My Other Home is a Mesocosm:

A Water Quality Analysis of Three Different Rearing Treatments for *Rana pretiosa*

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ABSTRACT

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Rana pretiosa populations have been in decline for many years. As a result, there are many captive rearing programs that are working to boost the local populations by rearing these frogs in captivity and releasing them back into the wild. Despite the hundreds of healthy young frogs that have been released into the wild, there has not been a corresponding increase in egg mass numbers during the survey season. It is theorized that this may be due to over-stimulation in captivity, and as a result the scientists working on the *R. pretiosa* recovery plan wanted to explore the possibility of rearing the young frogs in a Mesocosm environment. Mesocosms are meant to be a self-sustaining ecosystem unit, with balanced chemical and nutrient cycles that mimic the natural environment as closely as possible. It is hoped that this type of environment will reduce the human contact with the frogs during captivity, reducing their stimulation and keeping their predator-evasion response times fast. However, there are still many factors of the mesocosm environment that we must learn before rearing *Rana* pretiosa in them. This research project looked into gathering baseline data for water quality parameters, comparing the three potential rearing habitats for the Oregon Spotted Frog: Traditional, Mesocosm and Prairie Wetland. The water quality measurements taken and compared were pH, dissolved oxygen, temperature; and nutrient concentrations of chloride, sulfate, phosphorous and nitrate. In each of these parameters both captive rearing treatments (Traditional and Mesocosm) were significantly different than the Prairie wetland environment. These results indicate not only that the Prairie wetland environment is much more variable than either captive rearing environment, but this may also mean that more variability can be applied to the captive rearing environments to better prepare the juvenile frogs for their release into the wild. More extensive research is needed to explore the water quality—especially measurements of chemistry—fluctuations of a Mesocosm environment, however providing a more variable rearing environment for *Rana pretiosa* may prove beneficial..

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CHAPTER 1. LITERATURE REVIEW

Introduction

Oregon Spotted frog Ecology

Rana pretiosa, the Oregon Spotted frog, is one of 46 amphibian species native to Washington State (Washington State DNR, 2009). *R. pretiosa* was once vastly distributed from northern California to southern British Columbia. However, it has since disappeared from 70-90% of its historic geographical range (Pearl & Hayes, 2005). Currently its range is restricted to isolated sites in western and south-central Washington and the east Cascades region of central and south-central Oregon. *R. pretiosa* is listed as an endangered Species both in Washington State and as threatened federally under the Endangered Species Act of 1973 (WDFW 2013).

R. pretiosa is medium-sized (roughly 4.4-10.5cm length vent to snout) and is classified as a highly aquatic frog that is closely associated with permanent water (Conlon et al., 2011). Females are typically larger than males and can reach up to 100 millimeters (4 inches) (Leonard et al. 1993).While there is variation due to age, this species is distinguished from other PNW Ranids by the following characteristics:

> "The dark spots have ragged edges and light centers, which are usually associated with tubercles or raised areas of skin; these spots become larger and darker and the edges become more ragged with age. Body color also varies with age. Juveniles are usually brown or, occasionally, olive green

on the back and white or cream with reddish pigments on the underlegs and abdomen. Adults range from brown to reddish brown, but tend to become redder with age; large, presumably older individuals may be brick red over most of the back. Red increases on the abdomen with age, and the underlegs become a vivid orange-red. This red coloration can be used to distinguish the spotted frogs from other native frogs," (USFWS, 2014)

R. pretiosa frequents heavily vegetated wetlands, though the mechanisms behind this habitat preference are not well understood (Watson, McAllister, & Pierce, 2003). Pearl, 2005), Pearl and authors (2005) observed Oregon Spotted frogs in typical basking positions, eyes above the water with body partially submerged, on or among floating vegetation mats consisting mainly of algae and bladderwort (*Utricularia sp.*). Studies conducted on the predator evasion techniques of *Anuran* species (including *R. pretiosa*) observed frogs in a "frozen" or motionless posture in the water column (Rand, 1952; Heatwole, 1961; Gans and Rosenberg, 1966; Hedeen 1972).

Current Population Decline Theories

Amphibians are now in greater peril than at any time in recent geologic history (Lannoo, 2005). These are perilous times for amphibians, as evidenced by fully one-third of all amphibians worldwide are now considered threatened (Stuart et al., 2004). Native herpetological diversity in northwestern North America is in part a result of the complex geological processes that formed the massive mountain ranges and large plains of the region and subsequently split historical species ranges, fragmented habitats, and altered climates (Nussbaum, Brodie, & Storm, 1983). It is thus speculated that northwestern

amphibian species are reflective of current landscape diversity (Olson, 2009). Due to species habitat preferences outlining distributions, a species may not occupy all suitable habitats within its range due to many factors including, but not limited to, stochastic events affecting current population dynamics and lingering after-effects of historical disturbance events (Olson, 2009). Habitat loss and extirpation from historic ranges necessitate species-specific conservation plans for at- risk species.

The scientific community, in an effort to understand why amphibian species—even those on protected lands—were disappearing, hypothesized a list of the six most influential causes of amphibian decline. In no particular order: habitat destruction/modification, commercial over-exploitation, non-native species introduced to native habitat, environmental contaminants, global climate change and emerging infectious diseases (the most concerning being chytrid fungus *Bactrachochytrium dendrobatidis* (Collins & Storfer, 2003).

Habitat loss and alteration/degradation are considered among the most likely causes of the decline of the *R. pretiosa* (McAllister & Leonard, 1997). The loss and degradation of shallow breeding wetlands are particularly concerning as *R. pretiosa* is reliant on habitat that stays inundated year-round. Watson et al. (2003) observed the Oregon Spotted frog during an animal behavior study using a variety of different aquatic habitats depending on the time of year. For example, they used shallow pools with stable water levels for egg deposition and tadpole development during mating season in the spring, deep pools for juveniles and adults in the dry seasons (suitable for temperature regulation during the hotter months and for predator evasion), and finally shallow water overlaying emergent vegetation during the winter rainy/icy season (Watson et al., 2003).

Due to their specific habitat uses and needs, they are very susceptible to habitat changes and as a result population declines

Pollution of groundwater by agrochemicals (chemical runoff from agricultural practices (Hayes & Jennings, 1986) is a prevalent problem in wetland habitats. Even now many people see wetlands only as wastelands and have to place their current needs over the preservation of important habitats for the future (Aber, Pavri, & Aber, 2012). As such, pollution that reaches the wetlands originates from many different sources that aren't controlled (i.e. agricultural run-off, storm water run-off, etc). *R. pretiosa* spends its whole life in the aquatic environment, and thus is vulnerable to direct exposure of chemicals that are in the water. The egg-stage is especially susceptible to siltation and water pollution (Bugg & Trenham, 2003). Due to their highly permeable skin, the transdermal movement of toxins—absorption of toxins through the skin—can happen easily. Deterioration in water quality can therefore have potentially lethal or sub-lethal effects on amphibians (Boyer & Grue, 1995).

Mesocosms

Ecology is studied across varying geographic scales: from whole system scales to mesocosms, to microcosms. Mesocosms are moderately sized (i.e., smaller than whole ecosystem studies, bigger than microscopic ecosystem studies) man-made ecosystems that are used as tools in ecological research—allowing a certain amount of control over natural complexity through smaller scale, simplified studies. They are also used in applied research and educational development (Kangas & Adey, 1996). They combine a technological component related to the form of container structures with environmental management and control of boundary exchanges with living populations (Kangas &

Adey, 1996). The mesocosm is an extension of the microcosm method, first developed by

EP Odum in 1960 (Beyers & Odum, 1993). It is a wonderful research tool as it allows the

researcher to replicate natural conditions as close as possible, while still exhibiting the

controls of a laboratory to aid replication and statistical validity. Kangas and Adey (1996)

put it best by stating that "mesocosms are special 'windows' along the spatial scale of

ecosystems for examining ecological questions." The mesocosm was used initially in the

1970s as a basic terrarium in school classrooms, and now it is now often used when

conducting semi-experimental studies in aquatic ecology (Odum et al., 1993). It is

important to stress, however, the importance of replicability when using mesocosms. This

factor will prove crucial for any evaluation of ecosystem dynamics, including those

related to the factors I researched for my thesis. A suitable balance between mesocosm

replicability and ecological realism of the mesocosm must be found when using

mesocosms in research, preferably at reasonable costs (Kraufvelin, 1998).

Amphibian Rearing Practices

Current Water Quality Standards

The water quality parameters that can have potentially negative effects on

amphibian species are dissolved oxygen, temperature, pH, salinity/water conductivity,

organic carbons and pollution. All of these factors and the way they interact with each

other can affect survival, growth, maturation and physical development of amphibian

species (Dodd, 2010).

Members of the family *Ranidae* (i.e. *Rana pretiosa*) assimilate oxygen through dermal uptake, gills, and lungs (Dodd, 2010). In hypoxic conditions, larvae with lungs swim to the surface and gulp air into the lungs. Though this risks increased predator exposure, the alternative is hypoxia which can induce similar physiological responses in amphibians as in other vertebrates including changes in blood pH, build-up of lactate in muscles, lethargy and death (Dodd, 2010). There are natural diurnal cycles of dissolved oxygen in wetland environments, however. These cycles correspond to the rates of photosynthesis versus respiration and varies seasonally as well as regionally (Dong, Zhu, Zhao, & Gao, 2011).

Amphibians are ectotherms meaning they rely on elements in their environment to regulate their temperature (i.e. shade when they're hot, sunlight when they're cold, etc). Therefore water temperature is extremely important in metabolic function, physiological processes and behavior. For most amphibian species, including *R. pretiosa*, between 10° and 40°C each 10° increase in ambient temperature increases metabolism by 1.4-2.4 times (Rome, Stevens, & John-Alder, 1992). Licht (Licht, 1971) tested the range of tolerance for *R. pretiosa* embryos in extreme temperatures. He reported a lethal minimum, the temperature at which the eggs' survival is less than 50% is approximately 6°C. There is also a general relationship between temperature and dissolved oxygen. Generally speaking as temperature increases dissolved oxygen decreases (Dodd, 2010).

The final water quality parameter that I would like to address with my research is pH. pH is the negative log of Hydrogen ion concentrations in water , scaling from 0-14, with 6.0-7.5 considered "safe" or "neutral" conditions for most freshwater aquatic species (Dodd, 2010). Natural factors affecting pH are the bedrock found in the area and the

concentration of organic acids (Dodd, 2010). However, anthropogenic sources of acidity are acid deposition from the production of nitrous oxides and sulfates during the processing of fossil fuels and acid mine drainage (Dodd, 2010). pH can affect successful amphibian development at all life stages. For example, at pH values lower than 4.5, embryonic development may cease entirely, whereas at high pH values development continues but hatching is disrupted (Dunson & Connell, 1982). The critical pH or that which can cause significant increases in mortality for amphibian embryos ranges from 5.0-3.5 (Freda & Dunson, 1985). The primary effects of being exposed to low pH waters are interference with ion transport, compromised immune systems, inability for embryos to hatch, reduced growth and delayed metamorphosis (Brodkin et al., 2003).

Issues with Captive Rearing

Captive rearing programs have been successfully raising *R. pretiosa* for a number of years and releasing them back into their native habitat in hopes of boosting local populations. Currently there are partnerships with government agencies (such as Washington Department for Fish and Wildlife) and private organizations such as the Oregon, Pt. Defiance and Woodland Park Zoos that foster captive rearing projects for the rearing and reintroduction of the now endangered *Rana pretiosa*. However, despite years of successful rearing and release, there has not been a physical confirmation of increased populations in the form of egg masses which leads to one question—what is happening to the frogs once they're released?

Kyle Tidwell, currently a doctoral student at Portland State University wondered the same question and began exploring the possibility of lowered predator responses being responsible for the Oregon Spotted frog not bouncing back after successful captive

rearing programs. Kyle Tidwell, as well as his companion researchers, theorized that perhaps the exposure to frequent contact during captive rearing is causing the frogs to be "over-stimulated" and thus less responsive to predator attacks resulting in decreased survivorship post-release. His study tested two separate populations of captively-reared Oregon Spotted frogs with a ball drop test to simulate a predator dropping on them and gauged their response. Though the rearing conditions for both populations were the same, Tidwell did find a significant difference in response times between them, which suggests that there are factors affecting the Oregon Spotted frogs ability to respond to a predator threat (Tidwell, Shepherdson, & Hayes, 2013). He suggests that "it is possible that husbandry activities such as cleaning and feeding made the frogs progressively warier," (Tidwell et al., 2013). Tidwell's results led scientists and husbandry personnel involved in the captive rearing program to wonder if a mesocosm environment might not be better suited than captivity for raising the Oregon Spotted frogs and hopefully keeping them properly "stimulated' and aware of predators.

Mesocosm History in Captive Rearing

The use of aquatic mesocosms to study amphibian ecology was explored beginning with the need to move away from purely descriptive field studies and correlative analyses and move towards more manipulative studies that would allow for hypothesis testing (Dodd, 2010). "Studies using aquatic mesocosms are a compromise between the variability of natural wetlands, confounding factors and the lack of control that is common in field experiments," (Dodd, 2010). Mesocosms rather than microcosms are used in amphibian rearing as they are self-sustaining once they are established, which allows all organisms kept in the mesocosm to complete the critical phases of their life

cycle (Dodd, 2010). The two most common forms of mesocosms for amphibian rearing are (1) cages or floating mesh tanks that are placed directly in the stream/lake/water body the eggs are taken from and (2) large containers (i.e. plastic cattle watering tanks) mimicking wetland systems. Either of these options give the researcher the ability to capture a pocket of the natural environment, while still being able to manipulate much of the environmental processes within the mesocosm either for study or for the support of endangered amphibian species. However, it is crucial that we understand the nutrient and chemical cycles that should occur within the mesocosms as a part of the natural processes as well as what cycles need to be avoided to protect the health of the animals. My research was designed to test for the differences between water chemistry and quality of traditional, mesocosm and natural wetland ecosystems used by Oregon Spotted Frogs.

Wetland Biochemistry in Pacific Northwest Wetlands

Wetland ecosystems as well as mesocosms mimicking wetlands have diurnal cycles of oxygen. These cycles revolve around the ratio of photosynthesis to respiration which in turn are affected by temperature and pH. Water solutions typically contain dozens of dissolved solids, such that overall charges balance for electrical neutrality (Aber et al., 2012). The degree to which the wetland biochemical cycles fluctuate varies by wetland and in some cases can have a very wide fluctuation either daily or seasonally.

The chemistry of wetlands depends on many factors including the influence of bedrock and soil, inflow and outflow of surface and groundwater, climate and vegetation, characteristics of surrounding terrain, and human impacts (Aber et al., 2012). "Key wetland elements include nitrogen, potassium, iron and manganese, sulfur, phosphorous and carbon," (Aber et al., 2012). The specific chemical status of these elements within the

wetland environment depends primarily on the presence of oxygen within the wetland waters (Aber et al., 2012).

"Nitrogen is a major nutrient and is often a limiting factor in flooded wetlands or peat lands. Once ammonium is formed, it may be used directly by plants or anaerobic microbes, which convert it again into organic matter. In marshes with algal blooms, pH may exceed 8, in which case ammonium is converted into ammonia (NH_3) and released into the atmosphere (Mitsch & Gosselink, 2007). Under aerobic conditions, ammonium may be converted into nitrite (NO_2^-) and then nitrate (NO_3^-)," (Aber et al., 2012)

Phosphorous, like nitrogen, is a limiting factor in wetlands. It's most bioavailable under neutral to slightly acidic conditions (Aber et al., 2012). Phosphorous is considered to be an essential element for life in the DNA molecule and adenosine triphosphate, which stores chemical energy. It is also an essential nutrient for growth and development of algae and other plants (Stewart & Howell, 2003).

Sulfur is generally abundant in the wetland environments and thus is not likely to be considered a limiting factor for plant growth. In the anaerobic zone, sulfur and sulfate are reduced into hydrogen sulfide. This can alter the pH and thus the acidity of the wetland water chemistry.

My research will begin to address the gaps in our knowledge about water chemistry both in wetlands and captive rearing environments.

CHAPTER 2

Introduction

The Oregon Spotted frog, *Rana pretiosa* is a medium-sized, aquatic amphibian native to Canada, Washington, Oregon and California (Watson, McAllister, & Pierce, 2003). *R. pretiosa* was once distributed from northern California to southern British Columbia. However, it has since been extirpated from 70-90% of its historic geographical range (Pearl & Hayes, 2005). Currently its range is restricted to isolated sites in western and south-central Washington and the east Cascades region of central and south-central Oregon. *R. pretiosa* is listed as an Endangered Species both in Washington State and federally under the Endangered Species Act of 1973 (WDFW 2013, Federal Registry 2014). *R. pretiosa* is distinguished from other Pacific Northwest ranids by its unique spotting pattern, higher eye placement and increased webbing on the feet (USFWS 2014). These characteristics are related to *R. pretiosa*'s life history and highly aquatic habits.

Due to its completely aquatic lifestyle, *R. pretiosa* not only requires specific habitat characteristics, but is also more vulnerable to various threats—both biological and chemical—throughout its lifetime. There are many theories suggested as reasons for *R. pretiosa* population declines. Top theories currently include habitat alteration, which can range from small hydrological changes to transforming wetlands, predation by invasive fish and amphibians, and possible physiological impairments caused by exposure to toxins in the water (Pearl & Hayes, 2005).

Habitat loss and alteration/degradation are considered one of the most likely causes of the decline of *R. pretiosa* (McAllister & Leonard, 1997). The loss and

degradation of shallow breeding wetlands are particularly concerning as *R. pretiosa* is reliant on inundated wetland habitat year-round. Watson et al. (2003) observed the Oregon Spotted frog making use of a variety of different aquatic habitats—which varied with the time of year—during an animal behavior study. For example, the frogs used shallow pools with stable water levels for egg deposition and tadpole development during mating season in the spring, deep pools for juveniles and adults in the dry seasons (suitable for temperature regulation during the hotter months and for predator evasion), and finally shallow water overlaying emergent vegetation during the winter rainy/icy season (Watson et al., 2003). Due to their specific habitat requirements, spotted frogs are susceptible to habitat changes and as a result population declines.

Pollution of groundwater by agrochemicals (chemical runoff from agricultural practices (Hayes & Jennings, 1986) is a prevalent problem in wetland habitats. Even now many people see wetlands only as "wastelands" and place development and conversion priorities over the preservation of these important wetland habitats for the future (Aber, Pavri, & Aber, 2012). As such, pollution that reaches the wetlands originates from many different sources that aren't controlled (i.e. agricultural run-off, storm water run-off, etc.). *R. pretiosa* spends its whole life in the aquatic environment, and thus is vulnerable to direct exposure of chemicals that are in the water. The eggs especially are extremely susceptible to siltation and water pollution (Bugg & Trenham, 2003). Due to their highly permeable skin, the transdermal movement of toxins—absorption of toxins through the skin—can happen easily. Deterioration in water quality can therefore have potentially lethal or sub-lethal effects on amphibians (Boyer & Grue, 1995).

Captive rearing programs have been successfully raising *R. pretiosa* for a number of years with the goal of releasing them back into their native habitat to boost local populations or establish new populations. As guided by recovery goals for this species, collaborative efforts between government agencies (such as Washington Department for Fish and Wildlife) and private organizations such as the Oregon, Pt. Defiance and Woodland Park Zoos (found in Portland, Tacoma and Seattle, respectively) that foster captive rearing projects for the rearing and reintroduction of the now endangered *Rana pretiosa*. However, despite years of successful rearing and release, there has not been a physical confirmation of increased populations in the form of egg masses which leads to one question—what is happening to the frogs once they're released (K. Tidwell, personal communication)? Kyle Tidwell, a doctoral student at Portland State University, as well as his companion researchers, theorized that perhaps the exposure to frequent contact during captive rearing is causing the frogs to be "over-stimulated" and thus less responsive to predator attacks resulting in decreased survivorship post-release (Tidwell, Shepherdson, & Hayes, 2013). Due to Tidwell's results, local ecologists involved in the *R. pretiosa* recovery plan theorized that perhaps using mesocosms as the captive rearing environment would have more positive benefits beyond the goal of increasing survival during development.

Ecology is studied across varying geographic scales: from whole system scales to mesocosms, to microcosms. Mesocosms are moderately sized (i.e., smaller than whole ecosystem studies, bigger than microscopic ecosystem studies) human-made ecosystems that are used as tools in ecological research—allowing a certain amount of control over natural complexity through smaller scale, simplified studies. Scientists also use

mesocosms in applied research and educational development (Kangas & Adey, 1996). They combine a technological component related to the form of container structures with environmental management and control of boundary exchanges with living populations (Kangas & Adey, 1996).

Despite the strong arguments in favor of rearing *R. pretiosa* in mesocosms, there are still many facts we do not know about methodology to inform rearing protocols or how individual frog will respond to the treatment. My research addressed the water quality needs of rearing *R. pretiosa* in mesocosms. My primary research question was "is there a difference in water quality parameters—pH, temperature, dissolved oxygen, nutrient concentration—between a traditional captive rearing environment and a mesocosm rearing environment. Secondarily, I explored the differences between two captive rearing treatments and the natural prairie wetland environment.

Methods

Study Design

I collected water quality measurements and samples from three different rearing habitats for *R. pretiosa*: 1) Traditional rearing habitat which is a regularly-cleaned tank environment with no plant material, 2) Mesocosm rearing habitat which is a lab-

Figure 1—the captive rearing tanks at Woodland Park Zoo. Four tanks were Traditional rearing environments (without any natural elements) and four were Mesocosm rearing environments (which included wetland plants collected from West Rocky Prairie)

contained tank environment that resembles the natural habitat through the presence of wetland plants and 3) Prairie wetland habitat which is a natural habitat of *R. pretiosa*. The traditional and mesocosm rearing habitats were 300 gallon cattle tanks (see **Figure 1**) kept at Woodland Park Zoo in Seattle, WA (hereafter referred to as WPZ). The wetland habitat sampled at West Rocky Prairie (hereafter referred to as WRP) consisted of four randomly chosen points. There were 4 separate tanks of the Traditional and Mesocosm rearing habitat, for a total of 8 tanks sampled each week at WPZ. To complete the sampling, 4 points were chosen at random in the WRP wetland to be the 4 replicates of the third rearing environment; these same points were sampled during every sample collection.

<u>**Sample Collection**</u>

Samples were collected once per week for six weeks between July 26 and August 30, 2014. Woodland Park Zoo samples were collected every Saturday between 10:00am and 12:00pm and West Rocky Prairie samples were collected every Sunday within the sampling period between 10:00am and 12:00pm. During each sample collection the following measurements were taken: pH, temperature, and Dissolved Oxygen. Additionally 500ml water samples were collected in plastic (PP) sample lab bottles from each replicate back to the lab for nutrient analysis. See **Table 1** below.

Table 1. Sampling schedule for each of the three rearing environments for *R. pretiosa*. 500ml samples were taken from each of the four replicates for each of the three treatments once per week during the three weeks of sampling

Habitat Type	N (# of Replicates)	Times Sampled	Volume Sampled
Traditional (Captive Rearing)	4	3	500ml
Mesocosm (Captive Rearing)	4	3	500ml
Prairie (Wild)	4	3	500ml

Lab Methods

All sample bottles were washed with hot, soapy water and rinsed three times with DI (Deionized) water prior to field collection, to prevent sample contamination. Additionally all lab equipment and glassware used for nutrient analysis (volumetric glassware, vacuum filtration apparatus) were washed with hot soapy water and rinsed with DI water prior to analysis. During the analysis process, the syringe to insert samples into the Ion Chromatograph was washed with hot soapy water and rinsed with DI water between every sample to prevent cross-contamination.

Materials List

For full materials list see **Appendix A**

Nutrient Analysis

The nutrient analysis portion of my research was conducted at The Evergreen State College using a DIONEX IC25A ion chromatograph. I followed the EPA methods 300.1 (Plaff, 1993) as a guideline for testing nutrient concentrations in each sample. Nutrient analyses were conducted during the same week samples were collected.

According to the EPA methods 300.1 (Plaff, 1993) the holding time for chloride and sulfate is 28 days; for nitrate, nitrite and phosphate the holding time is 48 hours. Based on these holding times, samples were stored at 4°C prior to analysis to maintain accurate nutrient levels until analysis. Stock standards for most anions are stable for at least 6 months when stored at 4°C. **Phosphate stock standards are only stable for 1 month when stored at 4°C.** Working standards for chlorine, nitrate and sulfate were prepared once per month and the working standards for phosphate were prepared fresh on the day of analysis in order to prevent instability of nutrient concentration.

Concentration/Dilution

McKibbin (2008) found these the nutrient concentrations in a local wetland during sampling in British Columbia:

Nutrient	Concentration (mg/L)
Chloride	1.03
Sulfate	0.5
Nitrate	0.5
Phosphorous	0.04

There is a lack of published research on nutrient concentrations in water quality tests of wetlands—thus I used these concentrations as a starting point for my analysis. These determined the concentration of my High, Medium and Low Standards during the testing. In the process of ion chromatography High, Medium and Low standards are created in an effort to bracket the actual nutrient concentration in the sample being tested. These three working standards give the ion chromatograph three calibration curves for each nutrient

being tested, giving it three points of reference when assessing the nutrient concentration of the sample.

Preparing Standards

1. *Stock Standards*

    a. Prior to creating stock solutions, each compound was placed in glass petri dishes and put into an oven at 105°C for 24 hours. Compounds were then transferred to a glass desiccator to cool and prevent moisture from re-absorbing into the solid compounds.

    b. A pre-determined amount of each compound (see **Table 2** below) was added to a 1L volumetric flask, DI water was then added for a total volume of 1L. Next, each stock standard was poured into a specific 1L plastic bottle and mixed well by shaking.

Table 2-Amount (g) of each analyte added to deionized water to create stock standards at a concentration of 1000mg/L. These amounts were taken from the EPA methods 300.1 (Plaff, 1993) for detecting nutrient concentrations in drinking water

Analyte Stock	Concentration	Weight of Compound (g)	Final Volume (L)
Chloride	1000 mg/L	0.1649g Sodium Chloride	1 L
Sulfate	1000 mg/L	0.1814g Potassium Sulfate	1 L
NO_3^--N	1000 mg/L	0.606g Sodium Nitrate	1 L
PO_4^{3-}-P	1000 mg/L	0.4394g Potassium Dihydrogenphosphate	1 L

c. Use Stock Standards to make mixed intermediate stock standards:

    a. *Mixed intermediate stock standards were made* by adding 10mL each of chloride, nitrate, phosphate and sulfate stock standard solution to a 1L volumetric flask and bringing to a volume of 1L

with DI water. Intermediate stock standards were used to make

working standards because the stock standards, as instructed by

EPA methods 300.1 (Pfaff, 2003) were made at much higher

concentrations than what was being tested for and it made the

creation of the working standards more accurate.

d. Quality Control Stock Standards, Intermediate Standards and Working

Standards were made following the aforementioned methods, using

different compound stocks.

2. *Working/External Standards*

Use the following pre-determined amount of the mixed intermediate (chloride, nitrate,

sulfate and phosphate) stock standard solution to create the specified **mixed** Low,

Medium and High working standards. Add calculated volume of mixed intermediate

stock standard for each analyte to a 500 mL volumetric flask and bring to volume with DI

water. Then transfer to a clean 500 mL plastic bottle for use in analysis.

MIXED LOW STANDARD			
Analyte	*Concentration (mg/L)*	*Volume Needed from Int. Stock (mL)*	*Final Volume(mL)*
Chloride	0.50	25	
Sulfate	0.20	10	
NO_3^--N	0.10	5	
PO_4^{3-}-P	0.02	1	
		Total: 31mL	**500mL**

MIXED MEDIUM STANDARD			
Analyte	*Concentration (mg/L)*	*Volume Needed from Int. Stock (mL)*	*Final Volume(mL)*
Chloride	1.0	50	
Sulfate	0.7	35	
NO_3^--N	0.5	25	
PO_4^{3-}-P	0.06	3	
		Total: 113mL	**500mL**

MIXED HIGH STANDARD			
Analyte	*Concentration (mg/L)*	*Volume Needed from Int. Stock (mL)*	*Final Volume(mL)*
Chloride	1.5	75	
Sulfate	1.2	60	
NO_3^--N	1.1	55	
PO_4^{3-}-P	0.2	10	
		Total: 200mL	**500mL**

Running Samples through Ion Chromatograph

The ion chromatograph components (oven, eluent generator and chromatograph unit) were turned on 2 hours prior to running samples to allow the various readings to stabilize. Samples were also taken out 2 hours prior to running to allow them to reach room temperature. This was an important step to ensure accuracy during analysis. The

syringe and filter apparatus was washed with hot, soapy water, rinsed with DI water and then rinsed three times with the sample being run for "pre-contamination" This last step was important to ensure that no one sample contaminated another sample reading. Syringe with approximately 2 milliliters was then loaded and injected into the ion chromatograph for analysis. While one sample was running, the next sample was prepared for injection following the same protocols previously stated. **See Appendix B for full Run List**

Statistical analysis was completed using non-parametric tests as the data set did not pass preliminary tests for normality and spread.

Results

pH, Dissolved Oxygen, Temperature

All three parameters measured in real time with probe instruments demonstrated a statistically significant difference between both the captive rearing treatments (Mesocosm and Traditional) and the Prairie wetland habitat. For pH, the data were mostly consistent in all three treatments across the full six weeks (p=0.0313, df=2), see **Figure 2**.

Though the dissolved oxygen and temperature results showed more variability across the sampling period, both parameters showed a statistically significant difference between the captive rearing treatments (Mesocosm and Traditional) and the Prairie wetland habitat (DO: p=0.313, df=2; Temp: p=0.313, df=2). For dissolved oxygen, see **Figure 3**, for temperature see **Figure 4**.
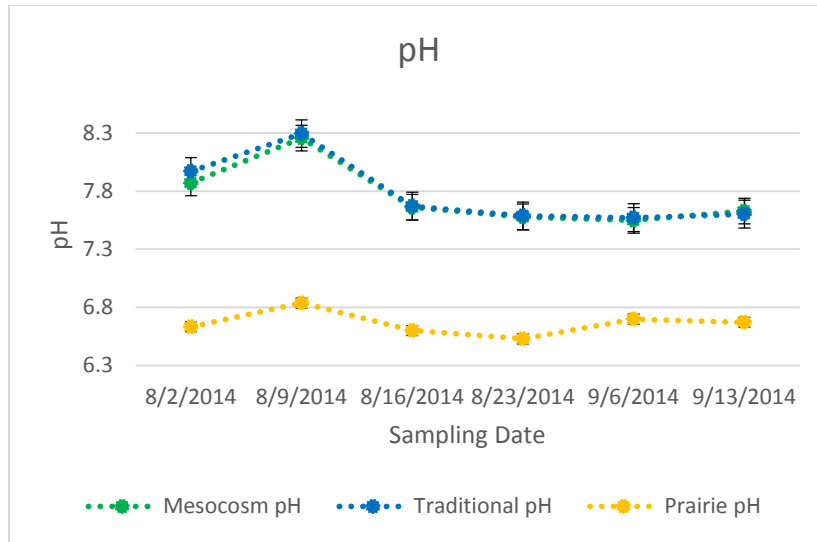
Figure 2—pH measurements for each rearing habitat (Traditional, Mesocosm and Prairie) taken over the sampling period of 6 weeks. Mesocosm and Traditional pH readings differed significantly from the Prairie habitat (p=0.313, df=2).
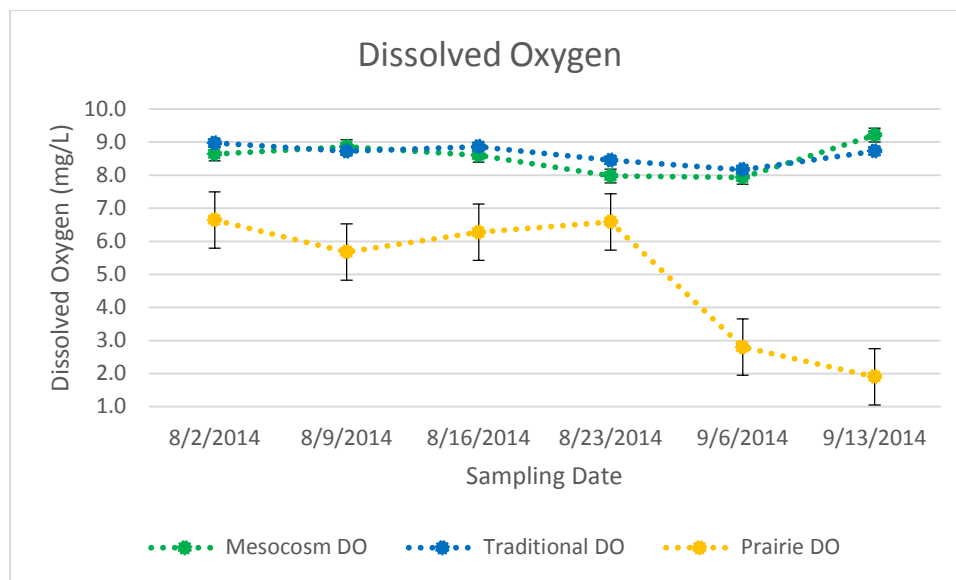

Figure 3—Dissolved oxygen measurements for the three rearing habitats across the sampling period. Statistical analysis showed a significant difference between both captive rearing habitats (Traditional and Mesocosm) and the natural Prairie habitat (p=0.313, df=2)
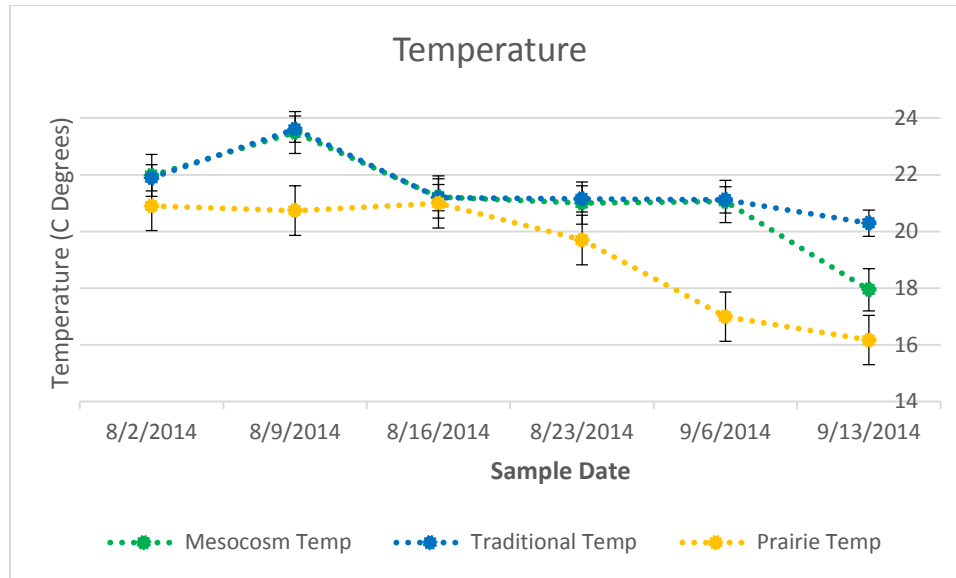
Figure 4—Temperature measurements across the sampling period. Statistical analysis showed a significant difference between both captive rearing habitats and the natural Prairie habitat (p=0.313, df=2)

Nutrient Analysis

All four nutrients measured showed significant differences between the treatments during the three sampling weeks. Phosphorous showed the most significant difference between the rearing environments across the sampling period. Week 1 of sampling showed a significant difference between the Mesocosm and Traditional rearing environments (p=0.029, df=2) as well as between the Mesocosm and Prairie rearing environments (p=0.019, df=2). Week 2 of sampling showed a significant difference between both captive rearing treatments (Mesocosm and Traditional) and the Prairie habitat (p=0.023, df=2; p=0.025, df=2, respectively). See **Figure 5**.
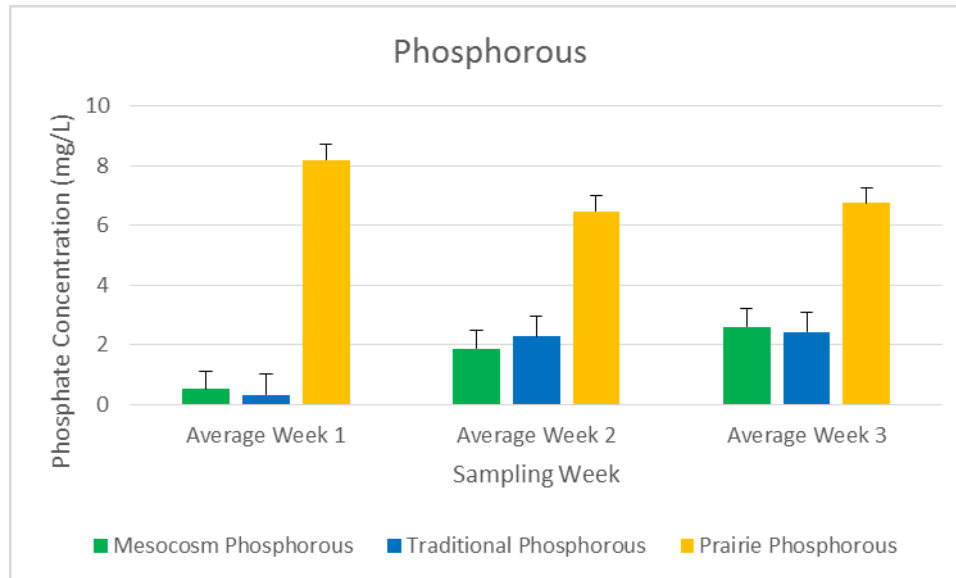
Figure 5—Phosphorous concentrations across the three sampling weeks for each rearing habitat. Here the data shows that there was a difference found between the Prairie wetland habitat and the two captive rearing habitats, statistical analysis determined that there was a statistically significant difference between both captive rearing treatments and the Prairie habitat (p=0.026).

Seen in more detail in **Table 3**, we can see the distinct difference in phosphorous concentration between the three rearing habitats for *R. pretiosa*.

Table 3—Data representing the weekly averages for the phosphorous concentration in each of the three rearing habitats (Traditional, Mesocosm and Prairie wetland). Though no significant differences were found during statistical analysis, the data to reveal a strong difference between the natural prairie habitat and the two captive rearing habitats.

Treatment	Average Week 1	Average Week 2	Average Week 3
Mesocosm	0.51075	1.87775	2.601
Traditional	0.32725	2.28425	2.43075
Prairie	8.1845	6.484	6.74575

Nitrate concentrations found in the three rearing habitats were on average much higher than all other nutrients measured, and showed significant differences between the

Mesocosm and Prairie rearing environment. Week 1, week 2 and week 3 sampling revealed a significant difference between the Mesocosm captive rearing treatment and the Prairie wetland habitat (Week 1: $p=0.029$, $df=2$; Week 2: $p=0.028$, $df=2$; Week 3: $p=0.029$, $df=2$).
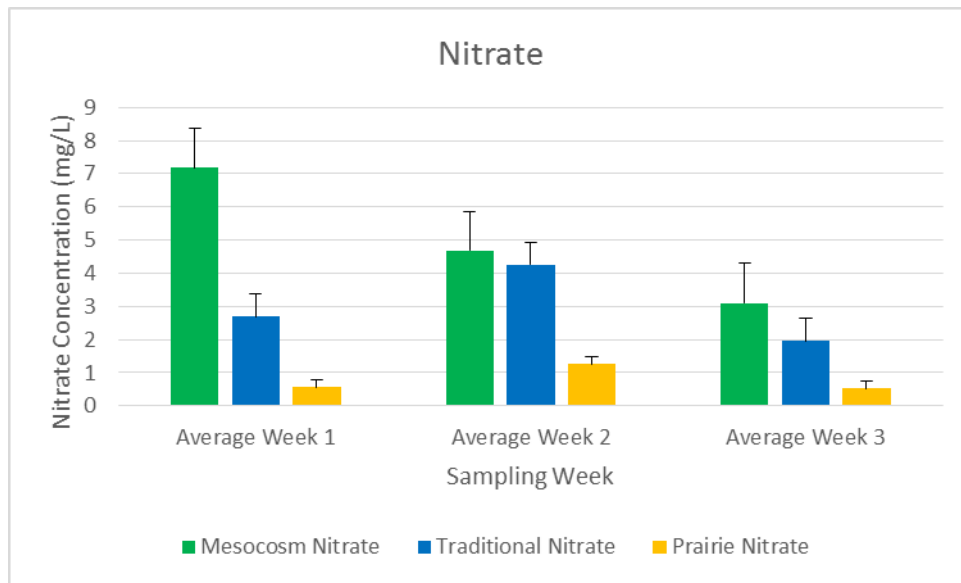

Figure 6—The nitrate concentrations across the three sampling weeks for the each of the three rearing habitats (Traditional, Mesocosm and Prairie). Statistical analysis revealed significant differences between the Mesocosm and Prairie environment for all three weeks of sampling

Additionally the Traditional rearing habitat showed higher nutrient concentrations over all three weeks of sampling when compared to the Mesocosm and Prairie habitat. Nitrate concentrations also showed the most variation from week to week compared to the other nutrients measured (see **Figure 6**, **Table 4**).

Table 4—Average data per sample week for each rearing habitat (Traditional, Mesocosm and Prairie). These data, though statistical analysis did not reveal any significant difference, show what appears to be a trend that may be discovered after additional sampling.

Treatment	Average Week 1	Average Week 2	Average Week 3
Mesocosm	7.188666667	4.676333333	3.103666667

| Traditional | 2.698 | 4.2545 | 1.957 |
| Prairie | 0.55475 | 1.2505 | 0.52 |

Chloride concentrations also showed significant differences between the captive rearing treatments (Mesocosm and Traditional) and the Prairie habitat. In Week 2 of sampling, chloride concentrations differed significantly between the Mesocosm captive rearing treatment and the Prairie wetland ($p=0.033$, df=2) as well as between the Traditional captive rearing treatment and the Prairie wetland ($p=0.031$, df=2). Similarly in Week 3 of sampling, chloride concentrations significantly differed from both captive rearing treatments and the Prairie wetland habitat (Mesocosm: $p=0.026$, df=2; Traditional: $p=0.026$, df=2). See **Figure 7, Table 5**.
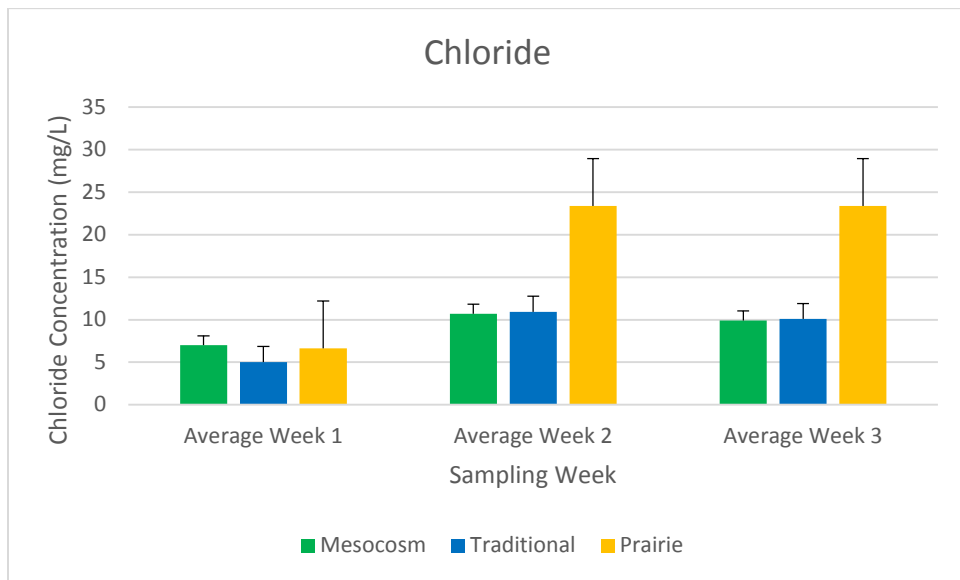

Figure 7—Chloride concentrations for each of the three rearing habitats across the three sampling weeks. This data shows that the Prairie wetland habitat had consistently higher concentrations of chloride, despite statistical analysis not revealing a significant difference between the mean values of each treatment.

Table 5—Average weekly values for the chloride concentrations in each of the three rearing habitats for *R. pretiosa.* Though statistical analysis did not reveal any significant differences or relationships, the data indicate that there are differences between the captive rearing environments and the natural Prairie environment. Further investigation may reveal more statistically significant relationships.

Treatment	Average Week 1	Average Week 2	Average Week 3
Mesocosm	6.99625	10.6985	9.898
Traditional	5.03125	10.931	10.0795
Prairie	6.635	23.377	23.379

Sulfate concentrations showed significant differences in the rearing habitats only one of the three sampling weeks. Week 1 of sampling showed a significant difference in sulfate concentrations between the Mesocosm and Traditional rearing treatments (p=0.046, df=2). This is the only nutrient concentration that did not show significant difference between the two captive rearing treatments (Mesocosm and Traditional) and the Prairie wetland habitat, however as seen in **Figure 8** and **Table 6**, there appear to be more trends in the data than those that were detected by statistical analysis.
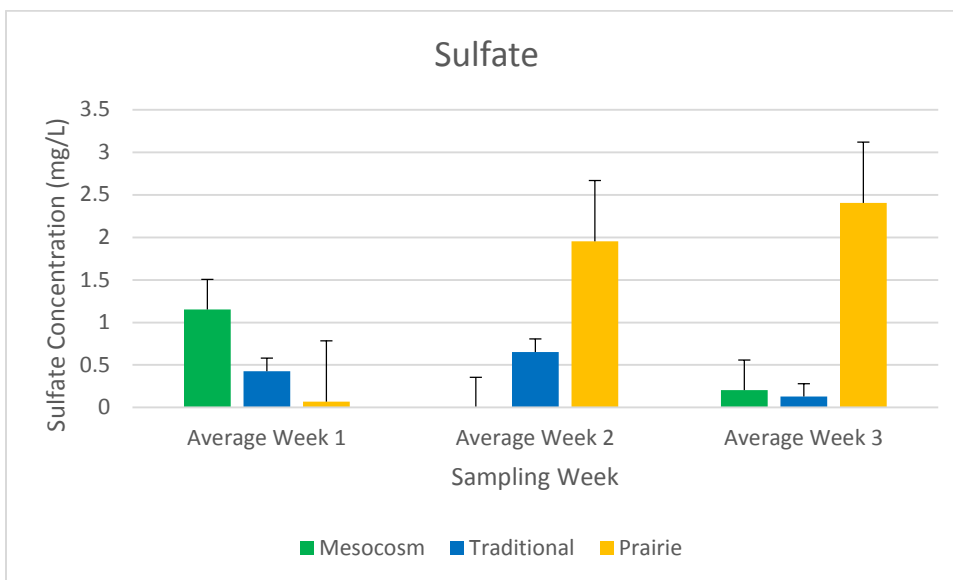

Figure 8—The concentrations of sulfate found in each of the three rearing environments. Each rearing habitat had 4 replicates whose results were averaged for analysis. Week 1 showed a significant difference in the sulfate concentrations of the Mesocosm and Traditional captive rearing treatment (p=0.046, df=2)

Table 6—Average sulfate concentrations for each of the three rearing environments across the three sampling weeks. *0 mg/L average was detected in the Mesocosm sample from week 2, however this may only indicate a sulfate concentration below the Ion Chromatograph's detectable range.

Treatment	Average Week 1	Average Week 2	Average Week 3
Mesocosm	1.15075	0*	0.203
Traditional	0.427	0.654	0.12875
Prairie	0.068	1.954	2.404

Discussion

pH is reflective of the hydrogen ion concentration in water and is a key characteristic in any aquatic habitat (Dodd, 2010). Acidic water affects survivability of juvenile and adult frogs, specifically disrupting the ionic balance within cells during developmental (Zug, 2001). Studies have reported a pH range of 6.0-7.5 to be neutral or at least within a range that should not harm organisms living in that aquatic environment (Dodd, 2010). The pH levels observed in this study, unsurprisingly, stayed right around 7.5 with slight fluctuation for both the Traditional and Mesocosm rearing treatments. These levels were maintained by frequent water changes (approximately every 3 days) which prevented organic build-up, which could have made the tanks more acidic. Low pH (or high acidity) can adversely affect osmoregulation in larval amphibians by substantially increasing rates of sodium loss through the skin (Wells, 2007); therefore, it is critical to maintain healthy pH levels in future mesocosm tanks for amphibian rearing. For example, the urea excreted by the frogs in the captive rearing tanks can make the tanks highly alkaline if the water is never changed, therefore we need to find a balance of a self-sustaining mini ecosystem and healthy water quality parameters for the frogs.

Given that the captive rearing tanks were kept within the pre-determined "pH neutral" bracket, it is curious that the Prairie wetland habitat pH readings would differ significantly from the both captive rearing treatments across the sampling period (p=0.313, df=2). Could this indicate high adaptability by *Rana pretiosa* in both its native and simulated environment, or are these reading reflective of the time of year during which sampling occurred? More research is needed to fully explore the pH limits of *R. pretiosa* and what those individuals will need during their captive rearing treatment, especially considering that those in captive rearing have no ability to move to different water conditions like those in the wild.

Aquatic environments can present less favorable conditions for oxygen uptake and oxygen levels are often more variable than terrestrial environments (Wells, 2007). Additionally, the oxygen content of closed bodies of water (i.e. captive rearing tanks) are greatly affected by the respiration of aquatic organisms and even daily fluctuations in respiration can result in major changes in the availability of oxygen (Wells, 2007). Previous studies have indicated that under laboratory conditions, oxygen concentrations below 4mg/L are deemed stressful to amphibian larvae and aquatic adults (Dodd, 2010). However, each species is different and may have the ability to acclimate to varying conditions depending on its niche in the local ecosystem (Dodd, 2010). In both the captive rearing treatments—Traditional and Mesocosm—the dissolved oxygen hovered between 8 and 9mg/L. The dissolved oxygen readings of both captive rearing treatments differed significantly from the Prairie wetland habitat (p=0.323). The average dissolved oxygen measurements for the Prairie wetland habitat had higher variability from week to week; starting around 6mg/L and ending closer to 2mg/L (see **Figure 3** on page 22). The

higher variation and on average lower dissolved oxygen values found in the Prairie habitat are likely due to the increased decaying plant matter and lowering water levels. Though the sampling period was only for six weeks during the summer, the Prairie wetland habitat demonstrated a much higher variability than both the captive rearing environments. These results could indicate that *R. pretiosa* has become adaptive to varying dissolved oxygen concentrations as a result of leading an entirely aquatic existence, or that perhaps all three treatments would show different dissolved oxygen fluctuation patterns given a longer sampling period.

One thing to consider when considering the dissolved oxygen results is that dissolved oxygen levels vary throughout the day depending on photosynthetic rates in response to local weather patterns. As such, dissolved oxygen concentrations in ponds are often lowest at dawn and increase throughout the day as the plants begin to photosynthesize (Dodd, 2010). This indicates the possibility that though dissolved oxygen readings appeared to be decreasing over the sampling period, it could have been linked to the amount of photosynthetic activity prior to sampling as all data was collected between 10am and 12noon. Regardless of fluctuations, though the captive rearing habitats—Traditional and Mesocosm—differed significantly from the Prairie ecosystem, they did support healthy growth throughout their captive rearing period. This indicates that *R. pretiosa* seems well adapted to fluctuating dissolved oxygen concentrations in captivity and in the wild, and therefore would not be oxygen-inhibited in a mesocosm environment under these conditions.

It is crucial to consider, however, that in a true mesocosm environment (i.e. one with less frequent water changes that more closely resembles a self-sustaining ecosystem)

oxygen levels may become lower due to the accumulation of organic matter. For example, hypoxia can be fairly severe in smaller bodies of water due to the respiration of organisms quickly depleting the available O_2 supply (Wells, 2007). As a result of potentially hypoxic conditions, the metabolic rates of developing larvae or tadpoles could decline as a result of decreasing oxygen availability, which could inhibit healthy growth (Wells, 2007). Regardless of any possibility of an amphibian species ability to adapt to a less oxygen-rich mesocosm environment, it will be important to monitor each species during captivity to ensure their health is not declining as a result.

Temperature also is a key factor in providing healthy growing habitat for amphibians in captive rearing environments. For ectotherms—such as frogs—temperature may be the single most important physiological variable because all cellular processes are temperature dependent (Zug 2001). Temperature influences the amphibians directly as well as other biological processes in their environment such as pH and dissolved oxygen concentration. In the case of this thesis research, temperature was not a limiting factor in the captive rearing environments as it was consistently regulated to be within a healthy range for the developing frogs. However, as indicated in the results (**Figure 4**, page 23) we see that the Prairie ecosystem showed a decrease in temperature across the sampling period. This is likely in response to the nighttime temperatures dropping with the approach of fall. Though the frogs developing in the Prairie ecosystem would have more freedom of movement than in captive rearing, this difference in temperature between the three rearing habitats could indicate a wider range of adaptability by *R. pretiosa* that could be explored in future captive rearing habitats.

Phosphorous is an essential plant nutrient but excessive amounts can cause water quality to deteriorate (Stewart, B. A., & Howell, T. A., 2003). The results indicate that the Prairie habitat had consistently higher concentrations of phosphorous than did either of the captive rearing treatments. This difference was further supported by the statistically significant difference found among the nutrient concentration data: there was a statistically significant difference between both the captive rearing treatments and the Prairie habitat all three weeks. Wetland environments, as the local weather gets warmer and drier from spring through summer, show changes in nutrient concentration as well as other water quality parameters such as dissolved oxygen and pH. These changes are a result of increased water evaporation leaving little standing water by the end of the summer months. This pattern was also observed during sampling at West Rocky Prairie between August and September—very little water remained in the large wetland. It has also been documented that in Pacific Northwest wetlands, concentrations of nutrients such as nitrate and phosphorous are much higher in sediments and belowground biomass than in overlying waters and aboveground plant tissues (Washington State Department of Ecology, 1986). This may be an explanation behind the increased levels of phosphorous in the Prairie environment as samples were taken at the end of the summer when there was a significantly less amount of water in the wetlands which could have been more influenced by nutrient concentrations in the sediments.

Nitrate concentrations were consistently highest in the Mesocosm rearing habitat. The Prairie habitat having the lowest concentrations of nitrate across the sampling period was unexpected due to it being a natural environment and more influenced by groundwater runoff and biological accumulation. The nitrate concentrations of the

Mesocosm captive rearing treatment differed significantly from the Prairie wetland habitat during all three sampling weeks, and showed the Mesocosm environment having consistently higher nitrate concentrations over the Prairie wetland habitat. Nitrates are readily absorbable and are quickly taken up by plant material. However the plants in the Mesocosm environment were not adapting to their new environment very well and as such were not performing (photosynthesizing, respiring, taking up nutrients) at maximum capacity and therefore not absorbing the nitrates in the water very quickly. This combined with the frogs excreting urea explains the increased nitrate levels in the Mesocosm environment. Several studies have shown negative impacts of increased ammonium levels (a nitrogen containing waste product excreted by most aquatic amphibians (Wells, 2007)) on amphibian species. For example a study conducted in Oregon by Adolfo Marco, Consuelo Quilchano & Andrew R. Blaustein (1999) observed that prolonged exposure to higher concentrations of nitrates proved fatal to *R. pretiosa*. Additionally, a similar study from Spain demonstrated a reduction of growth rate as a typical direct effect of ammonium nitrate on amphibian larvae (Ortiz-Santaliestra, et al. 2012). In contrast, the results of this pilot study demonstrated that *R. pretiosa* can survive well in the captive rearing treatments which had much higher nitrate concentrations than their natural prairie environment showed. This could indicate, similar to the other nutrient concentrations, *R pretiosa*'s ability to adapt the varying nutrient concentrations that the levels of nitrate may have had a lower overall average across the entire captive rearing period. More research needs to be conducted into the nitrate concentrations associated with captive rearing mesocosms.

Chloride ions, along with sodium and potassium ions, are a part of aquatic amphibian's active ion uptake to maintain electroneutrality between themselves and their environment (Wells, 2007). Ions are also reabsorbed within the kidneys to prevent large ion loss and thus increase the need to actively intake these ions from their surrounding environment. There is little research completed on specific needs of amphibians in captive rearing in terms of ion concentrations, however like any organic element within an ecosystem, moderate concentrations—not too low, not too high—will most likely be the best suited for amphibian captive rearing. However, this also varies by species. The chloride concentrations were consistently higher in the Prairie habitat across the sampling period. A study conducted in British Columbia (McKibbin et al., 2008) examined water quality as parameters for embryonic survivorship in three known *R. pretiosa* populations. They measured chloride levels between .063 mg/L and 1.83 mg/L across three sampling sites. In comparison, these rates seem rather low to the chloride levels detected during this thesis research, however their research was conducted earlier in the year and only one sample per year was collected for analysis. In addition, the chloride levels in the Prairie wetland habitat were higher due to their natural presence accumulating as the water levels dropped toward the end of the summer. There is a significant lack of published information regarding the nutrient levels of wetland ecosystems and what one can deem as "normal"; as such, it is difficult to interpret what my thesis results may indicate.

Sulfate concentrations were highly variable for all three rearing habitats across the sampling period. The Prairie environment showed the highest concentrations in the final two weeks of sampling and the Mesocosm rearing treatment showed the highest sulfate

concentration during the first week of sampling. Though there was only on significant difference detected during the sampling period—Week one showed a significant difference in sulfate levels between the Mesocosm and Traditional captive rearing treatments (p=0.046)—as we can see on **Figure 8** on page 27 there appears to be a large difference in sulfate concentration between the captive rearing habitats and the Prairie habitat. Changes in concentrations of nutrients such as sulfate can vary dramatically over the course of amphibian breeding and rearing season (Gerlanc & Kaufman, 2005). More research is needed to determine what specific effects these changes might have on amphibians in captive rearing environments.

This thesis research was part of a pilot study in partnership with Woodland Park Zoo in Seattle, WA. The sampling period was not ideal in length, and as such the data results may be skewed or not show an accurate account of the water quality parameters of each of the rearing habitats. Additionally, the top priority during this captive rearing pilot project was the health of the developing frogs. As such the caretakers at WPZ did weekly tests and changes to the aquatic environments—both Traditional and Mesocosm treatment—to ensure the continued health of the growing frogs. Tests for ammonia levels were conducted once per week, as well as visual checks for algae accumulation which may have inhibited the ability for the keepers to see the frogs clearly—to determine if they were in distress. If ammonia levels were outside healthy standards or if an algal mat had formed inhibiting visual scope of the animals, the water in the tank was changed. It was the original goal of my thesis research to test the differences in water quality between the Traditional treatment and the Mesocosm treatment, however frequent water changes in both treatments may have skewed my ability to accurately describe the

differences between these two environments. For example, based on the log of water changes supplied by Woodland Park Zoo, the water in both the Traditional tanks and Mesocosm tanks was changed roughly every 3 days. This was done to maintain the health of the animals during development, but may have inadvertently skewed the results. The data gathered are still important in beginning to describe baseline conditions for future mesocosm and captive rearing aquatic environments.

Captive Rearing Management Conclusions and Recommendations

The results of this pilot project were not as I had originally imagined, however they provide an interesting baseline for gaining insight into the inner workings of using mesocosms to captive rear amphibians and how closely those mesocosms need to resemble the natural environment or how closely they can resemble traditional captive rearing tanks as well. As we have seen from the results here, the water quality parameters in the captive rearing treatments (Mesocosm and Traditional) differed from the Prairie environment—some statistically significant, some not—and these differences open up a wide array of new questions that need to be answered.

Do the differences in the captive rearing treatments and the Prairie ecosystem indicate that the captive rearing environments could be more constant or less variable in terms of water quality parameters? For example, West Rocky Prairie houses a known *Rana pretiosa* population that is able to survive and reproduce every season in these conditions that were identified during this research project. If they can survive in current conditions, would it be more beneficial to expose captive-reared tadpoles to varying

conditions to closer simulate the natural environment? What is the balance of self-sustaining mesocosm environments and continued health of the growing frogs? These are all questions that should be addressed with future research.

I set out on this project to begin to understand whether or not mesocosms were a good idea for the captive rearing of *R. pretiosa*. Based on the data gathered, mesocosms show amazing potential for captive-reared individuals of all amphibian species, particularly those like *R. pretiosa* whose numbers are quickly declining. Currently, however, more information must be gathered before we can take true strides forward in adding mesocosms to the captive rearing plan for *R. pretiosa*. Based on the results of this thesis research, there is still a lot left to interpretation in terms of explaining the difference between the Prairie environment and the captive rearing treatments. There is a lack of research describing what is "normal" in any given wetland, and as such it may be difficult to pinpoint and replicate precisely what the frogs require while in captivity.

Suggestions for future research include looking into mesocosms as captive rearing environments for threatened/endangered amphibian species in the Pacific Northwest, specifically by examining biochemical cycles within a mesocosm environment across the entire captive rearing period (roughly February through September). It is recommended that future studies work to keep the water changes for mesocosms notably different from the control rearing environment so as to observe a more clear difference for all parameters being monitored. Mesocosms show amazing promise in the world of amphibian captive rearing, given continued research to assess how captive rearing environments can promote the rearing frogs that can survive and thrive once released into natural environments.

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Appendix A: Materials List

Materials List

Ion Chromatograph (Model: DIONEX IC25A), Plastic (PP) Sample Bottles (500 mL), Volumetric Pipettes (1, 1mL pipette, 2, 5mL pipettes, 1, 10mL pipette, 1, 15mL pipette, 4, 25mL pipettes, Bulb), Millipore Filtration Apparatus (47mm, 500mL capacity), 1 liter vacuum filtration flask, 1, 500 mL vacuum filtration flask, 70, 47mm 0.45 micropore filters, 1 rubber stopper with two openings, 2 vacuum rubber hoses, 2 ring stands, 2 ring clamps: large enough to hold 1L and 1 500mL vacuum filtration flasks, 1 small rubber hose "pincher", 0.2 micrometer syringe filters for the IC, Lab Tape for labeling supplies, Glass Dessicator, 8-100mL beakers, 4 spatulas, 10 1L plastic bottles, 4-1L volumetric flasks, 5-500mL volumetric flasks, 12, 1L plastic bottles for individual stock standards, mixed intermediate standards and mixed working standards, Analytical Balance for measuring salts, Chem-grade cooler for cold transport of samples for preservation

Standard Preparation Ingredients:

Nutrient Testing For	Ingredients Needed to Make Standard Soln. (EPA Method 300.1)
Chloride	1000mg/L: 0.1649g sodium chloride (CASRN 7647-14-5)

Sulfate	1000mg/L: 0.1814g potassium sulfate (CASRN 7778-80-5)
NO_3^--N	1000mg/L: 0.6068g sodium nitrate (CASRN 7631-99-4)
PO_4^{3-}-P	1000mg/L: 0.4394g potassium dihydrogenphosphate (CASRN 7778-77-0)

Appendix B: Full Run List for Ion Chromatography Analysis

Run List

1. Rinse Blank

2. Second Rinse Blank (must match first rinse blank before proceeding)

3. Standards (Low to High)

   a. This generates the calibration curve

   b. Check the R^2 (EPA standard R^2 value = 0.9998) to judge the fit of the line

4. Rinse Blank

5. Quality Control

   a. +/- 5% Retention Time

   b. +/- 15% Concentration

   c. Compare these first quality control values to 1) medium working standard retention times and 2) lab notebook concentrations for Quality Control standards.

6. Up to 10 samples

7. Quality control

   a. +/- 5% Retention Time

      b. +/- 15% Concentration

      c. **Compare these values to first quality control. If they do not match

            within the approved interval the previous samples run are null**

8. Up to 10 more samples

9. Quality Control

      a. +/- 5% Retention Time

      b. +/- 15% Concentration

**Compare these values to first quality control. If they do not match within the approved interval