had smaller sample sizes (n=33 and n=12, respectively) and lower assignment accuracy (75.7% and 75.0%, respectively). Elemental signatures were stable throughout the summer reproductive season of sampling (Fig 4), which is consistent with seasonal stability in elemental signatures found in previous research (Fordie et al. 2011; Becker et al. 2005; Carson et al. 2013).

Elemental signatures of *O. lurida* in Puget Sound were distinguished based on eight elements. Each region varied in the composition of these elements. North Sound had the highest values of Fe and Zn, Central Sound was high in Ba and Pb, and Sequim was highest in U values. Sequim Bay was more similar to North Sound than Central Sound in elemental composition, possibly because North Sound and Sequim are more influenced by the Strait of Juan de Fuca. While the mechanisms driving differences in trace elemental uptake into calcified structures of mollusks remain largely unknown, this knowledge is not required to apply elemental fingerprinting as a technique for tracking natal origins.

The geographical regional scale at which unique elemental signatures were found align with other research of *O. lurida* in Puget Sound. Evidence of population structure and possible

local adaptation of O. luirda was found for Central Sound and Hood Canal populations (Hood Canal oysters were sampled for this study, but no brooded larvae were collected), but evidence for location adaptation wasn't observed from the North Sound population (Fidalgo Bay) (Heare et al. 2017). Similarly, there is genetic evidence of distinct populations between oysters collected in North Sound, Central Sound, and Hood canal populations (White et al. 2017). Significant stock enhancements have been added to each of these regions and with such evidence of distinct populations at these regional scales, quantifying dispersal between these regions is a valuable addition to understanding population structure of *O. lurida* in Puget Sound.

Trace elemental fingerprinting, employing variation in shell microchemistry by location as a method to directly quantify larval dispersal, comes with a few caveats. Individuals can only be tracked back to locations where elemental signatures have been determined, relying on the assumption that all possible sources of larvae have been included in the reference map. Our sampling efforts covered a large geographical spread including locations of most available sites of *O. lurida* and found little reproduction. Geochemical signatures are often found to be stable within a reproductive season, however it has been shown that signatures do not remain stable year to year requiring resampling of possible source populations during each reproductive year (Carson et al. 2013). This limits the application of elemental signatures of potential source populations to the year the sampling was done. The non-lethal method of sampling adult oysters and collecting brooded larvae seems to have influenced the elemental signatures (Fig 6), however the elemental signatures of replicate analysis of brooded shells collected from the same parent oyster were much more similar to each other than the differences detected between collection methods (Table 4) suggesting any differences imposed by the sampling method would be overshadowed by the differences between individuals and site.

Population connectivity of *O. lurida* was previously quantified with trace elemental fingerprinting in Southern California. A significant portion of larvae were exchanged between estuaries along the open coast traveling as far as 75 km, the furthest distance detectable in the study design (Carson 2010). Larval dispersal is driven by biological and physical processes, where the physical oceanography plays a major role (Cowen and Sponaugle 2009), therefore dispersal distances determined and high degree of larval connectivity seen in Southern California cannot be extrapolated to Puget Sound. Furthermore, in the current study the furthest regions, North and Central Sound, are much further apart (115 km) than the maximum dispersal distance observed in Southern California. Replication of larval dispersal studies of the same species in different regions will help to generalize *O. lurida* larval dispersal patterns.

The model built in this research will be used to quantify immigration into and emigration out of populations, determine larval dispersal distances, and ultimately determine population connectivity patterns on ecologically relevant time scales. Regional elemental signatures will allow assessment of large scale connectivity patterns but not small scale (< 20 km) dispersal. This approach determines levels of connectivity on the individual scale, rather than population scale, and does not require assumptions about the dispersal pathway. The quantifications made with this model should be combined with approaches to determining population connectivity, such as biophysical modeling and genetics to achieve a complete understanding of connectivity patterns.

It is imperative to ensure population connectivity for the successful management and restoration of marine species. The level of connectivity within a population will inform whether targeted stock enhancement to improve demographics of existing populations or establishing several small populations will establish a self-persistent metapopulation (Puckett and Eggleston

2016). The ultimate goal of restoration efforts is to establish a network of self-sustaining populations of Olympia oysters in Puget Sound. The ability to track larval dispersal patterns of this species will greatly improve the ability to reach that goal.

3.5 FIGURES AND TABLES

Figure 3.1 Map of Puget Sound, WA, USA, representing sites of existing *Ostrea lurida* populations that were sampled for brooded larvae. Sites with orange circles represent where brooded larvae were collected for this study and sites with grey circles were sampled but no brooded larvae were collected.

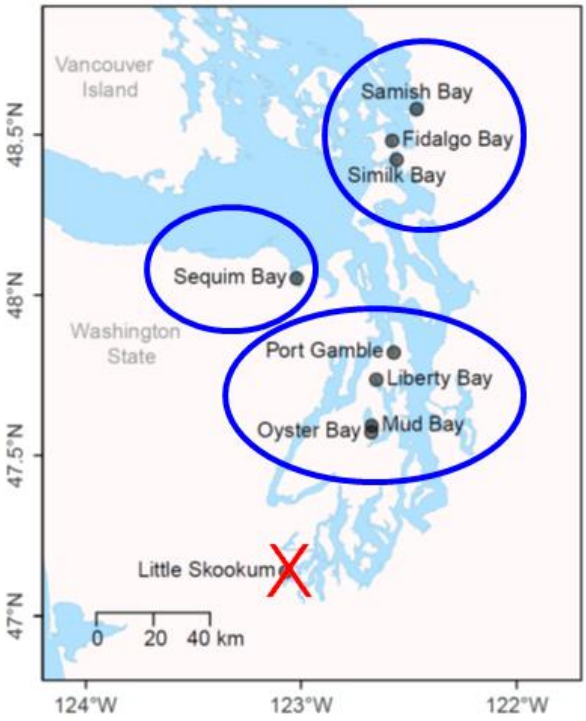

Figure 3.2 Map of Puget Sound, WA, USA representing locations of *Ostrea lurida* where brooded larvae were collected for elemental fingerprinting and the three regions in which unique elemental signatures were determined.

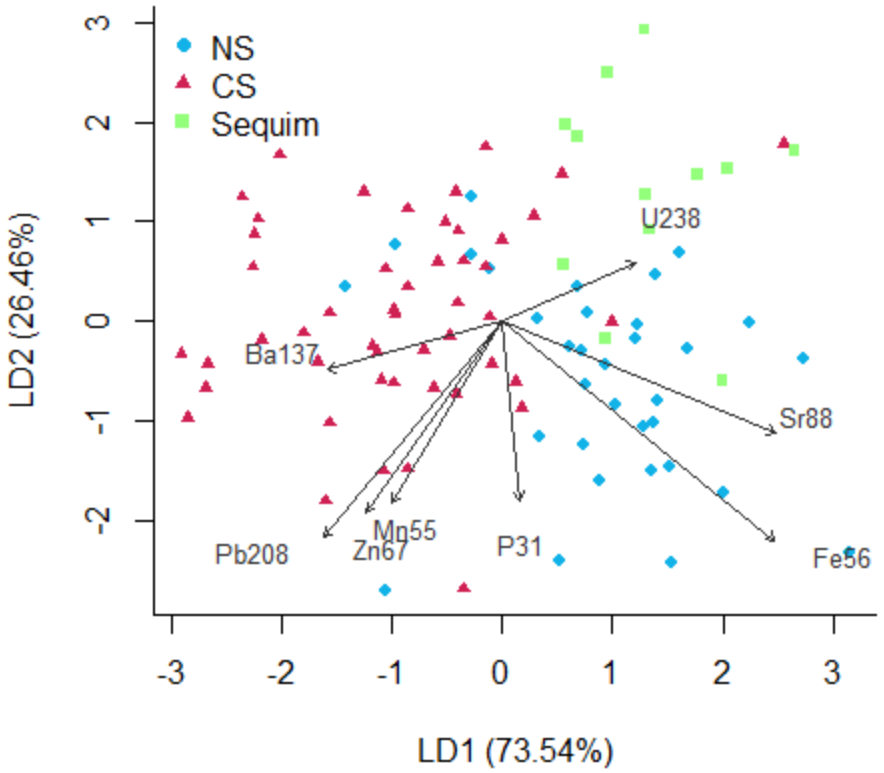

Figure 3.3 Canonical score plot of linear discriminant function analysis of *O. lurida* brood samples classified to region. Each data point represents the average elemental concentrations of brooded larvae collected from one adult oyster. Each region is shown with a different color and symbol. Factor loadings for all elements included in the LDA analysis are shown as vectors.

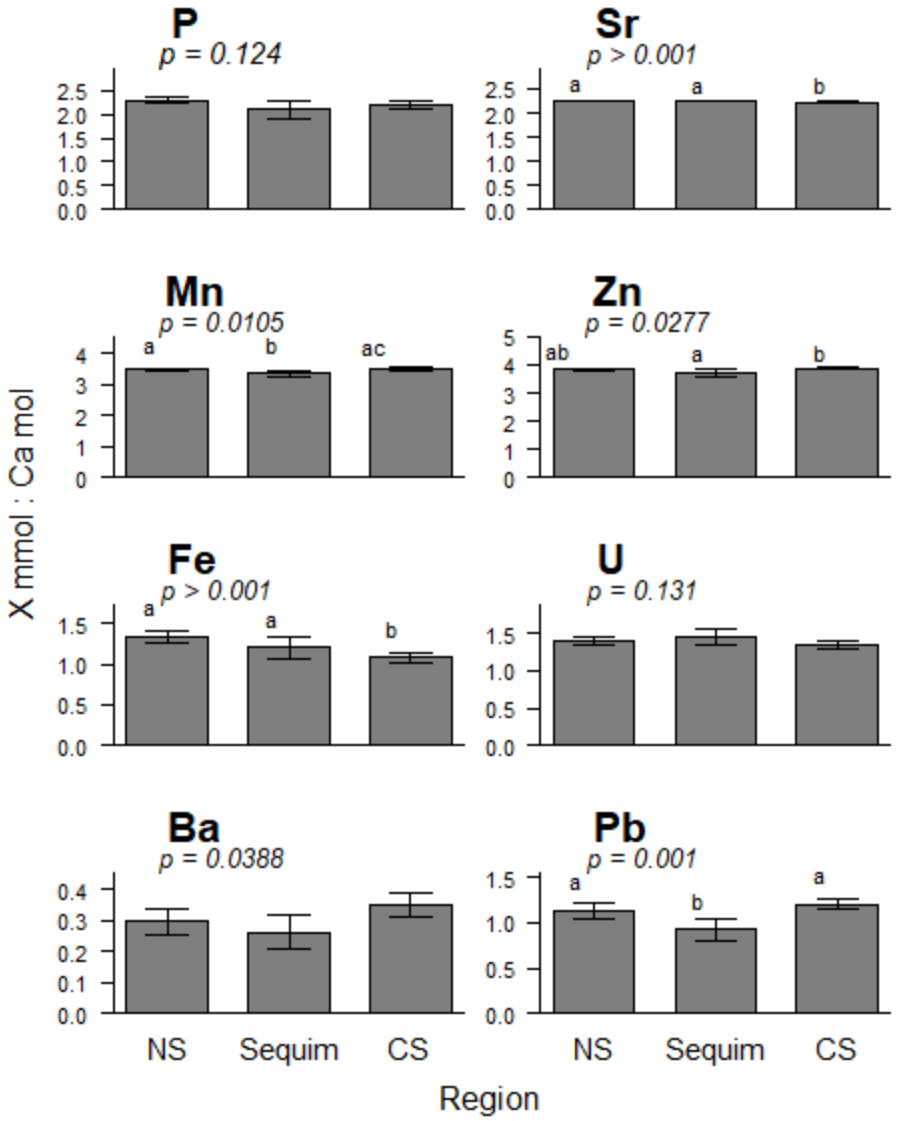

Figure 3.4 Elemental ratios to calcium for the 8 elements used to classify brooded larval shells of *O. lurida* with linear discriminant analysis (LDA) from the three regions North Sound (NS), Sequim Bay (Sequim) and Central Sound (CS) in Puget Sound, WA. Bars represent regional means with 95% CI error bars. The results of individual ANOVAs (df=2) are shown as p value and different letters above bars indicate significant differences ($p < 0.05$) from Tukey post hoc tests.

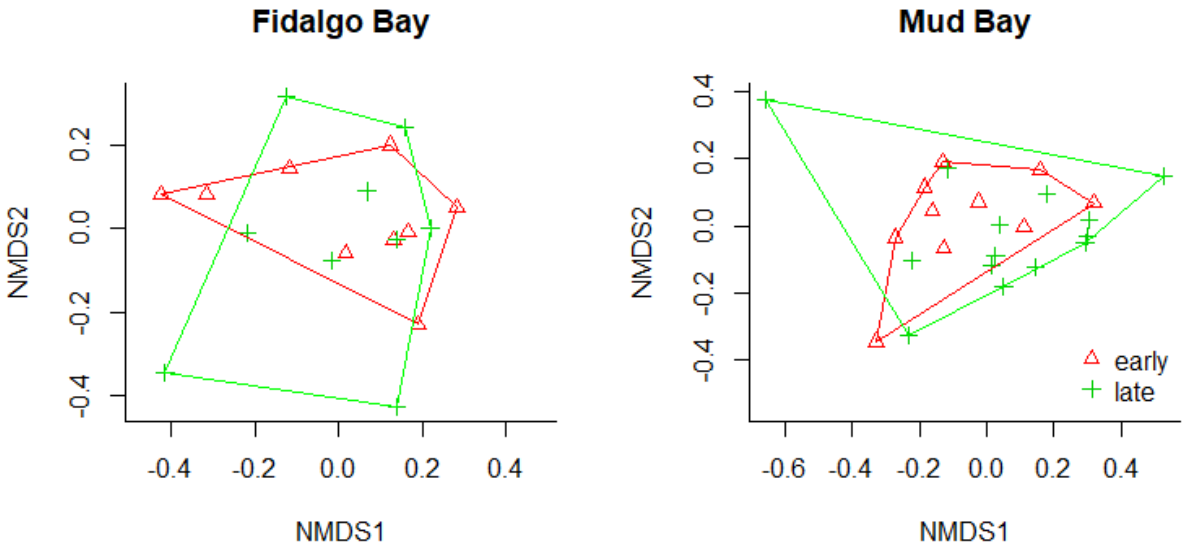

Figure 3.5 NMDS ordination of elemental concentrations in brooded *O. lurida* larval shells compared for temporal variation between early (6/15/2015-7/5/2015) and late (7/5/2015-8/15/2015) reproductive season for two locations, Fidalgo Bay and Mud Bay. Brooded larvae collected early in the season are depicted by red triangles and those collected late in the season by green plus signs.

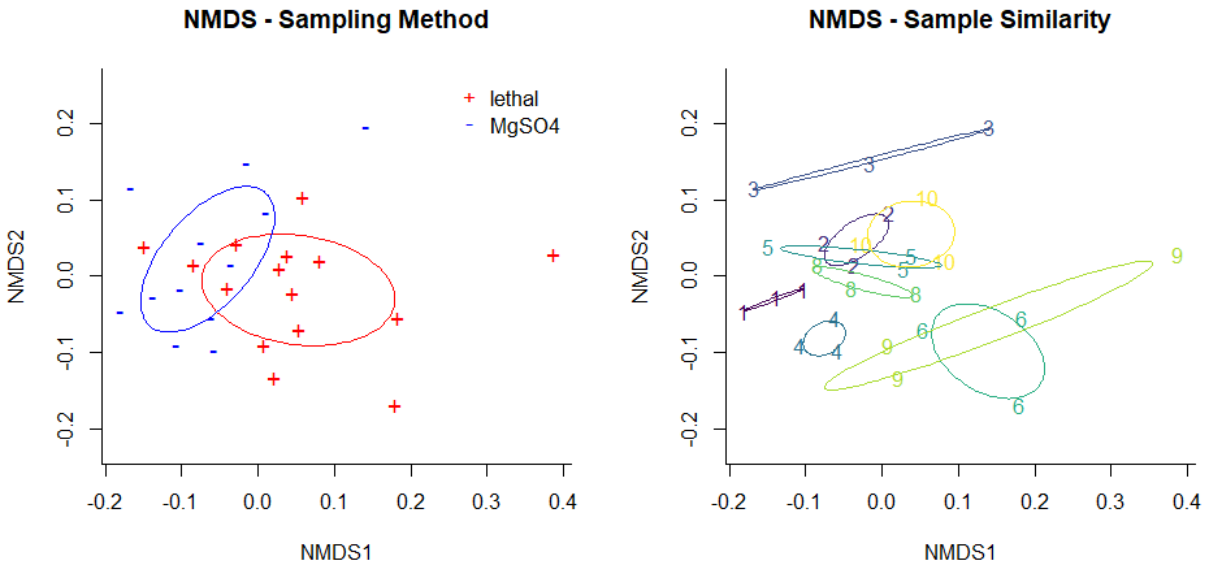

Figure 3.6 NMDS ordination of elemental concentrations in brooded larval shells collected using non-lethal $MgSO_4$ anesthetic and lethal collection, left plot analysis are labeled to sample collection method and the right plot analysis are labeled according to the Olympia oyster the larvae were collected from.

3.6 TABLES

Table 3.1 Summary of late stage brooded *O. lurida* larval samples collected for elemental fingerprinting.

		early							late										
Region	Oyster Collection Site	6/15	6/17	6/19	6/29	6/30	7/1	7/2	7/6	7/7	7/9	7/14	7/16	7/17	7/22	7/28	7/29	7/30	8/4
Central Sound n = 49	**Mud Bay**			2				8			1		6					7	
	Oyster Bay							5					6						
	Port Gamble	3							3										
	Liberty Bay												4	2				2	
North Sound n = 33	**Fidalgo Bay**		1			3	5			3							5		1
	Similk Bay									4		2			1	1			4
	Samish Bay												1				2		
Sequim n = 12	Sequim Bay						12												
X	Little Skookum Inlet				5														

Table 3.2 Summary of trace elemental classification accuracy of *Ostrea lurida* to site sampled and sites grouped into regions for two classification models, Linear Discriminant analysis (LDA) and Random Forest (RF). Classification success for LDA analysis is shown as cross-validated jackknifed assignment accuracy. RF analysis reports the prediction error on the out-of-bag (OOB) portion of the data, and the classification success reported is 1- OOB error rate. Every classification model was performed on all combinations of variables (elements) and the combination with the highest accuracy is reported.

Resolution	**Classification Model**	**Classification Success**	**n elements**	**elements**
Site	LDA	0.4949	6	Mg, Fe, Cu, Ba, Pb, U
	RF	0.4545	8	Mg, P, Mn, Fe, Ga, Sr, Pb, U
Region	LDA	0.8191	8	P, Mn, Fe, Zn, Sr, Ba, Pb, U
	RF	0.7253	4	Fe, Cu, Sr, Pb

Table 3.3 Confusion matrix of LDA and RF analysis of *Ostrea lurida* larvae samples classified to region. The rows represent the actual region of the larval sample and the columns show the number of larvae samples predicted to each region and the percentage that were predicted to their actual region of origin correctly, regions are abbreviated: NS, North Sound; CS, Central Sound; and Seq, Sequim.

		Predicted Group			
Actual Group	n	CS	NS	Seq	% correct
LDA					
Central Sound	49	43	4	2	87.76%
North Sound	33	7	25	1	75.76%
Sequim	12	0	3	9	75.00%
RF					
Central Sound	49	40	8	1	81.63%
North Sound	33	7	24	2	72.73%
Sequim	12	2	4	6	50.00%

Table 3.4 Comparing elemental concentrations between brooded larvae collection methods, non-lethal $MgSO_4$ anesthetic and lethal collection. Summary of NMDS ordination analysis and follow up ANOOSIM, Non-metric fit R2 value reported to measure goodness of fit.

	NMDS		ANOSIM		
	Stress	R^2	Trial	R statistic	P-value
	0.1995255	0.92	Method	0.1126	0.028
$MgSO_4$			Samples	0.6184	0.001

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