Hormone Profile Monitoring and Reintroduction Site Water Quality Assessment to Support Conservation of the Oregon Spotted Frog (*Rana pretiosa*)

by

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Abstract

The main goal of this research was to enhance the endangered Oregon spotted frogs (OSF) captive rearing methodologies and better understand the sensitivity of early life stages to various water quality changes. A key finding was that significant seasonal variations in estradiol and testosterone levels were evident in OSF between two British Columbia captive rearing facilities, indicating different captive rearing conditions may be influencing reproductive status in captive OSF. Larval OSF are more sensitive to the lethal effects of acute, waterborne copper exposure than any early life stage amphibians or fish studied to date. Finally, larval survival was significantly lower in OSF reared in captive waters followed by transfer to reintroduction site water, suggesting an increased survival rate may be achieved by acclimating OSF to reintroduction site water prior to release into the wild. This research provides critical insights for enhancing OSF captive breeding programs and reintroduction strategies.

Keywords: Captive breeding; Copper; Conservation Physiology; Estrogen; Testosterone

Dedication

I would like to dedicate my thesis to all the following: to the 300 tadpoles euthanized during the experiment and to the 20 adult frogs at the Greater Vancouver Zoo and Vancouver Aquarium used in the experiments for this project; to all the conservation practitioners worldwide who work tirelessly to save endangered species from extinction; to my 9th grade biology teacher, Mr. Raiesian, who was the first person to introduce me to conservation biology; and finally, to my parents, who have supported me endlessly since day one and continue to do so.

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List of Acronyms

℃	Degree Celsius
ANOVA	Analysis of variance
BC	British Columbia
CCME	Canadian Council of Ministers of the Environment
CI	Confidence interval
cm	Centimeter
DO	Dissolved oxygen
EC50	Effective concentration for the 50th percentile
EIA	Enzyme immunoassays
ESA	Endangered Species Act
g	Grams
GS	Gosner Stage
GVZ	Greater Vancouver Zoo
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamus-Pituitary-Gonad
HPI	Hypothalamic-Pituitary-Interrenal
ICUN	International Union for Conservation of Nature
IQR	Interquartile range
km	Kilometer
L	Litre
LAGDA	Larval amphibian growth and development assay
LC50	Lethal concentration for the 50th percentile
m	Meter
mg/L	Milligram per litre
ml	Millilitre
mm	Millimetre
No.	Number

ng/ml	Nanogram per litre
ng	nanogram
OECD	Organisation for Economic Cooperation and Development
OSF	Oregon Spotted Frog
Q1	Quartile 1
Q3	Quartile 3
SARA	Species at Risk Act
sd	Standard deviation
SVL	Snout-to-vent length
TL	Total length
US	United States
VA	Vancouver Aquarium
ww	Wet weight
µg	Microgram
µg/L	Micrograms per litre

Opening Image

Oregon Spotted Frog (*Rana pretiosa*). Photo: Pourya Sardari©

Chapter 1.

General Introduction

Amphibian Extinction

Amphibians are the most diverse group of vertebrates and, as a result, appear to play key ecological roles in freshwater habitats globally. Approximately 41% of amphibian species are categorized as threatened on the IUCN Red List of Threatened Species (Luedtke et al., 2023). The second Global Amphibian Assessment revealed that all amphibians are declining, particularly the salamanders of the Neotropics (GAA2, 2023). These declines are caused by a combination of factors including habitat destruction, climate change impacts (including ocean acidification), pollution, disease, and invasive species (Luedtke et al., 2023). Previous research indicates that the principal causes of population decline between 1980 and 2004 were disease and habitat loss affecting 91% of species (Butchart et al., 2007). From 2004 up to the present, climate change emerged as a major concern causing 39% of status declines among various species, followed by habitat loss, a close second with 37% (Luedtke et al., 2023). Human activities that lead to environmental pollution play a big part in habitat loss, along with farming expansion and infrastructure development (Luedtke et al. 2023). The most affected areas include Caribbean islands, Mesoamerica, the Tropical Andes, and parts of Africa and Asia. While some species are increasing their populations, others face considerable problems, and, most importantly, we need urgent conservation action to reverse these worsening trends (Rodrigues et al., 2006; Mallon & Jackson, 2017). Currently, addressing and managing climate change, habitat loss, environmental pollution, and diseases are key priorities for amphibian conservation (IUCN, 2023).

The role of conservation physiology in amphibian conservation

Increasing concerns regarding threats, such as habitat loss, invasive species, and climate change, have spawned the relatively new field of study called conservation physiology to address the challenges faced by amphibians as well as other taxa. This field of research incorporates physiological principles and methods to address conservation problems and improve biodiversity planning (Cooke et al., 2013; Park & Do, 2023). It examines how living organisms respond to environmental stressors (e.g.,

habitat loss or pollution), which can provide insights into the resilience, adaptability, and susceptibility of such organisms. Studying the physiological mechanisms allows scientists to find new strategies for species conservation, habitat restoration, and ecosystem management (Madliger et al., 2018). Studies within the field of amphibian conservation physiology cover a wide range, including how pollutants and pathogens affect stress responses, reproductive success, and survival. These studies commonly focus on major physiological aspects such as hormone levels, immune function, metabolic rates, and behavior that can indicate the health or resilience of the measured subjects (Walls and Gabor, 2019; Tornabene et al., 2021; Park and Do, 2022). Amphibians are good indicators of changes in temperature, moisture, and water quality parameters (such as pH, dissolved oxygen, and total dissolved solids) because they have permeable skin. These may affect their survival, growth, and reproduction (Ficetola & Maiorano, 2016; Nowakowski et al., 2017; Rollins-Smith, and Le Sage, 2023). It is essential to determine how amphibians are able to respond physiologically because this provides us with clues on what responses could be expected when facing global environmental changes (Madliger et al., 2018; Zaffaroni-Caorsi et al., 2023).

Conservation physiology also aids in studying captive populations to enhance captive breeding programs, providing valuable information on the physiological needs and responses of species in controlled environments (Currylow et al., 2017). Conservation physiology greatly improves amphibian reproduction management through various non-invasive techniques that monitor stress and reproductive health, which are crucial for managing these populations (Narayan, 2013). By utilizing non-invasive methods to monitor reproductive hormones such as estradiol, progesterone, and testosterone from urine samples, researchers can assess reproductive statuses and optimize breeding interventions. These techniques also involve measuring stress hormones like corticosterone, knowledge of which helps evaluate health and stress levels, critical for making informed management decisions in both captive and wild settings (Narayan, 2013). This approach is particularly beneficial in enhancing captive breeding programs by ensuring that amphibians reproduce effectively under conditions that cater to their physiological needs. Moreover, by analyzing how environmental factors and diseases such as chytridiomycosis impact hormone levels, conservation physiologists are able to tailor management strategies to better support the reproduction and overall health of amphibian populations. This in turn aids in their conservation and

sustainability, providing a hopeful outlook for the future of these vulnerable species (Narayan, 2013).

Conservation physiology also facilitates studies of captive populations to improve the success of breeding programs by providing a comprehensive understanding of physiological requirements and responses in captive environments (Currylow et al., 2017). Conservation physiology provides non-invasive means to monitor stress and reproductive health (see Narayan, 2013, for a review). Researchers can analyze reproductive statuses and calibrate breeding responses by non-invasively monitoring urine hormones such as estradiol, progesterone, and testosterone. These techniques include the use of stress hormones like corticosterone levels, which provide information on health and welfare (Narayan, 2013) that is vital for making appropriate, informed management decisions in both captive and wild settings. This approach is particularly useful in improving captive breeding programs by ensuring that specific conditions required for the physiological traits of amphibians during reproduction are met. Additionally, by understanding how environmental challenges and diseases such as chytridiomycosis affect hormone levels in amphibians, conservation physiologists can help develop management strategies that enhance the reproductive fitness and health of amphibian populations. This, in turn, aids their conservation and sustainability, guaranteeing a hopeful future for endangered amphibian species (Narayan, 2013).

Amphibian Ex-situ Conservation and Reintroduction Programs

Two strategies to protect endangered species are to safeguard their natural habitats (in-situ conservation) and to protect them in controlled settings like zoos and aquariums (ex-situ conservation). In the past, conservationists preferred in-situ conservation over breeding in captivity (Snyder et al., 1996). But now more conservationists recognize how zoos and aquariums help with conservation (Conde et al. 2011; Gusset and Dick 201; Olive and Jansen 2017). Zoos and aquariums play a critical role in wildlife research and conservation by keeping species captive for breeding and reintroduction programs (Lewis et al., 2019). These programs aim to boost the species' breeding success and release captive-born offspring into the wild. This helps to increase population numbers and gives a chance to reduce threats to their natural homes (Palm et al. 2003; Osborne et al. 2006). One example of ex-situ programs at zoos and aquariums is the amphibian breeding and reintroduction programs. The World

Association of Zoos and Aquariums, the IUCN (International Union for Conservation of Nature)-SSC (Species Survival Commission) Conservation Breeding Specialist Group, and the Amphibian Specialist Group created these programs. These programs aim to address the captive components of the Amphibian Conservation Action Plan in response to the global amphibian extinction crisis (Gascon et al., 2007).

Over the past two decades, there has been a notable increase in ex situ amphibian collections as a response to their global extinction crisis, which is primarily due to complex factors of human origin (Conde et al., 2011; Dawson et al., 2016; Gascon et al., 2007; Green et al., 2020; Stuart et al., 2004). To address these declines, various conservation action plans, including both in situ and ex situ activities, have been developed, with many focusing on the establishment of captive breeding and reintroduction programs as assurance strategies for the survival of wild populations that are no longer self-sustaining (Gascon, 2007; Bishop et al., 2012; Silla and Byrne, 2019). One such program is focussed on Oregon Spotted frog (OSF), an endangered species in Canada extant in only five locations in Southwestern British Columbia (OSF Recovery Team, 2019). Similar to other endangered amphibians, OSF has distinct life cycles requiring tailored care protocols, and breeding success can be challenging due to their dependence on various behavioral and environmental signals (Tapley et al., 2015; Bradfield et al., 2022; Bloxam and Tonge, 1995; Kouba et al., 2009; Carrillo et al., 2015; Ulloa et al., 2019). Although assisted reproductive technologies are increasingly used for individual well-being and genetic management, they should not be considered substitutes for replicating natural environments (Kouba et al., 2009; Graham et al., 2018; Calatayud et al., 2018; Silla et al., 2021).

Amphibian Conservation Breeding Program Challenges

Amphibian reintroduction and captive breeding programs (conservation breeding) present complex challenges that can only be tackled through multidisciplinary approaches. Genetic considerations are paramount since reintroduced populations will require genetic diversity to be maintained to ensure the population remains viable; however, careful planning is necessary when managing for genetic diversity due to risks of inbreeding and a loss of fitness via genetic drift, often worsened by having few individuals available for breeding captive populations (Frankham et al., 2017; Williams and Hoffman, 2009). Disease management is another major obstacle, with

Batrachochytrium dendrobatidis and *B. salamandrivorans* being potential pathogens, requiring strict biosecurity and health screening of all animals in managed populations to reduce the risks associated with these diseases (Scheele et al., 2014). The selection of release sites requires careful consideration of habitat suitability, with changing environmental conditions such as climate change and habitat degradation impacting the long-term suitability of many reintroduction areas (Harding et al., 2016). Post-release management, particularly long-term monitoring, is fundamental to determine reintroduction success and the necessary adaptive management of introduced populations, but these processes with amphibians have been difficult in some cases due to their cryptic nature (Germano et al., 2015). Ultimately, the involvement at a sociopolitical level of various stakeholders, starting from local communities up to government agencies, requires adequate engagement and support for such projects to work (Linhoff et al., 2021). The development of clear objectives and incorporating different management practices within these objectives is necessary for these restoration strategies to be effective in ongoing conservation programs to reintroduce amphibian species using captive breeding.

The Oregon Spotted Frog

Oregon spotted frogs (OSF) are native to the Pacific Northwest. These frogs are highly aquatic and can be found in shallow marshland habitats from close to sea level to more than 1525m elevations (Pearl et al., 2005). This species' historical range was between northern California and southwestern British Columbia (BC) (Pearl et al., 2005). Most of the current populations are found in Oregon's Cascade Mountains at elevations above 1200 metres, where growing seasons are brief, and winters are frequently harsh Licht, 1969. However, lowland locations in Washington state and British Columbia provide the majority of the information about the ecology of the OSF, including its life cycle, breeding and feeding habits (Licht, 1969, 1971).

Oregon Spotted Frog Recovery Program

Due to habitat loss, invasive species, environmental pollutants and disease, OSF has lost 90% of its historical distribution (Hammerson and Pearl, 2012) leaving only five populations in Canada, each with fewer than 200 breeding pairs (Kendra Morgan, pers. comm.; Kissel et al., 2017). OSF is federally listed as 'Endangered' under both the U.S. ESA and Canada's SARA and as 'Vulnerable' by the IUCN (Hammerson and Pearl,

2004; Environment Canada, 2015; Hallock, 2013). In particular, these frogs have lost critical breeding habitat due to habitat destruction (Environment Canada, 2015; OSF Recovery Team, unpublished data). These amphibians, known for their tendency to remain in water and consistently choose the same shallow, thermally stable sites for egg laying year after year (McAllister and Leonard, 1997; Phillipsen et al., 2010), typically have males arriving at breeding grounds first, where males vocalize to attract females for up to three weeks following the thaw of spring ice (Phillipsen et al., 2010). The quantity of eggs laid varies widely, with communal clusters containing anywhere from 5 to 75 egg masses (Licht, 1969; McAllister and Leonard, 1997). Also, the introduction of the non-native bullfrog (*Lithobates catesbeiana*), non-native vegetation (i.e., *Phalaris arundinacea*), infectious disease (i.e., *Batrachochytrium dendrobatidis*) and toxic chemicals (including trace metals) in OSF habitats have been linked to population declines (Pearl et al., 2005; McKibbin et al., 2008; Pearl 2007; Kapust et al., 2012).

Conservation breeding and reintroduction initiatives for OSF in Canada began in 2010, prompted by its designation as Endangered in Canada in 1999, a classification reaffirmed in 2000 and 2011 (COSEWIC, 2011). Presently, two zoos and one aquarium conduct separate yet coordinated efforts for captive breeding and reintroduction of OSF, and have successfully undertaken these efforts breeding and releasing tadpoles since 2010. However, all three facilities have historically encountered challenges such as low reproductive rates, high instances of reproductive issues (especially egg binding) and lack of knowledge on survival of OSF tadpoles after reintroduction (Hunter, 2023).

Thesis Objectives

This research aims to investigate methods for enhancing captive breeding and reintroduction programs in British Columbia, Canada. The first objective is to investigate the impact of the two current types of husbandry conditions, indoor aquaria and outdoor ponds, on stress and reproductive hormone levels in adult frogs. I hypothesize that variations in housing conditions, such as the type of enclosure, will affect baseline stress hormone (corticosterone) levels and the frogs' response to handling stress. Additionally, I anticipate that reproductive hormone levels will fluctuate throughout the breeding season in outdoor pond rearing conditions to a larger extent than in indoor aquaria rearing conditions, reflecting more dramatic changes in visible reproductive activity under the more natural outdoor environmental conditions. The second objective of this

research is to explore how differences in water quality, specifically between zoo and natural site water, influence tadpole growth, development, and survival. Finally, I seek to understand the impact of exposure to an environmental pollutant common at OSF reintroduction sites, copper, on OSF early life stage health. By examining these factors, this study aims to provide valuable insights into the ecological requirements and stressors affecting the OSF, thereby informing conservation strategies and management practices for this and similar amphibian species.

Chapter 2.

Using Non-invasive Hormone Monitoring to Characterize Stress and Reproductive Physiology in Two Captive Oregon Spotted Frog Populations

2.1. Abstract

Conservation physiology plays a critical role in assessing the health of both wild and captive animal populations by measuring their physiological responses to environmental changes and stressors. In this study, I aimed to evaluate the stress response and reproductive hormone levels in Oregon spotted frogs (*Rana pretiosa*, OSF) reared at two captive breeding facilities in British Columbia, Canada: The Greater Vancouver Zoo (GVZ) and the Vancouver Aquarium (VA). I measured corticosterone, estrone conjugate (E1C), estradiol (E2), and testosterone levels from water samples collected from the OSF across different seasons. I did not detect corticosterone in the water samples nor did I found a significant seasonal variation within each facility or between facilities for E1C levels. However, for E2 levels, I found significant seasonal variations at both facilities. At GVZ, E2 levels increased from early overwintering to late overwintering, peaking before decreasing significantly by the pre-breeding season and remaining low during the breeding season before increasing post-breeding. At VA, E2 levels followed a similar pattern but were significantly different from GVZ during the pre-breeding and post-breeding seasons. Testosterone levels in male OSFs showed significant seasonal variations, with the highest levels observed during early overwintering at GVZ and during breeding at VA. There were significant differences in testosterone levels between the two facilities during the pre-breeding season. Overall, I found that environmental conditions at the two facilities influenced the seasonal hormone profiles of the OSF. My results provide insights into the optimal conditions for captive breeding programs of not only OSF but other endangered amphibians. These findings underscore the importance of non-invasive hormone monitoring in understanding the physiological status of endangered species and improving conservation strategies.

2.2. Introduction

Conservation physiology is an emerging field that plays a crucial role in assessing the health of both wild and captive animal populations by measuring their physiological responses to environmental changes and stressors (Wikelski & Cooke, 2006). As such, conservation physiology includes addressing the adverse effects of natural abiotic and biotic stressors, as well as human-induced environmental stressors such as pollutants, habitat destruction, and fragmentation, on adults and developing animals, which can influence their health and survival (Madliger et al., 2021). Conservation physiology is particularly effective in predicting population declines and identifying vulnerable groups (Ames et al., 2020). Several studies have identified indicators of stress and impeded reproduction associated with endocrine system dysfunction, such as abnormal levels of hormones, hormone receptors and enzymes, reduced fertility and reproductive success (Madliger and Love, 2014; Sorenson et al., 2017; Shidemantle et al., 2022; Marlatt et al., 2022).

Amphibians and fish are particularly impacted by environmental stressors during early life stages due to their complex postembryonic development, which typically occurs in free-swimming larvae. This is different from other taxa, such as mammals, birds, and reptiles, where embryonic development occurs within eggs or in utero (Denver, 2021). Consequently, conservation efforts aimed at better understanding the implications of various natural and anthropogenic stressors on early life stage development of amphibian and fish populations is of paramount importance, alongside characterizing the physiological status and behaviors of healthy reproducing adults (Burraco et al., 2016; Navas et al., 2016). However, for most endangered species due to the inability to conduct live animal experiments, often there is a lack of information available to identify the impact of stressors on individuals which may be causing adverse impacts at the population level (Katzner et al., 2020). Despite this, a growing body of literature on non-invasive methods to detect disruptions of the various aspects of the endocrine axes that are critical for regulating growth, development and reproduction in various taxa exists. These methods can be applied and adapted for numerous endangered species without impacting population numbers (Narayan, 2013).

One amphibian species with poorly characterized responses to natural and anthropogenic stressors, which is of particular relevance to current conservation efforts

in British Columbia, Canada, is the Oregon spotted frog (*Rana pretiosa*). Oregon spotted frog (hereafter OSF) has lost 90% of its historical distribution due to habitat loss, invasive species, environmental pollutants and disease (Hammerson and Pearl, 2012). The OSF is federally listed as 'Endangered' under both the U.S. ESA and Canada's SARA and as 'Vulnerable' by the International Union for Conservation of Nature red list (Hammerson and Pearl, 2004; Environment Canada, 2015; Hallock, 2013). For the OSF, conservation breeding and reintroduction initiatives in Canada began in 2010, whereby adults are reared in captivity, bred and live offspring reintroduced into natural wetlands. Currently there are two captive breeding facilities for OSF in British Columbia; one at the Greater Vancouver Zoo (GVZ); and the second at the Vancouver Aquarium (VA). These facilities employ distinct husbandry techniques for the care and breeding of frogs. During the breeding season at GVZ, frogs are housed outdoors in groups within large Rubbermaid tubs, while at the VA, frogs are kept indoors in green houses in glass aquaria also with multiple males and females per aquarium. Differences in breeding success and offspring survival have been documented over the years at these facilities, with the GVZ experiencing more success compared to the VA. However, few studies examining the stressors associated with captive rearing, breeding and reintroductions back into the wild have been conducted. Ultimately, research on the hypothalamic-pituitary-interrenal (HPI) axis regulating the stress response and early life stage development as well as on the hypothalamic-pituitary-gonad (HPG) axis regulating reproduction in OSF under captive and wild conditions would provide invaluable insights that have the potential to enhance the existing conservation strategies for this species.

In amphibians, the stress response is primarily regulated by the HPI axis (Moore and Jessop, 2003), which is analogous to the hypothalamic-pituitary-adrenal (HPA) system in mammals (Herman et al., 2016). This axis plays a critical role in mediating physiological responses to stress through the secretion of corticosterone, the primary glucocorticoid in amphibians. Activation of the HPI axis begins with the perception of stress such as presence of a predator or change in temperature which stimulates the hypothalamus to release corticotropin-releasing hormone (CRH). In turn, CRH then stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH), which in turn stimulates the internal gland to produce corticosterone and the thyroid gland to produce thyroid hormones, respectively (Moore and Jessop, 2003; Denver, 2009). This cross-regulation of the adrenal and thyroid gland

activity by CRH through pituitary hormone induction leads to a complex interplay between hormones that control the individual's response to stressors, including the production and release of glucocorticoids and mineralocorticoids by the adrenals, as well as growth rate and development mediated by the production and release of thyroid hormones.

Numerous studies have shown the sensitivity of the HPI axis to a range of stressors (e.g., environmental contaminants, changes in habitat, and predators) resulting in increased corticosterone production (Dahl et al., 2012; Lee and Sawa, 2015; Carbaja et al., 2019). For example, a study showed how immediate visual and olfactory stimuli from predators trigger an acute corticosterone rise, leading to changes in both stress response and behavior (Narayan et al., 2013). While short-term and transient corticosterone is essential for survival, sustained elevation of this hormone has diverse negative effects, including inhibiting immune function, impairing reproduction, reducing growth rates, prolonging developmental periods, and diminishing sensitivity to endocrine signals (Sapolsky et al., 2000; Romero and Wikelski, 2001; Cockrem and Silverin, 2002; McEwen and Wingfield, 2003; Thaker et al., 2009; Homyack, 2010; Koolhaas et al., 2011; McCormick and Romero, 2017). Previous studies have developed non-invasive methods for measuring hormones, including corticosterone, that can be applied to compare natural/wild and captive animal stress responses to gain insights into stressors that may be of concern for captive breeding programs (Romero and Reed, 2005; Madliger et al., 2018).

Reproductive processes and behaviors in anuran amphibians, such as OSF, are controlled by hormonal cues originating from within the hypothalamus-pituitary-gonad (HPG) axis (Kikuyama et al., 2019). The HPG axis is stimulated by environmental factors like temperature, daylight, and rainfall that then trigger the production of key hormones in the hypothalamus, such as gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone then stimulates the pituitary gland to release gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Browne and Zippel, 2007; Vu and Trudeau, 2016). These hormones regulate gonad development and initiate reproductive behaviors such as egg-laying and mating calls. In female anurans, LH and FSH promote the maturation of oocytes and the synthesis of reproductive hormones, such as estrogen and progesterone, which are essential for ovulation and subsequent egg development (Browne and Zippel, 2007). Estrogen also stimulates the production of

vitellogenin by the liver, a yolk protein vital for egg development and subsequent embryonic nourishment. In males, LH primarily drives the production of testosterone, which is necessary for spermatogenesis and the exhibition of secondary sexual characteristics and behaviours conducive to mating, such as vocalizations and amplexus, the mating grasp of frogs (Czuchlej et al., 2019; Di Fiore et al., 2020). Collectively, the HPG axis and its products coordinate numerous, complex and integrated biological processes critical for reproductive success, or an organism's capacity to produce offspring that will reproduce in the subsequent generations.

Understanding the HPG axis is particularly important for captive breeding programs. This type of multiple gene evolution is also likely responsible for the origin of at least one GnRH paralog in all species studied thus far, including non-amniote vertebrates such as amphibians. In amphibians, the major forms of GnRH are chicken GnRH-II (cGnRH-II) and mammalian liable decapeptide-GnRHR. Although mGnRH is the principal regulator, with respect to control of gonadotropin release by stimulation (Brown et al., 2009), cGnRH-II appears more closely associated with the regulation of reproductive behaviors in relation to reproduction. Many conservation breeding programs and research studies rely on exogenous hormone-induced reproduction in captive anurans. The most frequently used hormones are mainly synthetic analogs of GnRH and human chorionic gonadotropin (hCG) that mimic the natural regulators to trigger gamete production and ovulation or spermiation (Comizzoli, 2019; Comizzoli, 2022; Pham et al., 2022). These assisted reproductive technologies have been used to manage genetic diversity and demographic stability in a number of threatened amphibians, typically involving IVF followed by captive-rearing and reintroduction into the natural wetlands (Trudeau et al., 2013; Vu et al., 2017). Hormone treatments, for example, can work differently among species and often need specific doses to induce the desired physiological responses in different anuran lineages. This highlights the necessity of continuing discipline in species-specific research into their reproductive physiology and optimization of these techniques to make them more effective for anuran conservation breeding programs.

Non-invasive methods to evaluate hormones in adult frogs have been developed in various studies, typically relying on urinary steroid metabolites (Germano et al., 2009; Narayan et al., 2010; Germano et al., 2012). Indeed, sampling water for hormones excreted via aquatic species is a non-invasive hormone measurement method initially

developed for fish (Félix et al., 2013; Scott and Ellis, 2007), and was expanded to include amphibians (Baugh et al., 2018, 2021; Gabor et al., 2013, 2016). Specifically, fish and amphibians release hormones, such as corticosterone, androgens, and estrogens, through the gills, skin, and urine into ambient water which can then be extracted (Gabor et al., 2013; Scott and Ellis, 2007). Water hormone sampling methods have the advantages of minimal or no animal handling and can be applied to fish and amphibians of any size, offering a non-lethal and non-invasive method to measure hormone levels in these aquatic species. The correlation between water concentration of hormones released and circulating plasma levels of respective hormones has been validated in numerous fish and amphibian species (Scott and Ellis, 2007; Gabor and Contreras, 2012). Further demonstrating that the non-invasive water hormone measurement method is a robust method for hormone quantification.

For the endangered OSF, characterizing indicators of stress and reproductive status under different captive rearing conditions will aid in better understanding optimal rearing conditions and potential culprits for differences in reproductive success in various captive populations. In British Columbia, Canada, the VA uses indoor aquaria that simulate natural conditions with controlled temperature, humidity, and light cycles, while the GVZ uses outdoor tubs that expose the frogs to natural environmental conditions and seasonal variations. Comparing these environments will provide insights into the impact of natural versus simulated conditions on the frogs' growth, behavior, and overall health, informing best practices for captive breeding and reintroduction programs.

In this study the two main objectives are: 1) to assess stress response by measuring excreted corticosterone after human handling of adult OSF from two captive breeding facilities in British Columbia, Canada; and 2) measure reproductive hormones throughout one year during different physiological stages (before, during, and after the breeding season) in adult OSF from the two captive breeding facilities in in British Columbia, Canada. The main hypothesis for the first objective is that frogs kept in the indoor environment will have higher baseline corticosterone levels than those in reared in the outdoor environment due to less natural environmental conditions in the indoor enclosures compared to the outdoor enclosures. The hypothesis of the second objective is that reproductive hormone levels, such as testosterone in males and 17B-estradiol in females, will be highest just prior and during the breeding season, and this hormone

profile will be more prevalent in the OSF reared in the outdoor environment compared to the indoor environment.

2.3. Material and Methods

2.3.1. Animal Husbandry

The VA and the GVZ are the two primary facilities for the captive breeding of OSF in British Columbia, each employing distinct husbandry techniques. At VA, the frogs are housed indoors in climate-controlled greenhouses with 25 to 30-gallon glass aquariums. The breeding environment is controlled to mimic natural temperature and humidity levels, with each tank typically containing 4 males and 2 females during the breeding season from March to August. During the overwintering period from September to January, males and females are housed separately in different tanks, with temperatures gradually reduced to simulate natural cooling. This indoor regime provides a stable environment but may not replicate natural fluctuations as precisely as an outdoor setup.

In contrast, GVZ employs a more naturalistic approach by housing the frogs outdoors in large 100-gallon Rubbermaid tubs during the breeding season. This outdoor setting allows the frogs to experience natural photoperiods and temperatures, accurately reflecting ambient conditions. The frogs are housed communally, with each tub accommodating 20 males and 40 females, fostering more natural interactions and behaviors. This hands-off approach has led to a higher breeding success rate, evidenced by earlier egg laying compared to VA. During the overwintering period, GVZ continues to utilize outdoor conditions, housing males and females separately in different tubs. The natural temperature fluctuations and gradual cooling period from September to January closely mimic the frogs' natural habitat, providing a more accurate physiological preparation for the breeding season.

The key differences between VA and GVZ lie in the control and accuracy of environmental conditions. VA's indoor setup offers a highly controlled environment with precise manipulation of temperature, but it may lack the full spectrum of natural fluctuations. On the other hand, GVZ's outdoor setup provides a more natural environment, with frogs experiencing real ambient conditions, which appear to lead to

higher breeding success. Understanding these distinct husbandry techniques and their implications on frog health and breeding outcomes is crucial for optimizing captive breeding programs for the OSF. The naturalistic approach at GVZ, which more closely mirrors the frogs' natural habitat, could serve as a model for improving breeding success in other facilities.

2.3.2. OSF Stress and Reproductive Hormone Characterization experiments

Non-invasive Stress Hormone Experimental Design and Collections

The stress hormone experiment involved collecting water samples from adult OSF, with five males and five females from each facility, at four distinct time intervals: 30, 60, 90, and 120 minutes after handling and confinement stress, whereby animals were removed from their respective tanks and were then placed in a beaker with 100 ml of VA tap water. This generally adhered to methods by Gabor et al., (2013) with the body immersed in water, but not the mouth and head. The beakers were then positioned in a water bath, and each beaker was fitted with a tygon tube so that at the end of each time interval water could be removed from the beakers using a syringe without disturbing the animals. All 100 ml of water was removed at each time interval and was placed on ice and then stored in plastic bottles at -20°C for long term storage. A volume of 100 ml of water was replaced at the end of each time interval. At the beginning of each sampling event each frog was weighed and snout-vent length (SVL) recorded. All samples were collected between 10:00 and 14:00 hours to minimize the impact of circadian rhythm influence on corticosterone levels.

Non-invasive Reproductive Hormone Experiment Design and Sample Collections

The OSF seasonal reproductive hormone characterization experiment was conducted using the same five male and five female adults that were randomly selected from GVZ and VA captive breeding facilities throughout the entire experiments. The time of year selected as a sampling time point was based on capturing the hormone status of the overwintering, breeding and post-breeding periods of OSF. All samples were collected in 2023 and the specific dates and description of OSF activities were as follows: December 6, early overwintering; January 8, late overwintering; February 3, pre-breeding; February 25, breeding; April 10, post-breeding; These dates also reflect

various season, including the onset of winter (December), late winter (January), the transition from winter to early spring (February), the onset of spring (April). Hormones were collected using the non-invasive method published by Gabor et al. (2013). Briefly, frogs were placed in beakers containing 100 ml of VA tap water, whereby mouth and head were not immersed in water. The beakers were immediately placed in a water bath to maintain the water temperature that reflected the original rearing conditions animals were collected from. After one hour, the frogs were removed, and the water from the beakers was poured into plastic bottles and stored at 4°C for maximum an hour and then transferred to -20°C for long term storage.

2.3.3. Hormone Extraction Methods and Analyses

Hormone Extraction

All hormone extractions generally adhered to the procedure described by Ellis et al. (2004). Briefly, to obtain waterborne corticosterone, testosterone, estrone and 17β-estradiol from each 100 ml water sample, hormones were extracted from the entire water sample volume for all hormones (i.e., 100 ml). This was achieved by passing the water through Tygon tubing (Saint-Gobain formulation 2475) into C18 solid-phase extraction columns (SepPak Vac 3 cc/500 mg; Waters, Inc., Milford, MA, USA) under vacuum pressure. Prior to addition of the water sample to a column, the column was first primed by the addition of 6 ml of HPLC-grade methanol and then the addition 6 ml of Millipore water. Water samples were then added to the C18 solid-phase extraction columns via the tygon tubing and after the entire 100 ml was filtered through, 4 ml of methanol was added to the C18 solid-phase extraction column to elute the hormones into borosilicate vials as described by Gabor and Grober (2010). Subsequently, to prepare the samples for shipment to the Toronto Zoo where the enzyme immunoassays (EIAs) for each hormone were performed, the methanol was evaporated using a gentle stream of nitrogen gas using an Evap-O-Rac (Cole-Parmer) placed in a 37°C water bath. Lyophilized samples were stored at -20 °C until overnight shipment on ice packs to Toronto Zoo.

Extraction Efficiency

To obtain the extraction efficiency for corticosterone, 17β-estradiol, and testosterone, solutions with known concentrations of each hormone were prepared and

extracted as described in the Hormone Extraction section. Different solutions of corticosterone (0, 0.0078, 1, 10 and 20 ng/ml), 17β-estradiol (0, 0.01, 0.1, 1 and 10 ng/ml) and testosterone (0, 0.01, 0.1, 1 and 10 ng/ml) were prepared in methanol and then added to 100 ml of municipal tap water to cover a range of concentrations. Each hormone solution was subjected the same extraction process, storage and shipment regimes described above and hormones were also analyzed at the Toronto Zoo in hormone enzyme immunoassays as described below.

Hormone Enzyme Immunoassays (EIAs)

Lyophilized samples were resuspended in enzyme immunoassay (EIA) buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of bovine serum albumin per litre). Reconstitution volume varied per hormone (volume of 300 μl designated as the 1:1 dilution) as described below. Samples were vortexed for 5 s, covered for minimum 1 hr at room temperature (RT), then vortexed again for 5 s. Samples were tested in duplicate technical replicates for all hormone EIAs.

Corticosterone EIA

For corticosterone analysis, samples were reconstituted in 200 μl EIA buffer (i.e., 1.5X concentration). Corticosterone was quantified using modifications of an enzyme immunoassay previously described (Baxter-Gilbert et al., 2014; Stewart et al., 2020; Veitch et al., 2021). Microtitre plates were coated with 0.25 μg/well goat anti-rabbit IgG polyclonal antibody (R2004 Sigma-Aldrich, Mississauga, ON Canada; 1:200,000 in coating buffer, 50-mM bicarbonate buffer, pH 9.6) and incubated overnight at room temperature. Plates were washed with 0.05% Tween 20, 0.15 M NaCl solution and blocked with 250 μl EIA buffer for 1 hr at room temperature. Plates were then loaded with 50 μl corticosterone standard (Steraloids Q1550; 39-20,000 pg/ml), reconstituted sample and controls, followed by 100 μl horseradish peroxidase conjugate (1:1,000,000) and 100 μl corticosterone antiserum (1:300,000; antibody lot: CJM006; C. Munro, University of California, Davis CA, USA), all diluted in EIA buffer. Plates were incubated overnight at room temperature, and then washed and loaded with 200-μl of substrate solution (0.5-ml of 4-mg/ml tetramethylbenzidine in dimethylsulphoxide and 0.1-ml of 0.176-M H_2O_2 diluted in 22-ml of 0.01-M sodium acetate trihydrate [$C_2H_3NaO_2 \cdot 3H_2O$], pH 5.0). After 30-min incubation, colour reaction was stopped with 50-μl H_2SO_4 (1.8M)

and absorbance was measured at 450-nm using a spectrophotometer (Epoch 2 microplate reader, BioTek, Winooski, VT, USA). The corticosterone antibody (CJM006) cross-reactivities are: corticosterone (100%), desoxycorticosterone (14.25%), and other metabolites (<3%) (Watson et al., 2013). Intra-assay CV was 4.4%, and inter-assay CVs were 5.7% and 17.9% at 50% binding and 70% binding, respectively.

Estrogen EIA – E1C and E2

For this study, waterborne estrone-3-glucuronide, estrone-3-sulfate and estrone (hereafter urinary estrone conjugate, E1C) and 17β-estradiol (E2) analyses were performed on the OSF water samples. For E1C and E2 analysis, samples were reconstituted in 300 µl EIA buffer (i.e., 1:1 dilution), serially diluted and then assayed. For E1C analysis, dilutions used were 1:2, 1:8, and 1:64. E1C was quantified using a double-antibody enzyme immunoassay (EIA) adapted from Munro and Stabenfeldt (1984), modified by Edwards et al. 2019. Microtiter plates were pre-coated with secondary goat-anti rabbit IgG antibody (GARG; A009, Arbor Assays, Ann Arbor, MI) described by Edwards *et al*. 2019. Briefly, secondary antibody, 150 µl (10 µg/ml) in coating buffer (10 mM Phosphate buffer, pH: 8.0), was added to each microtiter plate well (Nunc Maxisorp, VWR, Mississauga, ON, Canada) and incubated at RT for 15–24 h. After incubation, unbound antibody in the coating solution was aspirated from each well using a BioTek ELx 405VR microplate washer (BioTek Instruments, Winooski, VT). Blocking solution (250 µl; 10mM Phosphate, 15mM NaCl, 1% Sucrose, 2% BSA, and 0.01% Tween 20, pH:7.5) was added to each well and incubated for minimum of 4 hours and no more than 24 h at room temperature. Blocking solution was then aspirated from each well using a microplate washer and plates were dried at room temperature in a desiccator cabinet (Dry Keeper cabinet, SP Bel-Art, Wayne, NJ USA) until relative humidity (RH) was below 20%. These Goat-anti Rabbit IgG coated plates remained in desiccant cabinet at or below 20% RH until immediately before use.

For E1C EIA, GARG pre-coated plate was removed from the desiccant cabinet, and loaded with 50 µl E1C standard (Steraloids E2321; 12-3000 pg/ml) and 50 µl controls diluted in assay buffer (0.1 M Tris buffer, pH 7.5, containing 0.15 M NaCl, 10mM EDTA, 0.1% bovine serum albumin, and 0.1% Tween 2), as well as 50 µl already reconstituted samples. Next, 50 µl horseradish peroxidase conjugate (1:160,000) and 50 µl E1C antiserum (1:320,000; antibody lot: R522-2; C. Munro, University of California,

Davis CA, USA) diluted in the assay buffer were loaded. Plates were incubated for 2 h in the dark at room temperature. Plates were then washed with 5 mM Phosphate, 7.5 mM NaCl, 0.05% Tween 20, 0.01mM EDTA (pH: 7.2) and loaded with 200-µl of substrate solution (0.5-ml of 4-mg/ml tetramethylbenzidine in dimethylsulphoxide and 0.1-ml of 0.176-M H_2O_2 diluted in 22-ml of 0.01-M sodium acetate trihydrate [$C_2H_3NaO_2 \cdot 3H_2O$], pH 5.0). After a 30-minute incubation in the dark at room temperature, the colour reaction was stopped with 50-µl H_2SO_4 (1.8M) and absorbance was measured at 450-nm using a spectrophotometer (Epoch 2 microplate reader, BioTek, Winooski, VT, USA). The E1C antibody (R522-2) cross-reactivities are: estrone-3-glucuronide (100%), estrone-3-sulfate (66.6%), estrone (238%), 17β-estradiol (7.8%), estradiol-3-glucuronide (3.8%), estradiol-3-sulfate (3.3%), and other metabolites (<3%) (Munro et al., 1991). Intra-assay CV was 7.8%, and inter-assay CVs were 6.5% and 11.1% at 40% binding and 65% binding, respectively.

For E2 analysis, dilutions used were 1:1, 1:2, 1:16, and 1:128. E2 was quantified using modifications of an EIA previously described (Kummrow et al., 2011; Pimm et al, 2015; Terwissen et al., 2014). Microtitre plates were coated with 50 µl E2 antiserum (1:29,500 in coating buffer, 50-mM bicarbonate buffer, pH 9.6; antibody lot: R4972/R0008; C. Munro, University of California, Davis CA, USA. After overnight incubation at 4°C, plates were washed with 0.05% Tween 20, 0.15 M NaCl solution, and loaded with 50 µl E2 standard (Steraloids E0950; 19-5000 pg/ml), reconstituted sample and controls, along with 50 µl horseradish peroxidase conjugate (1:100,000), all diluted in EIA buffer. Following a two-hour room temperature incubation, plates were washed and 100 µL substrate solution (50mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0; Munro et al., 1991) added, and then absorbance was measured at 405 nm using a spectrophotometer (Epoch 2 microplate reader, BioTek, Winooski, VT, USA). The E2 antibody (R4972/R0008) cross-reactivities for this EIA are: 17β-estradiol (100%), estrone (3.3%), progesterone (0.8%), testosterone (1.0%), androstenedione (1%), and other metabolites (<0.1%) (deCatanzaro et al., 2003). Intra-assay CV was 5.3%, and inter-assay CVs were 13.3% and 13.9% at 25% binding and 65% binding, respectively.

Testosterone EIA

For testosterone analysis, samples were reconstituted in 200 μl EIA buffer (i.e., 1.5X concentration) and assayed 1.5X concentration or diluted 1:4. Testosterone was quantified using modifications of an EIAs previously described (Kummrow et al., 2011; Pimm et al, 2015; Terwissen et al., 2014). Microtitre plates were coated with 50 µl testosterone antiserum (1:10,500 in coating buffer, 50-mM bicarbonate buffer, pH 9.6; antibody lot: R156/7; C. Munro, University of California, Davis CA, USA. After overnight incubation at 4°C, plates were washed with 0.05% Tween 20, 0.15 M NaCl solution, and loaded with 50 µl testosterone standard (Steraloids A6950; 48-12,500 pg/ml), reconstituted sample and controls, along with 50 μl horseradish peroxidase conjugate (1:20,000), all diluted in EIA buffer. Following a two-hour room temperature incubation, plates were washed and 100 μL substrate solution (50mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0; Munro et al., 1991) was added, and then absorbance was measured at 405 nm using a spectrophotometer (Epoch 2 microplate reader, BioTek, Winooski, VT, USA). The testosterone antibody (R156/7) cross-reactivities for this EIA are: testosterone (100%), 5α-dihydrotestosterone (57.4%), androstenedione (0.27%), and other metabolites (<0.05%) (deCatanzaro et al., 2003). Intra-assay CV was 7.2 %, and inter-assay CVs were 3.3% and 11.8% at 35% binding and 65% binding, respectively.

2.3.4. Statistical Analyses

Parallel displacement between the standard curve and serial dilutions of reconstituted water sample pool was used as an indirect measure of assay specificity. For E1C, E2 and testosterone individual samples were pooled. For corticosterone, individual samples had low values, so a pool from a six frog water samples (included males and females) was used. The pooled samples were serially diluted two-fold in EIA buffer and compared to the respective standard curve. The data were plotted as log (relative dose) vs. percent antibody bound in Microsoft Excel. The slopes of the lines within the linear portion of the curves were determined using linear regression analysis and compared (Soper, 2021) where $p>0.05$ indicates that the slopes are not significantly different and thus interpreted as parallel.

Corticosterone, testosterone, E1C and E2 concentrations were standardized for each water sample by dividing by the mass of the respective individual frog. There was a moderate to strong correlation between SVL (snout-vent length) and mass in adult OSF (Linear regression: $r^2 = 0.53$, n = 99, F = 110.65; P < 0.0001), therefore, mass was used to accommodate in our analysis. The standardized hormone level is expressed as ng/ml/mass(g), making hormone data directly comparable across subjects of varying sizes.

All statistical analyses were conducted using RStudio version 2023.06.1+524 (RStudio Team, 2023). Outliers were detected using Tukey's method (Inter-quartile Range), if the data points were below Q (Quartile) 1 - 1.5 IQR or above Q3 + 1.5 IQR, they were removed from the dataset (Sullivan et al., 2021). Data was tested for normality using a Shapiro-Wilk test and for homogeneity of variance using Levene's test, based on Zuur et al. (2009) guidelines. For data that passed these two tests and thus met the criteria for parametric analyses, a one-way repeated measures analysis of variance (repeated measures ANOVA; $p<0.05$) was performed. This was followed by a Tukey's HSD post-hoc test to determine if there were significant differences between season. If the dataset was not normally distributed and failed Levene's test, Log10 or arcsine transformations were applied, and the parameters were retested. If the transformed data did not meet parametric test assumptions, a Friedman non-parametric test was conducted ($p<0.05$). A Dunn's test with Bonferroni correction was performed to assess the effects of different seasons on hormone concentration. Specifically, we controlled for multiple comparisons on five time points with the Bonferroni correction (alpha = 0.05). For the five time points, we used ten pairwise comparisons (e.g., early overwintering vs. late overwintering, pre-breeding vs. breeding, etc.) to control the risk of type I errors by adjusting the alpha level for multiple comparisons within each time point. To compare hormone concentrations between the two facilities in different seasons an independent t-test or a Wilcoxon Mann-Whitney test was used. A Spearman's correlation was conducted to evaluate the relationship between E1C and E2 across different seasons in female frogs. Same statistical pipeline was used to determine if mass and SVL of frogs was different between season or facilities.

A power analysis was performed to determine the sample size required for detecting significant differences in hormone levels across different seasons using a one-way ANOVA (Cohen, 1992). The analysis involved calculating the means and standard

deviations of hormone levels for each season. The power analysis was conducted for two facilities, GVZ and VA. The analysis indicated that the study design was adequately powered to detect seasonal variations in hormone levels, considering an alpha level of 0.05 and a desired power of 0.80.

2.4. Results

Corticosterone Extraction Efficiency

The concentration of corticosterone in all water samples collected from the handling and confinement stress exposure experiment with OSF adults were below detection limits of this assay. However, for samples spiked with corticosterone the extraction method demonstrated higher recovery than the initial amount at all other concentrations (Table 2.1). These data invalidate this assay, and therefore no further interpretations or conclusions can be derived from this data set.

E2 Extraction Efficiency

The results of E2 extraction are summarized in Table 2.2. The samples with 0 and 0.01 ng E2 were below the limit of quantification (LOQ). The extraction efficiencies for the higher concentrations showed that the method could recover estrogen with varying efficiency, notably about half of the initial concentrations.

Testosterone Extraction Efficiency

Testosterone extraction results are presented in Table 2.3. Similar to estrogen, samples with 0 and 0.01 ng testosterone were below the LOQ. The extraction method showed a good recovery for testosterone, especially at the higher concentrations.

Parallel Displacement in Hormonal Standard Curves of Water Samples

Serial dilutions of pooled water samples showed parallel displacement with the respective standard curves: corticosterone ($t=1.56$, $p=0.16$, $df=7$), E1C ($t=0.16$, $p=0.88$, $df=7$), E2 ($t=0.98$, $p=0.36$, $df=7$), and testosterone ($t=1.25$, $p=0.25$, $df=7$).

Morphometrics of OSF in GVZ and VA Rearing Facilities

There was no significant difference in mass and snout-vent length (SVL) between the seasons for both female and male frogs in both facilities (Kruskal-Wallis test, $p >$

0.05). Additionally, there was no significant difference in mass and SVL between facilities for both female and male frogs (Figures 2.1, 2.2, 2.3 and 2.4; Wilcoxon Mann-Whitney test, $p > 0.05$). The coefficient of variation (CV) was calculated to quantify the relative variability in the data sets. For male frog mass, the CVs were 21.11% and 11.57% for the VA and GVZ facilities, respectively, and for females 35.26% and 21.55% for the VA and GVZ facilities, respectively. For SVL, the CVs were 6.90% and 2.61% for the VA and GVZ male frogs, respectively, and 9.63% and 2.28% for the VA and GVZ female frogs, respectively. These values indicate that the relative variability in mass was higher than in SVL, with the females at VA showing the highest variability in mass.

Waterborne Corticosterone Excreted from Males and Females

The results of the corticosterone EIAs over-estimated the concentration of this hormone in the corticosterone spiked samples indicating some non-specific binding was occurring and interfering with the analysis. This was also supported by the corticosterone concentrations in the water samples exhibiting corticosterone concentrations higher than the highest concentration in the standard curve. Due to the non-specific binding of some component in the water samples, issue no further analysis of this data was performed.

Waterborne E1C Excreted from Females

There was no significant difference in the E1C levels between different seasons for the female frogs within both the VA and the GVZ (Friedman test, $X^2(4) = 8.96$, $p = 0.22$ and $X^2(4) = 9.12$, $p = 0.17$, respectively). Additionally, urinary E1C levels were not significantly different between the two facilities in each season ($p > 0.05$; Figure 2.5).

Waterborne E2 Excreted from Females

For female OSF reared at GVZ there was a significant difference in the E2 levels between different seasons (Friedman test, $X^2(4) = 9.12$, $p = 0.002$). The seasonal 17β-estradiol appeared to increase from early overwintering (mean= 0.012, SD = ± 0.01 ng/ml/g) to late overwintering (mean= 0.046, SD = ± 0.06 ng/ml/g) to reach its maximum, and then significantly decreased by the pre-breeding time point (mean= 0.001, SD = ± 0.00006 ng/ml/g, Dunn's test, p= 0.009) and remained significantly low for the breeding season (mean= 0.0008, SD = ± 0.0006 ng/ml/g, Dunn's test, p= 0.004) before increasing post-breeding (mean= 0.17, SD = ± 0.3 ng/ml/g).

For female OSF reared at VA there was a significant difference in the E2 levels between different seasons (Friedman test, $X^2(4) = 12.16$, $p = 0.016$). The seasonal E2 levels in early overwintering (mean = 0.17, SD = ± 0.2 ng/ml/g) decreased by late overwintering (mean = 0.054, SD = ± 0.07 ng/ml/g) and continued to decrease by pre-breeding (mean = 0.005, SD = ± 0.001 ng/ml/g). E2 levels remained low during the breeding season (mean = 0.0013, SD = ± 0.001 ng/ml/g) before significantly increasing post breeding (mean = 0.02, SD = ± 0.01, Dunn's test, $p = 0.02$). Moreover, E2 levels were significantly different between the two facilities in the pre-breeding season and the post-breeding season ($p = 0.007$ and $p = 0.03$, respectively), but in other seasons the facilities did not appear to affect E2 levels in these female frogs (Figure 2.6.).

Upon examination of E2 levels, several outliers were identified across different periods and locations. Post-breeding, one female from GVZ exhibited an E2 concentration of 0.82 ng/ml/g. During early overwintering, a notable outlier with an E2 concentration of 0.63 ng/ml/g was observed from VA. In the late overwintering period, two outliers were identified: one from VA with an E2 concentration of 0.18 ng/ml/g, and another from GVZ with an E2 concentration of 0.15 ng/ml/g.

Correlation Between Waterborne E2 and E1C Excreted from Females

To examine the relationship between E2 and E1C, Spearman's correlation analysis was performed to compare these two hormones within a season for each facility. Essentially, no significant correlation was observed and details are provided below.

GVZ females

Before breeding, there was a non-significant positive correlation between E1C and E2 ($r_s = 0.3$, $p = 0.68$). During breeding, there was a non-significant positive correlation between E1C and E2 ($r_s = 0.1$, $p = 0.95$). After breeding, there was a non-significant negative correlation between E1C and E2 ($r_s = -0.2$, $p = 0.92$). During early overwintering, there was a non-significant positive correlation between E1C and E2 ($r_s = 0.8$, $p = 0.33$). Lastly, during late overwintering, there was a non-significant positive correlation between E1C and E2 ($r_s = 0.6$, $p = 0.42$). Overall, none of the correlations at the GVZ were significant.

VA females

Specifically, before breeding, there was a non-significant positive correlation between E1C and E2 (rs = 0.6, p = 0.35). During breeding, there was a non-significant positive correlation between E1C and E2 (rs = 1, p = 0.083). After breeding, there was a non-significant positive correlation between E1C and E2 (rs = 0.9, p = 0.083). During early overwintering, there was a non-significant positive correlation between E1C and E2 (rs = 1, p = 1). Lastly, during late overwintering, there was a non-significant positive correlation between E1C and E2 (rs = 1, p = 0.083). Overall, none of the correlations at the VA were significant.

Waterborne Testosterone Excreted from Males

For GVZ male frogs, there was a significant difference in testosterone levels across different seasons (Friedman test, $X^2(4) = 11.68$, p = 0.019). Testosterone levels were the highest during the early overwintering period (mean = 0.081, SD = ± 0.053) and declined in the late overwintering (mean= 0.021, SD = ± 0.004), and remained low in pre-breeding (mean = 0.018, SD = ± 0.009). Testosterone increased during the breeding season (mean = 0.049, SD = ± 0.03) and further declined significantly in the post-breeding season (mean = 0.014, SD = ± 0.01, Dunn's test, p = 0.015). Similarly, there was a significant difference in the testosterone levels between different seasons for the VA male frogs (Friedman test, $X^2(4) = 11.68$, p = 0.019). Testosterone levels were low during early overwintering (mean = 0.047, SD = ± 0.02) and late overwintering (mean = 0.055, SD = ± 0.03), increased pre-breeding (mean = 0.084, SD = ± 0.04), reached their highest during breeding (mean = 0.099, SD = ± 0.04). Then testosterone levels decreased significantly post-breeding (mean = 0.010, SD = ± 0.004, Dunn's test, p = 0.03). Finally, there was a significant difference in testosterone levels between the two facilities in the pre-breeding season (w = 2, p = 0.031). However, there was no significant difference in testosterone levels between the facilities in other seasons (Figure 2.7.).

Power Analysis

Males (Testosterone)

The power analysis yielded Cohen's f of 0.954, indicating a strong effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the

required sample size per season is approximately 4 frogs from GV Zoo For VA the analysis yielded Cohen's f of 0.994, indicating a strong effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the required sample size per season is approximately 4 frogs.

Females (E2)

For GVZ the analysis yielded Cohen's f of 0.435, indicating a moderate effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the required sample size per season is approximately 14. For VA The analysis yielded Cohen's f of 0.606, indicating a moderate to strong effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the required sample size per season is approximately 8 frogs.

Females (E1C)

For GV Zoo the power analysis yielded Cohen's f of 0.382, indicating a moderate effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the required sample size per season is approximately 18 frogs. For VA, the analysis yielded Cohen's f of 0.613, indicating a moderate to strong effect size. To achieve a power of 0.8 at a significance level of 0.05 with five groups (k = 5), the required sample size per season approximately 8 frogs.

Table 2.1 Total corticostrone (ng/L) recovered from a single water sample spiked with a known concentration of corticostrone extracted via C18 cartridges according Gabor et al. (2013). Extraction efficiency was calculated by dividing the measured concentration by the spiked concentration and multiplying by 100.

Spiked Concentration (ng/L)	Measured concentration (ng/L)	Extraction efficiently (%)
0	8.57	-
0.078	20.59	26474.36
1	25.55	2555
10	25.74	257.4
20	47.21	236.05

Table 2.2 **Total 17β-estradiol (ng/L) recovered from a single water sample spiked with a known concentration of 17β-estradiol and extracted via C18 cartridges according Gabor et al. (2013). Samples with 0 and 0.01 ng/L were below the detection limit of the enzyme immunoassay. Extraction efficiency was calculated by dividing the measured concentration by the spiked concentration and multiplying by 100.**

Spiked Concentration (ng/L)	Measured concentration (ng/L)	Extraction efficiently (%)
0	Below LOQ	-
0.01	Below LOQ	-
0.1	0.05	50
1	0.55	55
10	5.58	55.8

Table 2.3 **Total testosterone (ng/L) recovered from a single water sample spiked with a known concentration of testosterone extracted via C18 cartridges according Gabor et al. (2013). Samples with 0 and 0.01 ng/L were below the detection limit of the enzyme immunoassay. Extraction efficiency was calculated by dividing the measured concentration by the spiked concentration and multiplying by 100.**

Spiked Concentration (ng/L)	Measured concentration (ng/L)	Extraction efficiently (%)
0	Below LOQ	-
0.01	Below LOQ	-
0.1	0.03	30
1	0.27	27
10	5.89	58.9

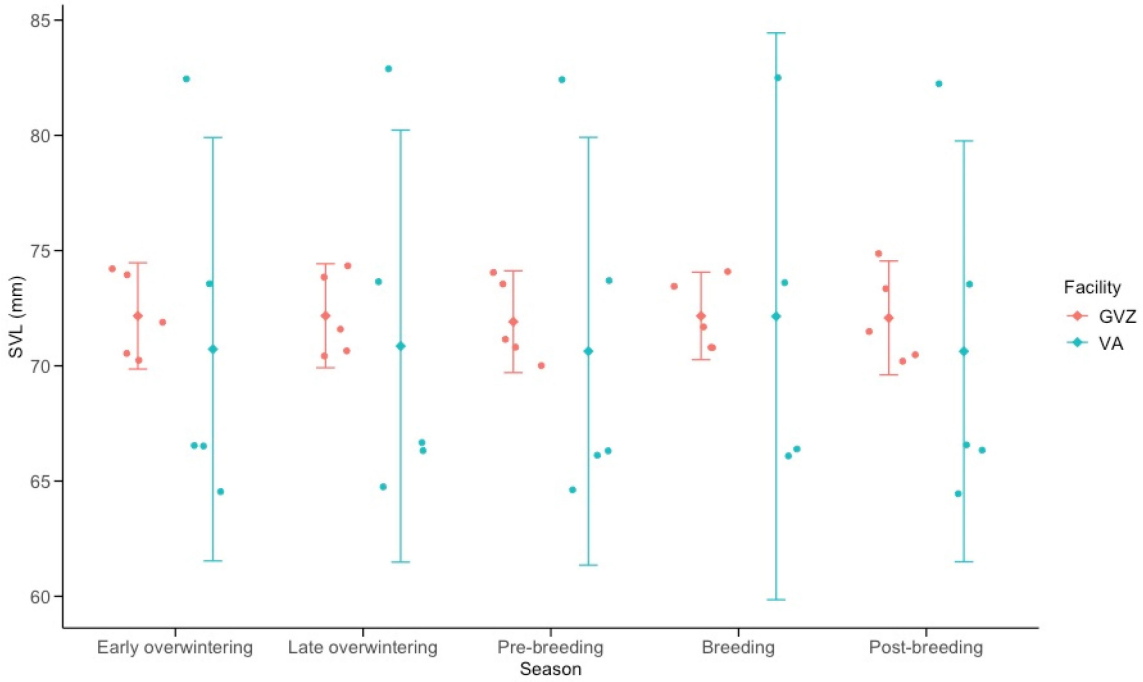

Figure 2.1 **Snout-vent length (SVL) of female Oregon spotted frogs (Rana pretiosa) at two captive breeding facilities, the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA), across different seasons: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. Each value represents an individual frog (n=5 frogs per time point) and error terms represent 95% confidenice intervals.**

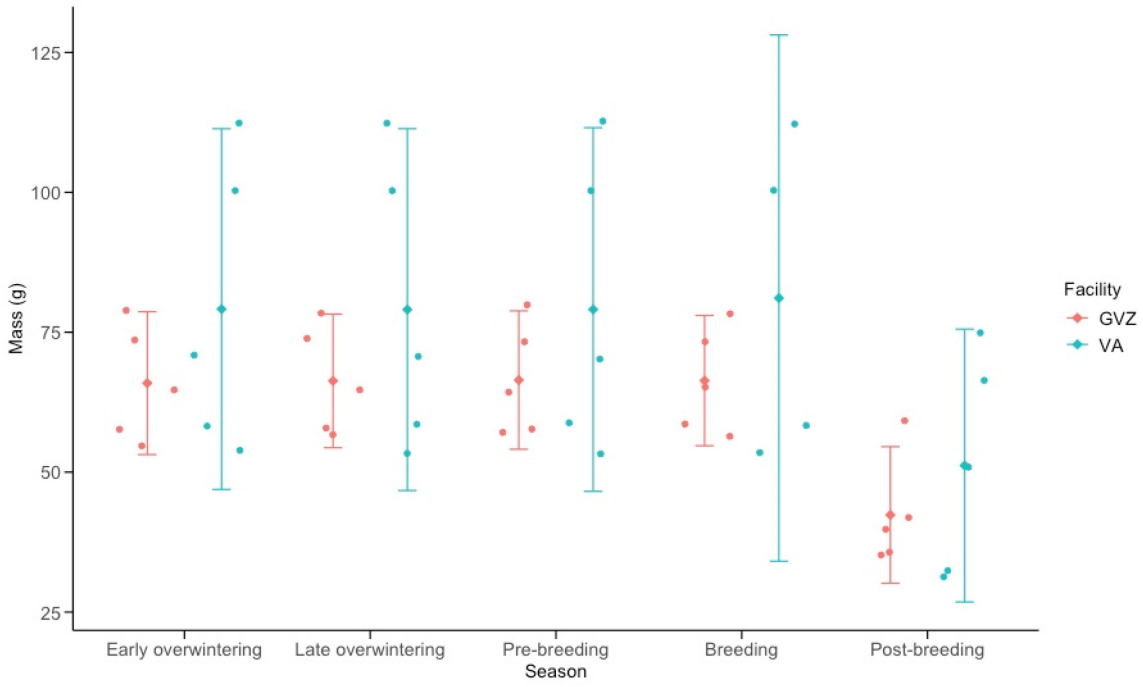

Figure 2.2 **Mass of female Oregon spotted frogs (*Rana pretiosa*) at two captive breeding facilities, the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA), across different seasons: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. Each value represents an individual frog (n=5 frogs per time point) and error terms represent 95% confidenice intervals.**

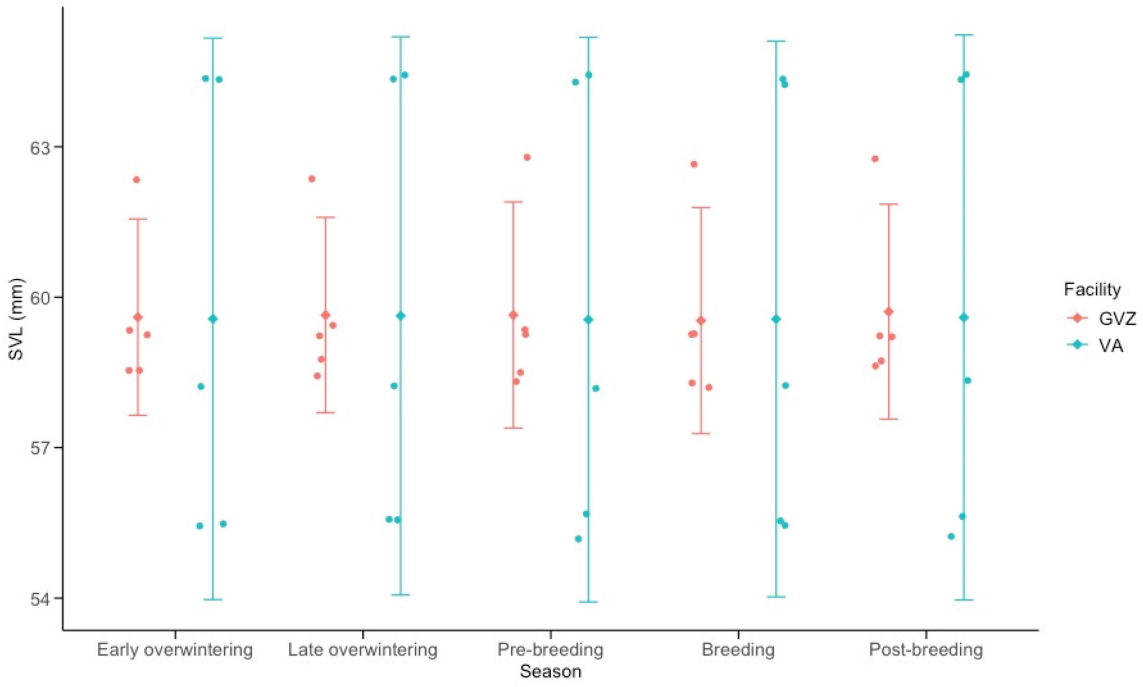

Figure 2.3 **Snout-vent length (SVL) of male Oregon spotted frogs (Rana pretiosa) at two captive breeding facilities, the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA), across different seasons: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. Each value represents an individual frog (n=5 frogs per time point) and error terms represent 95% confidenice intervals.**

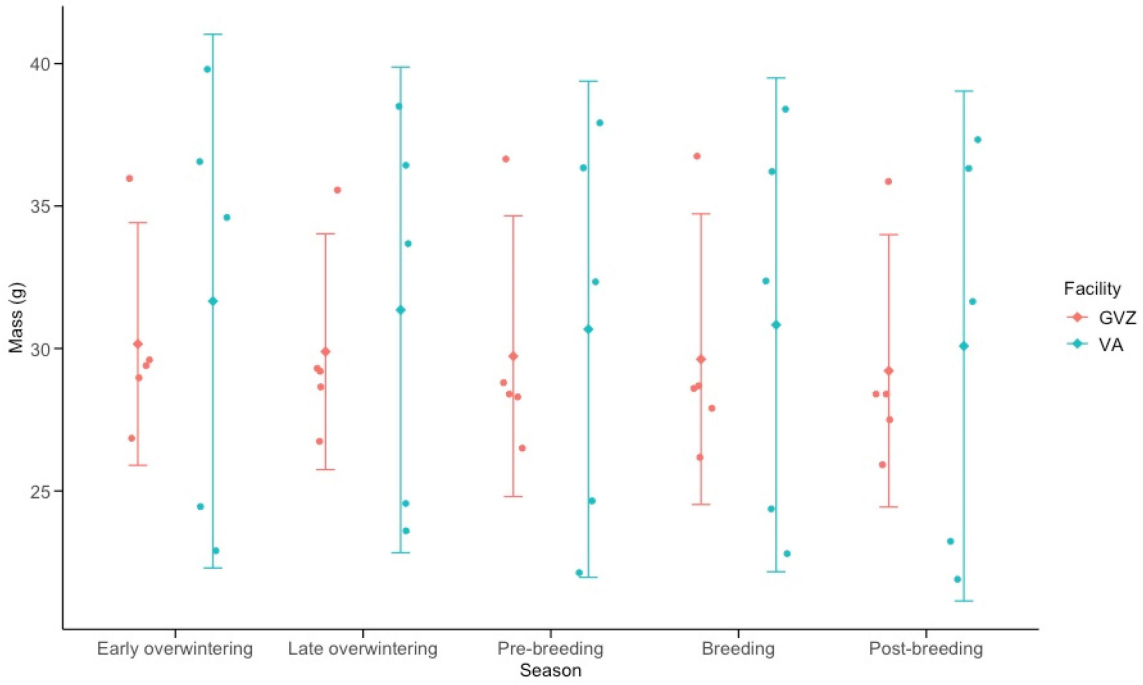

Figure 2.4 **Mass of male Oregon spotted frogs (*Rana pretiosa*) at two captive breeding facilities, the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA), across different seasons: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. Each value represents an individual frog (n=5 frogs per time point) and error terms represent 95% confidenice intervals.**

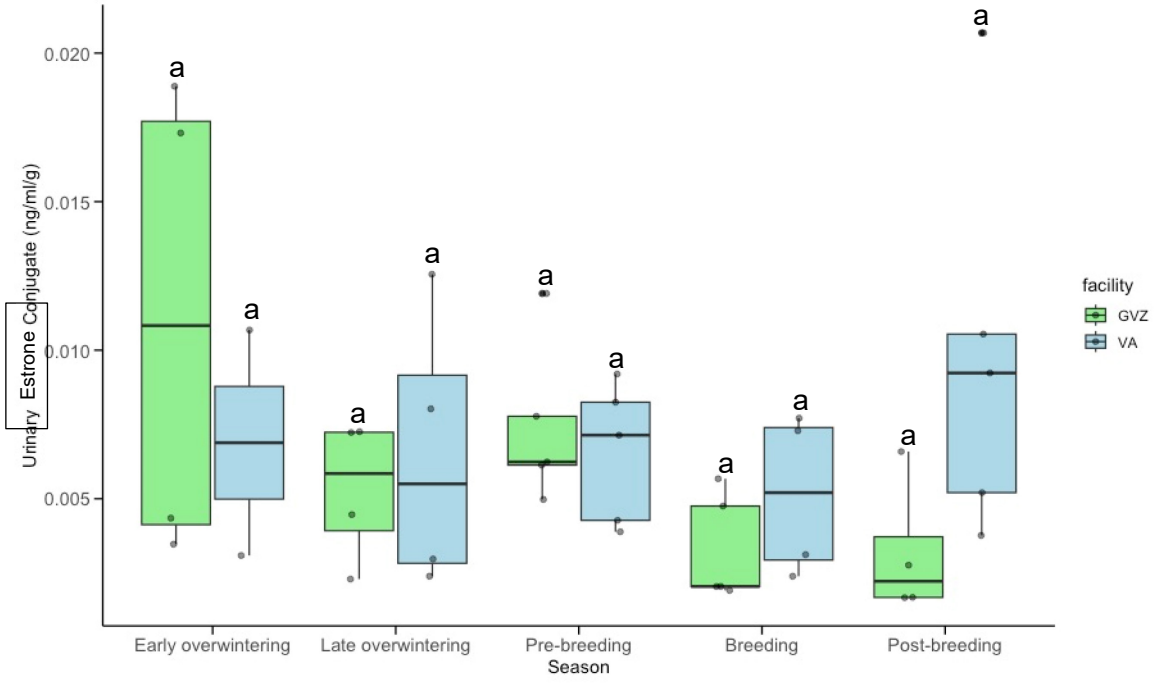

Figure 2.5 **Urinary Estrone Conjugate (E1C) levels in adult male Oregon Spotted Frogs (*Rana pretiosa*) at the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA). Box plots show E1C levels across seasonal stages: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. The central line indicates the median, the box edges represent the interquartile range (IQR), and whiskers denote 1.5 times the IQR. Raw data are shown in grey dots. Significant differences ($p < 0.05$) between seaon in each facility are indicated by different superscripts. Asterisks indicate significance between facilties ($p < 0.05$).**

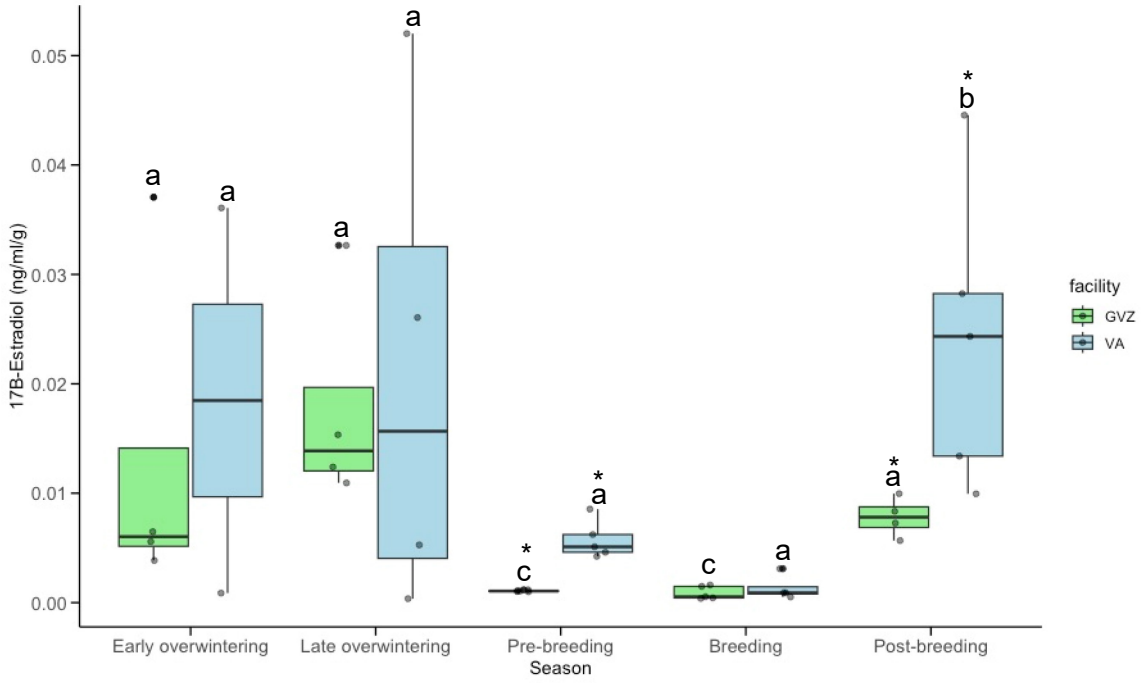

Figure 2.6 **17β-estradiol (E2) levels in adult male Oregon Spotted Frogs (*Rana pretiosa*) at the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA). Box plots show E2 levels across seasonal stages: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. The central line indicates the median, the box edges represent the interquartile range (IQR), and whiskers denote 1.5 times the IQR. Raw data are shown in grey dots. Significant differences ($p < 0.05$) between seaon in each facility are indicated by different superscripts. Asterisks indicate significance between facilties ($p < 0.05$).**

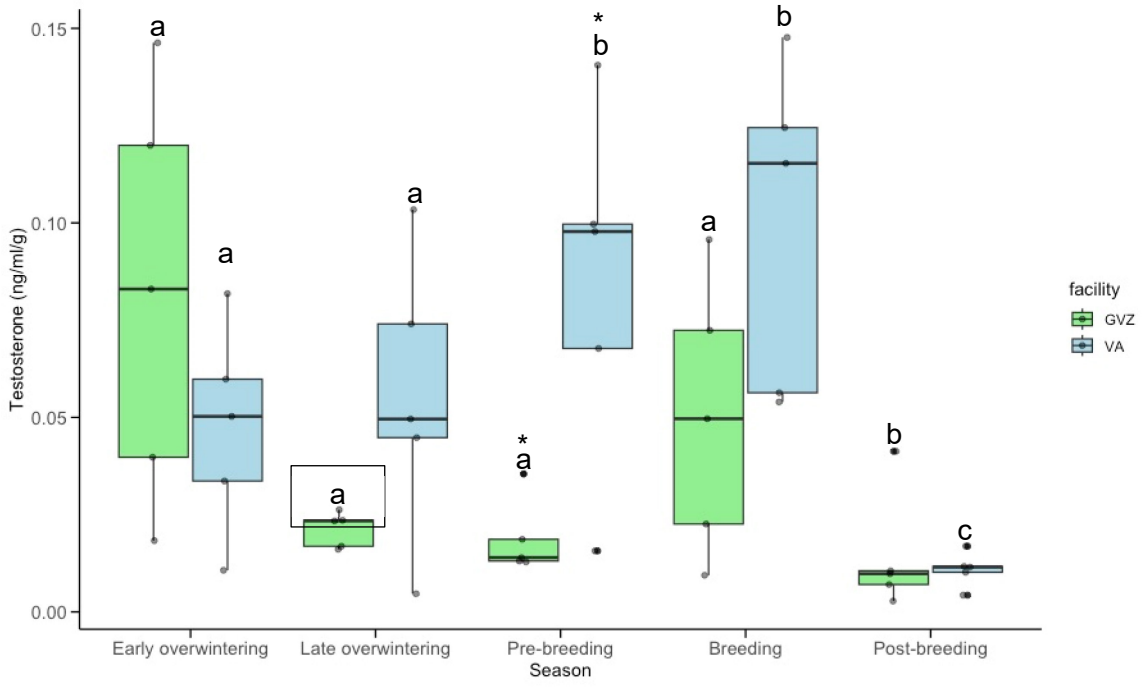

Figure 2.7 **Testosterone levels in adult male Oregon Spotted Frogs (*Rana pretiosa*) at the Greater Vancouver Zoo (GVZ) and Vancouver Aquarium (VA). Box plots show testosterone levels across seasonal stages: early overwintering, late overwintering, pre-breeding, breeding, and post-breeding. The central line indicates the median, the box edges represent the interquartile range (IQR), and whiskers denote 1.5 times the IQR. Raw data are shown in grey dots. Significant differences ($p < 0.05$) between seaon in each facility are indicated by different superscripts. Asterisks indicate significance between facilties ($p < 0.05$).**

2.5. Discussion

The reproductive cycle in amphibians, as in all vertebrates, is regulated by the endocrine and nervous systems via a multitude of neuropeptides, pituitary hormones and sex steroids (i.e., androgens and estrogens; Marlatt, 2022). Indeed, estrogens and androgens have been shown to play a pivotal role in controlling the reproductive cycle in several anuran species (Marlatt, 2022), and these complex physiological events are far from resolved. For the OSF, this is the first study to examine reproductive hormone profiles in adult male and female frogs and the findings suggest that seasonal profiles of E2 and testosterone do vary across the seasons, and there are differences in these profiles between the GVZ outdoor and VA indoor captive breeding facilities. This is not surprising based on observations of differences in reproductive success between the GVZ and the VA, with GVZ OSF population consistently producing multiple egg masses each year while the VA rarely produces viable egg masses. It was hypothesized that this would be attributed to the outdoor rearing conditions at the GVZ that more closely mimic natural environmental conditions, providing the essential cues such as appropriate temperature and photoperiod required for successful reproduction (Narayan et al., 2010; Calatayud et al., 2017). In contrast, the VA's artificial indoor environment may lack or have less optimal environmental cues, leading to disrupted reproductive cycles. Stress factors, highlighted by Narayan et al., (2012) and Narayan et al., (2011), such as handling and visitor presence, may also be suppressing reproductive behaviors and hormone levels at the VA. Interestingly, in the present study the seasonal profiles of E2 in female OSF did not peak during the reproductive season which is typical in numerous anurans, and may suggest females are not in optimal reproductive health at both GVZ and VA. However, testosterone levels in OSF males did exhibit the expected trend of peaking during breeding and declining post-breeding, although for GVZ males, testosterone levels were not significantly different between overwintering and the breeding season. Overall, OSF males appear to generally exhibit testosterone profiles more typical of several other studies of healthy reproducing anurans, and this was more pronounced at the indoor VA facility (Germano et al., 2009; Germano et al., 2012). Collectively, the reproductive hormone profiles obtained in the present study suggests that female reproductive health may be less than optimal at both facilities, and ongoing non-invasive reproductive hormone monitoring combined with other reproductive health

measures will aid in better characterizing OSF reproductive physiology and optimal captive breeding strategies.

2.5.1. Seasonal Variation in E2 and E1C

Contrary to studies conducted in numerous temperate and tropical anurans (Carr, 2011; Scaia et al., 2013; Ayad et al., 2020) in the present study E2 levels were lowest in OSF females during the pre-breeding and breeding season compared to the post-breeding and overwintering periods. This was consistent for both GVZ and the VA, with the VA having slightly higher levels than GVZ during the pre-breeding, breeding and post-breeding periods. This does not align with E2 levels measured in the blood of wild Gray Tree Frogs (*Hyla versicolor;* a temperate species) which peaked during the breeding season (Gordon and Hellman, 2015). Similar results were found in studies on the wild temperate European Common Frog (*Rana temporaria*; Calatayud et al., 2017), wild Perez's Frog (*Rana perezi*, Delgado et al., 1990), wild Edible frog (*Rana esculenta*, Pierantoni et al., 1984) and wild Bombay Night Frog (*Nyctibatrachus humayuni*; Joshi et al., 2018), whereby E2 levels were highest during the breeding season and low during the non-breeding season. Collectively, numerous studies have demonstrated that E2 plays a crucial role in regulating the reproductive cycle, preparing the female frogs for breeding and supporting oocyte development and ovulation during the breeding season (Lynch and Wilczynski, 2005; Germano et al., 2009; Narayan et al., 2010; Ayad et al., 2020; Scaia et al., 2019). There are several possible explanations as to why OSF exhibited the lowest levels of E2 prior to and during breeding in the present study that require follow up, such as: this is a unique species-specific endocrine physiology; or an artifact of measuring this hormone in dermal/urinary excretions in water as opposed to blood levels; or was due to less than optimal captive breeding environments at both GVZ and VA. In addition, the hormone extraction efficiencies using the C-18 solid-phase extraction column methods based on Ellis et al., (2004) may need optimization given the ~50% recovery of E2 during the extraction of samples spiked with E2 in the present study. Indeed, compared to the present study, Baugh et al., (2018) reported higher E2 extraction efficiencies from water samples (61.4% to 110.9%) using similar methods except for using Sep-Pak C18 columns, suggesting these latter columns may further optimize this method for measuring waterborne hormones. Nonetheless, the present study suggests that OSF from two captive breeding facilities may have reduced E2 pre-

and during the breeding season compared to all other seasons, and additional studies are needed to better understand the E2 profiles throughout the seasons in mature female OSF.

Interestingly, Hunter (2023) conducted ultrasound imaging in female OSF at VA and GVZ at specific intervals during the breeding season to assess follicular development. Delays in follicular development during the breeding season were observed in OSF at the VA but not GVZ (Hunter, 2023), which does correspond to the delayed E2 peak observed in female OSF at the VA in the present study that did not occur until the post-breeding season. In addition, compared to GVZ the VA has significantly lower numbers of viable egg masses and increased egg binding (Hunter, 2023). Together, these data may suggest that the cues required for OSF oogenesis and breeding at VA are not optimal or are asynchronous with the natural seasons and this may be causing delayed follicular development, egg laying and reduced numbers of viable offspring observed by Hunter (2023). However, some degree of the variation in E2 levels and successful reproduction observed within and between these two captive facilities may also be due to differences in the age and size of the animals, which although was not significantly different between facilities exhibited considerable variability. Based on the known age of animals at GVZ and VA, it is presumed that all are of reproductive age which ranges from 1-2 years to 6-8 years, however, the age of adults entering reproductive senescence is not well studied in OSF. Age and size influence hormone production, metabolism and regulation in most vertebrates studied to date with reductions in reproductive hormones during later life stages a prominent trend (Sever and Staub, 2011; Calatayud et al., 2022). In addition to a larger sample size and comparisons between influential abiotic factors between the outdoor and indoor environments (i.e., photoperiod, temperature, water quality, etc.; Duellman and Trueb, 1994; Rastogi et al., 2011) at these two captive breeding facilities, it is important to ascertain the age differences between these two populations as this likely contributes significantly to the reproductive hormone profiles exhibited in these two captive populations.

The present study further suggests that E1C does not exhibit significant seasonal variation in female OSF at both the VA and the GVZ. This stability suggests that urinary E1C may not be a sensitive biomarker for detecting seasonal reproductive physiological changes in these amphibians. However, the study by Germano et al., (2009) found that

urinary hormone analysis, including E1C, in Temperate Bell Frogs (*Litoria raniformis*) is an effective non-invasive method for monitoring reproductive hormones in amphibians. Specifically, Germano et al., (2009) found that concentrations of urinary estrone, testosterone, and progesterone increased during the breeding season for female Bell Frogs. In addition, the results for E1C do not align with Narayan et al., (2010) who showed E1C levels measured from the urine in wild and captive endangered tropical Fijian Ground Frogs (*Platymantis vitiana*) peaked during the breeding season. Future studies optimizing the extraction efficiencies of hormones from water and a larger sample size are necessary to more accurately characterize the seasonal estrogen profiles, including E1C, in the OSF. Nonetheless, the present study demonstrates that hormone monitoring studies using these non-invasive hormone profile monitoring methods combined with ultrasounds of follicular development have the potential to provide invaluable insights into reproductive health in captive and wild OSF.

2.5.2. Seasonal Variation in Testosterone

For both the GVZ and the VA male frogs, there was a significant fluctuation in testosterone levels throughout the seasons with the lowest levels observed post-breeding. However, for GVZ males no other significant differences across seasons were observed, while for the VA males, testosterone levels were significantly increased pre- and during the breeding season to levels that exceeded those during overwintering. Therefore, at these two facilities the male testosterone profiles in OSF exhibited some differences that may be attributed to different husbandry practices. Interestingly, the indoor environment at the VA resulted in an OSF testosterone profile that more closely aligns with previous studies of testosterone in anurans than the presumed more natural outdoor environment at the GVZ (Germano et al., 2009; Germano et al., 2012).

As with studies of E2 levels in female amphibians, testosterone levels in male amphibians typically peaks during the breeding season, decline immediately after, which is then followed by a gradual increase during overwintering (Moore & Jessop, 2003) (Wingfield and Sapolsky, 2003; Harvey et al., 1997; Gobbetti and Zerani, 1996; Marlatt 2024). For example, Delgado et al., (1989) studied the temperate Perez's Frog (*Pelophylax perezi*) and observed that testosterone levels were highest during the breeding season, supporting the development of secondary sexual characteristics and promoting reproductive behaviors such as calling and territoriality. Similarly, Raucci and

Di Fiore (2007) investigated testosterone changes in the temperate green frog (*Rana esculenta)* and observed seasonal changes in the expression of the c-kit receptor, a protein associated with germ cell maturation in the testis. During the reproductive period (March-June), c-kit receptor expression was highest, coinciding with peak testosterone levels and enhanced spermatogonial proliferation (Raucci and Di Fiore, 2007). In the pre-reproductive period (January-February), both c-kit receptor expression and testosterone levels began to rise, preparing the testis for the upcoming breeding season. Conversely, during the post-reproductive period (October-December), c-kit receptor expression and testosterone levels were significantly lower, corresponding with reduced spermatogonial activity (Raucci and Di Fiore, 2007). In a wild population of the temperate Maud Island Frog (*Leiopelma pakeka*) Germano et al. (2012) investigated the annual cycles of urinary reproductive steroid concentrations and found that testosterone levels peaked during the breeding season, facilitating mating behaviors and spermatogenesis. In addition, in the Temperate Bell Frog (*Litoria aurea*) Germano et al. (2009) reported significant seasonal variations in testosterone levels, with higher concentrations during the breeding season. Studies in the Temperate Bullfrog (*Lithobates catesbianus)* showed that in the winter during the non-breeding season androgen receptors and sex hormone-binding globulin are present in primordial spermatogonia, providing evidence that testosterone is maintained in the cells of the testis while animals overwinter (Canequim et al., 2013). Indeed, testosterone, has been shown to play a pivotal role in controlling spermatogenesis in several anuran species, and the levels of plasma testosterone are presumed to reflect testosterone production of the Leydig cells in the testis (Marlatt, 2024). The significance of testosterone in male gamete maturation and differentiation leading to mature sperm during overwintering and peak levels during the breeding season is further supported in the present study of OSF adult males and aligns with other anuran species (Marlatt, 2024).

Testosterone levels differed significantly between the two facilities during the pre-breeding, breeding and post-breeding seasons, with VA frogs exhibiting higher levels of testosterone than GVZ frogs. This did not support the hypothesis that the outdoor environment at GVZ would promote more optimal breeding conditions, as GVZ OSF testosterone levels remained fairly steady throughout these seasons with a significant decrease only during the post-breeding season. Whether the testosterone profile in OSF at GVZ do reflect males in optimal reproductive condition warrants further study since

the GVZ captive population consistently produces more viable offspring than the VA indoor facility. The differences in testosterone profiles between GVZ and VA could be attributed to differences in environmental conditions and management practices between the frog populations at these two facilities. For example, it is possible that the VA frogs might be exposed to conditions such as higher temperature that favor earlier cues for spermatogenesis and breeding behaviors based on the significantly higher testosterone levels during pre-breeding observed at the VA compared to GVZ males (Parua et al., 2011). The variation in testosterone levels between facilities may also be partly explained by differences in the age and size differences between the VA and GVZ. Age and size variability have been shown to influence hormone levels in most vertebrates including anurans (Sever and Staub, 2011; Calatayud et al., 2022). Finally, Baugh et al., (2018) reported similar testosterone extraction efficiencies from water samples (47.2 ± 4.3%), using similar methods except for using Sep-Pak C18 columns, suggesting that these methods are comparable but may benefit from further optimization. Nonetheless, the present study suggests that male OSF from two captive breeding facilities have reduced testosterone post-breeding as in other amphibians studied to date and that the VA indoor environment does not appear to impede the typical seasonal variation in testosterone levels. Ultimately, although ongoing studies are necessary with a larger sample size of males from each facility and over additional years, the testosterone profiles at the GVZ and VA both generally appear to reflect testosterone profiles observed in other healthy reproducing anurans studied to date.

2.5.3. Conclusion

In summary, this study provides insights into the reproductive hormone profiles of two captive OSF populations across different seasons and rearing environments to assist in assessing the reproductive health of these endangered frogs. As expected, significant seasonal variations in E2 and testosterone levels were observed. However, for female frogs, E2 levels did not peak during the breeding season as observed in other anurans, which may indicate potential suboptimal reproductive health in female frogs at both the GVZ and VA facilities. Male testosterone levels did align more closely to previous studies in anurans with peak levels occurring during the breeding season and reductions post-breeding, which suggests male reproductive health is good at both facilities. Nonetheless, future studies to verify these findings incorporating a larger

sample size and optimizing hormone extraction efficiencies from water are needed. Finally, using this non-invasive hormone monitoring method combined with other complimentary reproductive health assessments (i.e., ovarian ultrasound, egg number, sperm motility and number) to further assess OSF frog reproductive health holds considerable promise for optimizing OSF captive breeding programs.

Chapter 3.

OSF Reintroduction Site Water Quality and Sensitivity to Contaminants

3.1. Abstract

In aid of yearly zoo to wild reintroductions of the endangered Oregon spotted frog (OSF), I investigated the impact of reintroduction site water on early life stages of this species. Egg masses containing Gosner stage (GS) 15-16 embryos were seeded into one of the following three treatments (five replicate aquaria with ten individuals per treatment) for 103 days: water taken from the site where the tadpoles are eventually reintroduced; Greater Vancouver Zoo water (where the tadpoles were born) and "Z-R" water (where tadpoles are transferred from Zoo water to reintroduction site water at GS 25). No significant differences in growth (i.e., mean total length, snout-vent length, mean wet weight) were observed among treatments. There were also no significant differences in development trajectory between treatments based on number of surviving individuals reaching forelimb emergence (GS 42). However, survival in the Z-R treatment was significantly lower (50%) compared to both the Zoo (88%) and reintroduction site (78%) treatments ($p<0.05$, pairwise Log-Rank Test). This finding suggests that the transition from the Zoo water to reintroduction site water is a significant stressor that results in mortality. I recommend that reintroduction programs reintroduce OSF egg masses or larvae acclimated to site water before ~ GS 25 to maximize survival. This study underscores the importance of experiments determining appropriate acclimation periods with reintroduction site water before OSF reintroductions, and perhaps for other amphibian species where reintroductions are done. These types of experiments are crucial to enhance the success of such efforts to aid the survival of endangered amphibians.

3.2. Introduction

Amphibian declines have become a major conservation issue worldwide (Stuart et al., 2004). Amphibians are now the most endangered class of vertebrates worldwide and approximately 41% of their species are under threat (Luedtke et al., 2023). The main reasons for this decline are climate change, diseases, habitat loss, invasive species (Luedtke et al., 2023), and environmental pollution (Hayes et al., 2010). Although there have been some successful recoveries for some species (for example, Iberian Frog (*Rana iberica*) and Sierra Nevada Yellow-Legged Frog (*Rana sierrae*); Bosch et al., 2019; Knapp et al., 2016), largely due to effective habitat protection and restoration. However, for amphibians as a whole, populations continue their downward trend (Walls et al., 2016). This ongoing decline is particularly concerning given the vital ecological roles amphibians play and the broader implications their loss could have on biodiversity (Whitfield et al., 2007).

The decline of amphibian populations has led to the development of various conservation action plans that incorporate both *in-situ* and *ex-situ* activities, including the establishment of captive breeding and reintroduction programs, also known as conservation breeding programs (Gascon, 2007; Bishop et al., 2012; Silla and Byrne 2019). These programs serve as assurance strategies for the survival of wild populations that are no longer self-sustaining. One example is the captive breeding and reintroduction effort for the Oregon Spotted Frog (OSF), an endangered species found in only five locations in Southwestern British Columbia (OSF Recovery Team, 2019).

Amphibian reintroduction, such as the OSF reintroduction program, is a complex endeavor that requires a thorough understanding of multiple challenges, including disease management, habitat requirements, and the unique biology of the species (Griffiths, 2008; Harding et al., 2016; Donnelly et al., 2021). Additionally, post-release monitoring of amphibians' survival and health, including morphometrics and deformities, is essential to gauge the success of reintroduction efforts and to learn from any failures (Donnelly et al., 2021). The success of OSF reintroduction program heavily relies on the survival of the tadpoles released into the habitat (Kaye, 2008). However, monitoring these tadpoles after their release is challenging due to their cryptic nature, making it

difficult to track their survival in the wild. This underscores the need for effective, multi-faceted monitoring strategies to assess their survival post-release (Storfer, 2003; Donnelly et al., 2021).

The conservation of aquatic species in their native habitat is contingent upon the maintenance of water quality conditions suitable for the species. Water quality is widely recognized as one of the environmental factors contributing to amphibian population decline on a global scale (Blaustein et al., 1994; Alford and Richards, 1999). Temperature, pH, and dissolved solid conductivity are essential parameters of habitat suitability that determine the health status and possibility for survival of aquatic organisms (Pollard et al., 2017). Temperature and pH can affect the metabolic rate, leading to deviations in physiological processes; dissolved oxygen is a crucial element for respiration while conductivity could indicate the presence of harmful ionic compounds (Moreton and Marlatt, 2019). High-quality water chemistry data from release sites are therefore essential to assess the suitability of these habitats for supporting healthy populations (Ewen and Armstrong, 2007). Specifically, for amphibian reintroduction projects, biotic factors such as animal and plant communities must be targeted while abiotic factors—e.g., temperature, pH, dissolved oxygen, conductivity, and hardness—need to remain under control; different parameters (metals, pesticides, sewage, industrial wastewater) must also be monitored to facilitate species establishment (Jourdan et al., 2019).

Identifying habitat needs and preparing habitats so that they meet the particular requirements of reintroduced species, such as adequate food sources, shelter, breeding areas, and suitable water quality, are indispensable to examine before actual release (Cheyne, 2006; Stadtmann and Seddon, 2020). Site investigations may need to be conducted, or habitat restored or managed prior to implementing any reintroduction program (Helm, 2015). A final key consideration is an explicit recognition of the ecological and biological requirements needed for that species to survive (Mendelson et al., 2006). All amphibian species require different care, and a holistic strategy will not work universally (Ferrie et al., 2014). Each species will require an individualized reintroduction program, and this usually involves substantial amount of research with many trial-and-error attempts during the early reintroduction stages (Michaels et al., 2014).

Numerous studies have identified pollutants as a significant contributor to the global decline in amphibian populations (Collins and Storfer 2003; Kiesecker 2011; Brühl et al., 2013). A broad spectrum of these pollutants, such as pesticides, flame retardants, industrial chemicals, pharmaceuticals and metals, have been extensively studied for their adverse effects on various ecosystems (Edwards, 2002; Doyle et al., 2020; Rhind, 2009). For instance, various studies have reported significant effects of pesticides such as *organophosphates*, *chloropyridinyl, phosphonoglycines*, carbamates, and triazines, particularly on tadpole survival, growth and mobility across multiple species (Baker et al., 2013; Denoël et al., 2013; Islam and Malik, 2018). In addition, metal pollution is another widespread problem primarily caused by human activities, particularly in urban and industrial areas, including metal mines (Tchounwou et al., 2012; Jones, 2020). While certain metals such as Cu, Zn, Fe, Mo, Mn, Se, and Co are vital for animal biological processes in small quantities, their bioaccumulation, non-biodegradability, and potential health risks to aquatic life in metal-contaminated waters are concerning (Byrne et al., 2012; Taslima et al., 2022). There are environmental quality guidelines for some metals and pesticides, setting concentration limits in environmental matrices to protect ecosystems (e.g., Canadian water quality guidelines; Canadian Council of Ministers of the Environment (CCME 2007), which are mainly based on laboratory animal toxicity tests with individual metals or pesticides.

Recent studies have shown the effects of various pesticides on aquatic life, particularly focusing on vulnerable taxon such as amphibians (Schäfer et al., 2011; Kadiru el al., 2022). For example, a study evaluated the effects of cypermethrin, chlorpyrifos, endosulfan, glyphosate, and 2,4-Dichlorophenoxyacetic acid on the tadpoles of *Boana pulchella*, *Leptodactylus latrans*, *Rhinella fernandezae*, and *Rhinella arenarum*, from South America, using in situ enclosures in natural pond settings (Agostini et al., 2020). This study found that pesticide mixtures containing endosulfan (230.3 to 327.5 µg/L) or chlorpyrifos (176.9 and 256.6 µg/L) significantly reduced tadpole survival to less than 1% within 48 hours post-application. Similarly, mixtures with cypermethrin (45.6 to 413.9 µg/L) significantly decreased survival rates to around 10% in the same timeframe. Additionally, all tested pesticide combinations impaired tadpole mobility, indicating a broad spectrum of sublethal effects beyond mortality. Denoël et al., (2013) used video-tracking technology to evaluate the sublethal impacts of endosulfan on *Rana temporaria* tadpoles. Exposing tadpoles to 5 µg/L and 50 µg/L concentrations

of endosulfan for 48-h led to notable impairments in their locomotor behaviors. At the higher concentration, tadpoles exhibited reduced movement, swimming speed, and spatial usage, with similar, albeit milder, effects observed at the lower concentration. Notably, these behavioral changes were linked to decreased survival rates following long-term exposure. This research underscores how even low concentrations of endosulfan can significantly disrupt important behaviors in tadpoles, potentially affecting their survival and ecological roles.

The physiological effects of metal exposure on anuran amphibians have been well-documented in scientific literature as well, with several studies highlighting the specific mechanisms of harm. An example is how cadmium exposure disrupts the endocrine system of amphibians, leading to compromised reproduction and development (Lanctôt et al., 2024). For instance, Gross et al. (2007) reported that chronic cadmium exposure reduces survival and shifts growth across Northern Leopard Frog (*Rana pipiens*) tadpoles in a dose-dependent manner, with high concentrations (5.0 and 20.0 µg/L) markedly decreasing tadpole survival. Apart from cadmium, magnesium is a more prevalent metal that surpasses natural background levels regularly since magnesium chloride ($MgCl_2$) has become applied to the roadsides, particularly via de-icing practices (Hopkins et al., 2013). Lastly, Leggett et al. (2020) reported significant alterations of sex ratios of larval wood frogs (*Rana sylvatica*) in $MgCl_2$-exposed Cl^- concentrations of 200, 600, and 1000 mg/L, thus affecting population dynamics and overall species viability. They also added that the LC50 of larval wood frogs (*Rana sylvatica*) for magnesium chloride (7.11 g/L) is less acutely toxic compared to other de-icers. This information calls for stronger management of $MgCl_2$ applied in de-icing practices to avoid such effects.

Copper exposure has been linked to oxidative stress in amphibians, leading to cellular damage and eventually death in amphibians (Brix et al., 2022). Araújo et al., (2014) studied the effects of copper on three amphibian species from different climatic zones (a South American species, *Leptodactylus latrans*, a North American species, *Lithobates catesbeianus*, and a European species, *Pelophylax perezi*). They used a non-forced exposure system with a gradient of copper across seven compartments to allow organisms to move freely, assessing the tadpoles' ability to detect and avoid copper contamination (Araújo et al., 2014). Araújo et al. (2014) reported that all species avoided copper at concentrations as low as 100 µg/L. Avoidance was the sole factor for

population decline at the lowest (sublethal) concentrations (up to 200 µg/L), while at higher concentrations (>450 µg/L), mortality was observed. The median concentrations that caused mortality in the exposed animals were 93, 106, and 180 µg/L for *L. latrans*, *L. catesbeianus*, and *P. perezi*, respectively. A study by Aronzon et al. (2011) delved into the complex effects of copper toxicity on the Argentine Toad (*Rhinella arenarum*), revealing that susceptibility varies across different developmental stages. Their study assessed the impact of copper through continuous and 24-hour pulse treatments at 12 developmental endpoints, uncovering a stage-dependent vulnerability. Early stages, such as the blastula and gastrula, exhibit high resistance to copper, whereas the organogenic period (from the muscular response to the open mouth stage) showed significant susceptibility. Although larval stages generally displayed greater resistance, a clear stage-dependent susceptibility remained, highlighting the intricate nature of copper's toxic effects. The study also examined copper's teratogenic potential, documenting adverse developmental outcomes like reduced body size, malformations, and organ agenesis.

Research by Garcia-Munoz et al. (2009, 2010, 2011) observed the consequences of exposure to copper sulfate in a real-world scenario, detailing its adverse effects on growth, development, and behavioral changes among three Iberian amphibian species: *Bufo bufo*, *Epidalea calamita*, and *Pelodytes ibericus*. The LC50 values, which represent the concentration of copper sulfate at which 50% of the larvae are expected to die, were found to be 0.096 mg Cu/L for *B. bufo*, 0.113 mg Cu/L for *E. calamita*, and 0.125 mg Cu/L for *P. ibericus* (García-Muñoz et al., 2011). These values suggest that typical levels of copper in agricultural areas due to human activities may pose a risk to amphibians inhabiting wetlands near these activities. Regarding sublethal effects, larvae exposed to sublethal concentrations of copper sulfate exhibited significant behavioral changes (García-Muñoz et al., 2010). The number of stimuli needed to elicit activity increased, suggesting reduced sensitivity to external cues. Additionally, the distance moved after stimulation decreased, and abnormal movement patterns, including zigzagging or circling, were observed, implying changes in escape behavior efficiency (García-Muñoz et al., 2010). These behavioral changes could have ecological consequences in predator-prey relationships, affecting the larvae's ability to escape from predators. However, larvae transferred from contaminated water to clean water regained normal escape behavior within 96 hours, indicating that the effects of copper on behavior

can be reversible, at least partially (García-Muñoz et al., 2011). This demonstrates recovery potential in polluted scenarios if pollution sources are removed or mitigated.

Despite these studies there remains a substantial gap in our understanding of amphibians' susceptibility to various contaminants such as copper, specifically for endangered and understudied species such as OSF. These gaps underscore the need for further research on different amphibian species to elucidate how these pollutants impact them at different levels, from individual organisms to entire populations. Developing a deeper understanding of this susceptibility is crucial for implementing effective conservation strategies and mitigating the adverse effects of environmental pollutants on amphibian populations worldwide (Dutta, 2018). Protecting appropriate habitats for amphibians and monitoring these environments for contaminants is crucial for their conservation (Baldwin et al., 2009; Rowley et al., 2010). Additionally, assessing potential habitats for reintroduction and checking them for contamination and other threats is vital for the success of amphibian reintroduction efforts (Semlitsch, 2002; Griffiths and Pavajeau, 2008).

The embryonic survival of OSF in British Columbia, Canada, is significantly influenced by water quality parameters, particularly conductivity, chloride, and sulfate levels (McKibbin et al. 2008); embryonic survivorship also varies significantly among sites (McKibbin et al. 2008), with higher survivorship in areas with higher sulfate levels. This finding is consistent with the research by Beattie and Tyler-Jones (1992) and Leuven et al. (1986), which showed that water chemistry, including pH and ion concentration, can impact amphibian embryonic development and survival. Natural water chemistry conditions, particularly low chloride and conductivity, may be contributing factors to the low embryonic survivorship observed in OSF populations at one site, MD Aldergrove, BC (McKibbin et al., 2008).

The objectives of this study were to assess the sensitivity of early life stage OSF to: 1) changes in water quality expected between reintroduction site waters versus captive breeding facilities; and 2) to copper, a common environmental pollutant. To accomplish the first objective a laboratory study was performed to compare OSF early life stage survival, growth, and development when reared in reintroduction site water versus captive breeding facility waters. This first study entailed rearing egg masses collected from the GVZ under three different conditions in the laboratory setting until

metamorphosis: reintroduction site water (R); GVZ water (Z); and GVZ water until Gosner stage 25-26 followed by transfer to reintroduction site water (Z-R). To further understand reintroduction site water quality characteristics and potential pollutants present, a chemical analysis of reintroduction site water and GVZ water was performed. For the second objective to assess the sensitivity of OSF early life stages to copper, I conducted a laboratory waterborne acute toxicity test exposing OSF tadpoles to range of copper concentrations and recording the impact on survival.

3.3. Material and Methods

3.3.1. Animal Collection and Culture

All manipulations of animals in this experiment were permitted under the Simon Fraser University (SFU) Animal Care Committee Animal Care Protocol #1362B-23.

Three Oregon spotted frog (OSF) egg masses (at Gosner stages (GS) 15-16 (Harrison 1969)) were randomly collected from the captive breeding program managed by Wildlife Preservation Canada at the Greater Vancouver Zoo (Langley, British Columbia, Canada) and transported in pond water at a temperature of 12°C ± 1°C (the same temperature as the pond water where the egg masses were collected) to Simon Fraser University within two hours of collection.

3.3.2. Health Assessment of OSF Embryos and Tadpoles in Reared in Zoo Versus Reintroduction Site Waters

Upon arrival at SFU, the egg masses were placed in a bucket containing water from the zoo, which was gently aerated and maintained under a photoperiod of 12 hours light and 12 hours dark for 48 hours for acclimation. A 100% water renewal was done, and the egg masses were then transferred to 10-liter glass aquaria for the experimental treatments. The three egg masses were pulled apart in about eight clusters and half of the clusters were placed in zoo water and the other half were placed in site water in 10 L (30 cm × 15 cm × 20.5 cm) glass tanks. The water quality, including conductivity, dissolved oxygen, pH, and temperature, was monitored daily using an HQd portable meter from Hach Company in Loveland, CO until hatching. All the embryos hatched within seven to eight days after being transferred to the glass aquaria.

The experimental design and exposures were based on Test No. 231: The Amphibian LAGDA Assay (OECD 2009) with the following modifications to tailor the study to the unique characteristics of OSF, in contrast to *Xenopus spp*. The duration of exposure was extended to 103 days, as opposed to the 21 days, to capture the more protracted developmental stages in OSF. The water quality parameters were maintained to mimic rearing conditions employed by the GVZ OSF captive breeding and reintroduction program, where animals are reared in outdoor aquaria subjected to natural environmental conditions. To achieve this, the temperature in the experimental setup was adjusted to mirror local temperature fluctuations, which resulted in a gradual increase in temperature as the experiment progressed to mimic summer conditions.

After eggs hatched, ten tadpoles were seeded in five replicate tanks for each treatment at a loading density of 0.8 g/L of tadpoles at water temperatures of 12°C ± 2 according to the American Society for Testing and Materials Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (E 729–96,1996) in the one of the following three treatments. These treatments are consisted of: (*i*) *Reintroduction site water*, whereby egg masses were placed in water sourced from the reintroduction site (a natural wetland located near Mission, BC where tadpoles at 25-26 GS are released at after rearing at the GVZ; (*ii*) *GVZ water*, whereby egg masses were placed in water from the GVZ, where a successful captive breeding program has been conducted for ~12 years high with a high tadpole survival rate (i.e., over 90% (personal communication with Andrea Gielens)); (*iii*) *Zoo to reintroduction site (Z-R):* egg masses experienced a dynamic exposure regimen, initially placed in GVZ water to mimic captive-bred OSF tadpole conditions, then at GS 25-26 (swimming tadpoles) water was gradually changed over 40 minutes to site water, simulating their release into their natural habitat. This transition involved slowly adding site water to the tadpoles' existing zoo water, mimicking the acclimation process used during field releases.

Exposed sides of the tanks were covered with black plastic to shield tadpoles from visual disturbances. Tanks were monitored three times a day (once in the morning, twice in the afternoon) for deformities, changes in color, signs of infection, and mortality, and tadpoles were immediately removed upon detection of mortality. Water renewals of 80% were performed every 48 hours on all tanks and tadpoles were fed with a mixture of boiled kale and lettuce that was prepared by the captive breeding program at GVZ. A 12-

h light: 12-h dark photoperiod was maintained throughout the experiment. Water quality (conductivity, dissolved oxygen, pH, and temperature) was monitored daily using a HQd portable meter in one of five replicate tanks for each treatment and the water control throughout the exposure period. Total ammonia levels were measured weekly in one replicates of each treatment using Seachem MultiTest Total Ammonia Test Kit (Seachem Laboratories, Madison, USA; detection limit 0.05 mg/L), then based on the pH and temperature of the replicate unionized ammonia was calculated.

After the 103-day exposure period tadpoles were euthanized by immersion in 0.4 g/L of tricaine methanesulfonate buffered with sodium bicarbonate to pH 7.0–7.5. Total length (mm), snout-vent-length (SVL, mm), wet weight (g), and deformities were recorded including evidence of cannibalism (missing tail tips). Deformities were assessed using a scale adapted from one originally designed for fish (Holm et al., 2005). The evaluation, conducted under 10x magnification with a Fisherbrand™ Basic Stereo Zoom Microscope, categorized deformities into four types: edema, craniofacial, skeletal, and limb deformities. The severity of each deformity was rated on a scale from 0 (indicating no deformity), 1 (less than 30% change), 2 (30-50% change), to 3 (severe deformity with more than 50% observable difference compared to unaffected individuals) (Holm et al., 2005). Edemas were identified by fluid-filled sacs, typically located in the abdomen or under the eyes (Holm et al., 2005). Craniofacial deformities encompass abnormalities in the head, jaw, and eyes, such as reduction or protrusion (Holm et al., 2005). Limb deformities were noted for significant malformations of joints and reductions in limb segments (micromelia; Holm et al., 2005). Skeletal deformities included conditions like lordosis (inward curvature of the spine), kyphosis (outward curvature of the spine), scoliosis (lateral curvature of the spine), and alterations affecting swimming and movement capabilities (e.g., swimming in circle or upside down; Holm et al., 2005).

3.3.3. Reintroduction Site Water Quality and Chemistry

Water quality and potential contaminants at the OSF tadpole reintroduction site located near Mission, BC was measured from two samples, and these were compared to the water used for rearing at the Greater Vancouver Zoo. On April 24 and May 31, 2023, water and sediment samples were collected from the reintroduction site and a suite of metals were measured from surface water and sediment. In addition, pesticides were measured in water samples collected on May 31, 2023.

All water and sediment sample collection vials and preservatives were supplied and by ALS Environmental (Burnaby, BC). For analyzing pesticides using LC/MS/MS samples were collected in either 60 mL HDPE (High-density polyethylene) bottles with no preservation or 100 mL amber glass bottles with sodium thiosulfate and kept at ten °C or less. Organochlorine pesticides were prepared in two 40 mL glass vials, and samples were kept at ≤10°C. Neonicotinoids were collected in 250 mL amber glass bottles without preservatives. Semi-volatile organics were collected in either two 100 mL or two 500 mL amber glass bottles for routine and low detection levels, respectively, with no preservatives and kept at ≤10°C. For metals analysis in sediment samples, 120 mL glass jars were used, and the samples were chilled to 10°C or less. To analyse general parameters such as acidity, alkalinity, anions, and metals water samples were collected in a 250 mL HDPE bottle, with anions requiring a 60 mL bottle. For total suspended solids (TSS) and total dissolved solids (TDS) analyses 500 mL and 145 mL water samples were collected in HDPE bottles, respectively. For Biochemical Oxygen Demand (BOD) tests, a 500 mL HDPE bottle was used. To test chlorate, bromate, and chlorite, a 60 mL UV-inhibited HDPE bottle with ethylenediamine (EDA) preservative was used. Nutrients were collected in a 100 mL amber glass bottle with sulfuric acid preservative, while sulphide tests were collected in a 60 mL HDPE bottle with zinc acetate and sodium hydroxide preservatives. Cyanate required a 250 mL HDPE bottle with sodium hydroxide preservative, and cyanide required a 60 mL opaque HDPE bottle with the same preservative. For Thiocyanate testing, a 60 mL HDPE bottle with nitric acid preservative was used. Metals were collected in a 60 mL HDPE bottle with nitric acid preservative, while mercury was collected in a 40 mL glass bottle with hydrochloric acid preservative. Methyl mercury testing required a 250 mL amber glass bottle with either hydrochloric acid or sulfuric acid preservative, depending on the water source.

All water and sediment samples collected were shipped on ice to ALS Environmental (Burnaby, BC) for analysis of metals (both total and dissolved) and pesticides, as listed in table A1. Surface Water and sediment contaminant concentrations were compared to Canadian water quality guidelines (the Canadian Council of Ministers of the Environment (CCME) freshwater acute or chronic WQG and BC WQG) when available, or USEPA (United States Environmental Protection Agency) guidelines if no Canadian water quality guidelines were available. If the detection limit for an analyte exceeded WQGs it was noted in table A1.

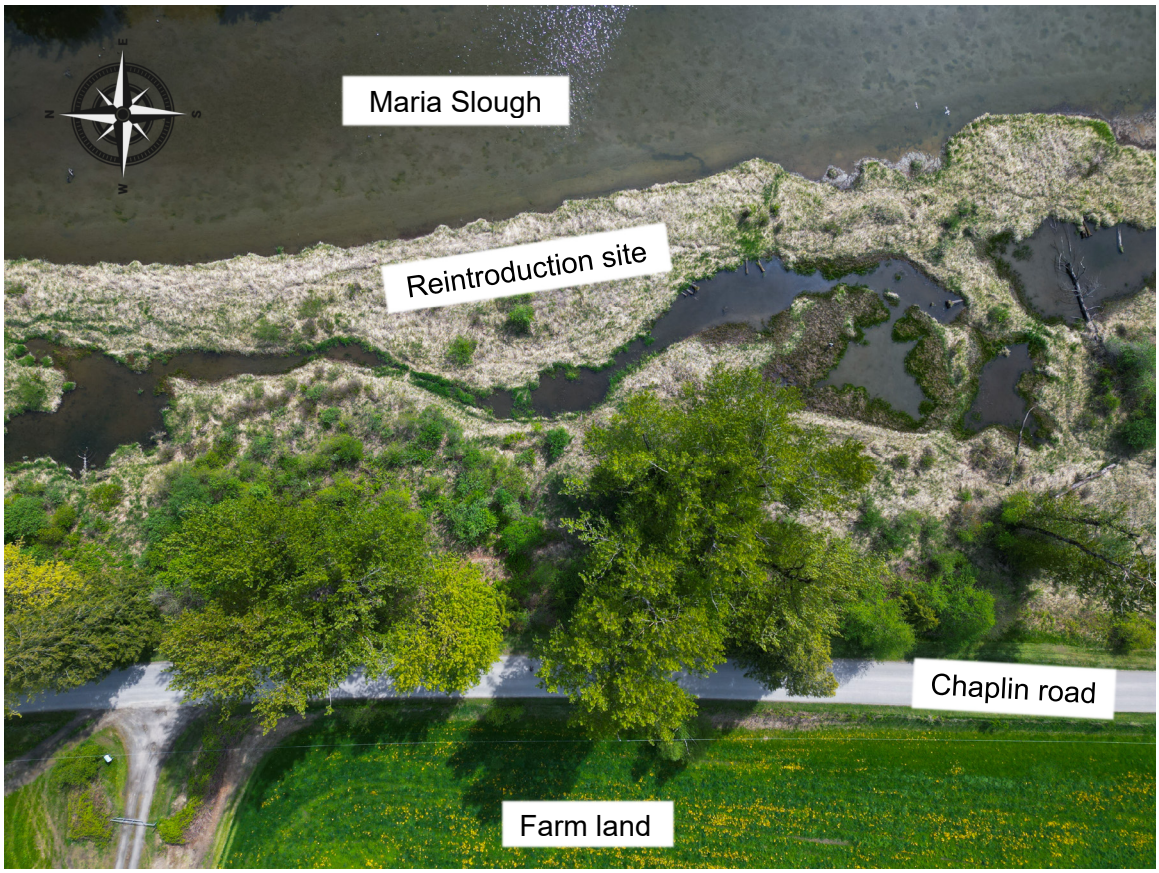

Figure 3.1 **Arial photo of the Oregon Spotted Frog (*Rana pretiosa*) reintroduction site near Mission, BC.**

3.3.4. 96-hour Acute Exposure of Oregon Spotted Frog Tadpoles to Copper

The experimental design and exposures for this test were adapted from Test No. 231: The Amphibian Metamorphosis Assay (OECD 2009), with modifications to use OSF tadpoles and terminate the study at 96 hours instead of 21 days. In the 96-hour acute exposure study, tadpoles at Gosner stages 25-26 from two distinct laboratory rearing conditions were used: one group was reared in GVZ water; and, a second group was reared in water from the OSF reintroduction site near Mission, BC.

For each rearing condition, a total of 60 tadpoles were used, resulting in 120 tadpoles across both rearing conditions. Tadpoles from each rearing condition were randomly assigned to one of five different waterborne copper treatments. These treatments included a dechlorinated municipal water control group and four groups with copper concentrations of 1, 10, 100, or 1000 µg/L, all dissolved in dechlorinated municipal water. Each treatment group consisted of four replicate glass beakers, with each beaker containing three tadpoles, for a total of 12 tadpoles per treatment for each rearing condition. This experimental setup was conducted separately for each water rearing background.

Copper concentrations were made from Copper (II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) which has a water solubility is 320 g/L (20ºC). At the beginning of the experiment, a stock solution of copper (II) sulphate pentahydrate with a concentration of 40,000 mg/L was prepared using salts with a purity of approximately 99% (CAS# 7758-99-8, Fisher Scientific, Hampton, New Hampshire, USA) and dechlorinated municipal water. The stock solution was stored at 4ºC in the dark throughout the experiment. All test concentrations added to the exposure vessels were prepared by serial dilutions from this stock solution. Water samples were collected from one replicate per treatment, including the controls, to verify the dissolved copper concentration, water hardness ($CaCO_3$), and dissolved organic carbon (DOC) content. The collected samples were filtered using a 0.45 µm glass syringe filter and sent to ALS Environmental (Burnaby, BC) to be measured using inductively coupled plasma mass spectrometry (ICP-MS)

(EPA, 1998; Martin et al., 1994). Table 3.6. shows the nominal versus measured concentrations of copper ions in treatment beakers.

Tadpoles were monitored twice daily for abnormal behaviour, changes in colour and signs of infection and mortality and were immediately removed upon identification. Water quality (conductivity, dissolved oxygen, pH, and temperature) was monitored daily using a HQd portable meter in one of five replicate beakers for each treatment and the water control throughout the acute (96 h). After 96 hours of exposure, the larvae were euthanized in an overdose of Tricaine mesylate (MS-222) (0.5 g/L buffered to pH = 7 with $NaHCO_3$; Canadian Council on Animal Care, 2021). Developmental stage was determined using a stereo microscope and the Gosner stage system (Gosner, 1960), and measurements were taken for total length (TL, mm), snout-vent length (SVL, mm), wet weight (WW, g), and any deformities were recorded, including evidence of cannibalism.

3.3.5. Statistical Analysis

All statistical analyses were conducted using RStudio version 2023.06.1+524 (RStudio Team, 2023). Outliers were detected using Tukey's method (Inter-quartile Range), if the data points were below Q (Quartile) 1 - 1.5 IQR or above Q3 + 1.5 IQR, they were removed from the dataset (Sullivan et al., 2021). Data was tested for normality using a Shapiro-Wilk test and for homogeneity of variance using Levene's test, based on Zuur et al. (2009) guidelines. For data that passed these two tests and thus was met the criteria for parametric analyses, a one-way analysis of variance (ANOVA; $p<0.05$) was performed. This was followed by a Tukey's HSD post-hoc test to determine if there were significant differences between rearing water treatments. If the dataset was not normally distributed and failed Levene's test, Log10 or arcsine transformations were applied, and the parameters were retested. When the assumptions required for parametric tests were not met by data transformations, a Kruskal-Wallis test was applied ($p < 0.05$). This was followed by Dunn's post-hoc test with Bonferroni correction for total length (TL, mm), snout-vent length (SVL, mm), and wet weight (WW, g). The Bonferroni correction was applied to account for multiple comparisons across the different treatment groups. Each measurement type (TL, SVL, WW) constituted a separate family, with pairwise comparisons made among the treatments. The significance threshold was adjusted by dividing the alpha level by the number of comparisons within each family to control the

risk of type I errors effectively. The same statistical test pipeline was used to determine the effects of copper treatments on survival. To compare the GVZ and reintroduction site reared tadpoles in the copper exposure a two-sample t-test or a Wilcoxon rank sum test was used.

A Kaplan-Meier survival analysis was conducted to assess metamorphosis and survival data. This was followed by a Log Rank Test and a pairwise Log Rank Test with Bonferroni correction to evaluate differences in event probabilities over time. The analyses were performed using the 'survival' package (Therneau, 2021; Therneau and Grambsch, 2000). I also employed the Cox Proportional Hazards Model to compare the risk of mortality and metamorphosis between treatments. The hazard ratios, along with their corresponding z-values, p-values, and 95% confidence intervals, were calculated to determine the significance of the differences observed.

To test the effect of rearing water treatment on the frequency of deformities on tadpoles, we applied a pairwise Fisher's exact test with Bonferroni correction on the categorical data ranging from 0 (no observation of deformities) and 3 (observation of severe deformities). Packages used for building plots included ‘ggplot2’ (Wickham, 2016), ‘ggpubr’ (Kassambara, 2021), ‘surveminer’ (Kassambara et al., 2021) and ‘cowplot’ (Wilke, 2020).

Concentration–response curves and estimated LC50s were created with the dose-response model function (drm()) in R by using the “fct” argument to fit 2, 3, 4 and 5 parameters logistic models using the dissolved concentrations as measured concentrations were different than the nominal concentrations. The models were then compared by calculating the AIC to find the best fitted model.

3.4. Results

3.4.1. Rearing in Zoo Versus Reintroduction Site Water Impacts OSF Embryonic and Tadpole Health

Water Quality in Reintroduction Site Versus Zoo Water

The measured water quality parameters during the 103-day chronic exposure test were generally similar across the three rearing water source treatments and within

the range specified by the OECD Test No. 241: LAGDA, including temperature, pH, dissolved oxygen, and conductivity (table 3.1).

The weekly measurements of unionized ammonia in the GVZ and Z-R treatments were 64.05 ± 74.02 ug/L and 23.40 ± 14.78 ug/L, respectively. Both these values were above the long-term freshwater (CCME) water quality guidelines for protecting aquatic life (19 ug/L). In the GVZ treatment, the levels of unionized ammonia reached as high as 217.93 ug/ml in one week. On the other hand, the mean (± standard deviation) unionized ammonia in the reintroduction site water treatment was below the guideline value at 15.44 ± 11.35 ug/L, for the majority of the experiment (Figure 3.2). However, no mortality or other adverse effects were observed during periods of elevated ammonia levels in any treatment.

Growth

Two data points were identified as outliers for total body length, one for snout-vent length, and three for wet weight. None of GVZ, Z-R, and reintroduction site chronic exposure treatments had observable effect on the mean total body length, which ranged from 41.35 to 69.61 mm ($F_{2, 12} = 0.184$, $p = 0.835$; Figure 3.3 A). Similarly, there was no significant difference in mean snout-vent length, which ranged from 18.04 to 25.95 mm ($F_{2, 12} = 1.014$, $p = 0.392$; Figure 3.3 B). Finally, the mean wet weight (range from 1.26 to 3.46 g) also showed no significant difference among treatments ($F_{2, 12} = 1.628$, $p = 0.237$; Figure 3.4 C).

Survival

All eggs hatched in all the replicates across the three treatments. However, a significant decrease in tadpole survival was observed in the Z-R treatment, the treatment that simulated the typical captive breeding program protocol whereby egg masses are reared in GVZ water followed by release at the reintroduction site at GS 25-26. However, when comparing GVZ treatment (solely reared from the egg stage to metamorphosis in GVZ water) with the reintroduction site treatment (solely reared from the egg stage to metamorphosis in reintroduction site water) no significant reductions in survival were observed. Together, this indicates higher survival when no transfer into a different water source is performed. Specifically, survival of OSF in the Z-R treatment significantly

decreased to 50% (CI = 38%–66%) over the course the experiment compared to a survival of 88% (95% CI = 79%–97%) in the GVZ and 78% (95% CI = 67%-90%) in the reintroduction site treatments. Most mortalities (~ 40 %) in the Z-R treatment occurred after day 72 (when metamorphosis started). Essentially, these results indicate a significant difference in survival between GVZ and reintroduction site with Z-R, but no significant difference between GVZ and reintroduction site (Log-Rank test followed by pairwise Log-Rank Test, $p < 0.05$). The OSF raised in the Z-R treatment were approximately 5 times more likely to experience mortality during the experiment than in the GVZ treatment (Cox Proportional Hazard Ratio = 5.403, z = 3.700, p= 0.000216, 95% CI = 2.2108–13.205). In contrast, when comparing the reintroduction site treatment to the GVZ treatment, there is not enough evidence to conclude a significant difference in mortality risk between these two treatments (Cox Proportional Hazard Ratio = 2.075, z = 1.438, p = 0.150346, 95% CI = 0.7674–5.612; Figure 3.4.).

Forelimb Emergence

The different water rearing treatments did not significantly affect forelimb emergence, nor achieving Gosner stage 42 in OSF (Log-Rank test followed by pairwise Log-Rank Tests P= 0.11). By day 97, all surviving tadpoles in the Z-R treatment had reached forelimb emergence. However, at this point, only 93.2% (95% CI = 80%-97.7%) in the GVZ treatment and 87% (95% CI = 71%-94.3%) in the reintroduction site treatment had reached this stage. By day 103, all surviving tadpoles in both GVZ and reintroduction site treatments reached forelimb emergence. The hazard ratio of Z-R treatment compared to GVZ treatment indicates a marginal and non-significant reduction in reaching forelimb emergence (Cox Proportional Hazard Ratio = 0.9218, z = -0.320, p = 0.7487, 95% CI = 0.56–1.51). In addition, comparing reintroduction site to GVZ treatment shows a potential decrease in reaching forelimb emergence in the reintroduction site treatment (Cox Proportional Hazard Ratio = 0.6484, z = -1.955, p = 0.0506, 95% CI = 0.42–1.001; Figure 3.5.).

Deformities

There were no significant differences between treatments for skeletal, craniofacial, and edema presence (Figure 3.6.). Regarding skeletal changes, pairwise comparisons indicated no significant differences between GVZ and reintroduction site

treatments (p = 0.99), GVZ and Z-R treatments (p = 0.64), and reintroduction site and Z-R treatments (p = 0.37). Similarly, for craniofacial, there were no significant differences observed between zoo and site treatments (p = 0.38), GVZ and Z-R treatments (p = 0.71), and reintroduction site and Z-R treatments (p = 0.99). Lastly, for edema presence, comparisons also indicated no significant differences, with all comparisons showing a p of 0.99. During the observations conducted at the GVZ treatment and reintroduction site treatment, no instances of cannibalism were recorded. However, in two of the Z-R treatment replicates, there were signs of cannibalism. Three incidents were recorded in one replicate, while another recorded two.

3.4.2. Reintroduction Site Water Quality and Chemistry

During the April 23 sampling event, water temperature was 12.4°C, pH was 7.14, dissolved oxygen was 9.79 mg/L, conductivity was 147.6 µS/cm, hardness was 71 mg/L as CaCO3, and the air temperature was 14°C. At the second sampling event on May 31, 2023, from 11:30 to 13:30, water temperature increased to 17°C, pH increased to 7.26, dissolved oxygen decreased to 8.83 mg/L, conductivity increased to 195.5 µS/cm, hardness increased to 97.6 mg/L as CaCO3, and the air temperature increased to 18°C (Table 3.2.). Surface water at the reintroduction site contained total and dissolved magnesium (Mg) concentrations exceeding the recommended US EPA guideline value of 0. 647 mg/L on both sampling dates. Total copper (Cu) exceeded the guideline values set by the CCME and BC Ambient Water Quality Guidelines (WQGs) on both sampling dates, while total iron (Fe) also surpassed CCME guideline values in the sample collected on May 31, 2023 (see Table 3.3. and Table 3.4.). All surface water concentrations of organochlorines, organophosphates, and neonicotinoids at the reintroduction site were below the detection limits reported by ALS Environmental (see Table A.1.). Where guideline values for pesticides exist, the analytical detection limits remained below these guidelines. In sediment samples collected on April 23, 2023, iron, nickel, and selenium exceeded CCME sediment guidelines. On both sampling dates, April 23, 2023, and May 31, 2023, manganese and uranium levels were above the CCME sediment guidelines (see Table 3.5. and Table A.1.).

3.4.3. 96-hour Acute Exposure to Copper

Water Quality

During the 96-hour experiment, the following ranges were observed for water quality parameters: dissolved oxygen levels, 9.60 to 10.81 mg/L; pH, 7.14 to 7.99; conductivity, 40.90 to 122.70 µS/cm; and water temperature, 11.30 to 15.50 °C (Table 3.6.). The mean (± standard deviation) hardness was 21.17 ± 0.79, and the mean (± standard deviation) dissolved organic carbon was 1.85 ± 0.15. All of these parameters were within the range of the LAGDA guidelines (OECD, 2015), except for the water temperature, which was adjusted to accommodate the OSF tadpoles at GS 25-26.

Survival

In the experimental setup, OSF tadpoles at GS 25-26 were sourced from two distinct rearing conditions. One group of tadpoles was reared in water from GVZ, while the other group was reared in water from the reintroduction site near Mission, BC. Acute copper exposure did not significantly affect survival up to 1 ug/L in either rearing water treatment, but caused significant mortality at higher concentrations across rearing treatments. Copper at 5 ug/L resulted in 41% (95% CI = 15.1 – 68.2) survival of the tadpoles reared in GVZ water and 75% (95% CI = 48.5–102) survival in the tadpoles reared in reintroduction site water, which is marginally significant ($p = 0.057$), but there was no significant difference in survival between the control group and the five ug/L concentration for either ($p = 0.62$ and $p = 0.99$, respectively). However, all tadpoles in the 50 ug/L and 620 ug/L concentrations died within 6 to 12 hours after exposure, and this level of survival is lower than both the control and 5 ug/L copper treatments (Figure 3.7). The AIC values for the 2, 3, 4, and 5 parameter logistic models were 37.7, 41.79, 45.79, and 49.79, respectively. Based on these values, the two-parameter logistic model, which had the lowest AIC, was used to calculate the LC50. The LC50 values for tadpoles reared in site and zoo water were 5.93 µg/L and 4.76 µg/L, respectively (Figure 3.8).

Table 3.1 **Water quality measurements (mean ± SD) across different treatments in a 103-day study of Oregon Spotted Frog (*Rana pretiosa*) exposed to different water types: Greater Vancouver Zoo (GVZ); Zoo to Reintorduction Site (Z-R); and reintroduction site. Measurements include temperature, pH, dissolved oxygen, conductivity, and unionized ammonia levels. For unionized ammonia, values were obtained weekly, while other parameters were monitored daily.**

GV Zoo Treatment	*N*	Mean	Min	Max	SD
Temp (°C)	152	17.57	12	22.6	3.26
pH	152	8.47	7.96	8.86	0.18
Dissolved oxygen (mg/L)	152	8.46	1.61	28.4	2.56
Conductivity (µS/cm)	152	354.71	261	402	23.42
Unionized Ammonia (µg/L)	11	64.05	11.01	217.93	74.02
Z-R Treatment					
Temp (°C)	143	17.50	12	23.7	3.39
pH	143	8.21	6.66	8.76	0.28
Dissolved oxygen (mg/L)	143	8.47	1.60	10.29	1.47
Conductivity (µS/cm)	143	239.97	138	370	62.75
Unionized Ammonia (µg/L)	10	23.40	8.82	48.95	14.78
Reintroduction Site Treatment					
Temp (°C)	158	17.74	12	22.7	3.29
pH	158	8.17	7.33	8.48	0.20
Dissolved oxygen (mg/L)	158	8.36	2.11	10.27	1.40
Conductivity (µS/cm)	158	221.15	139.1	370	52.55
Unionized Ammonia (µg/L)	11	15.44	4.81	41.74	11.35

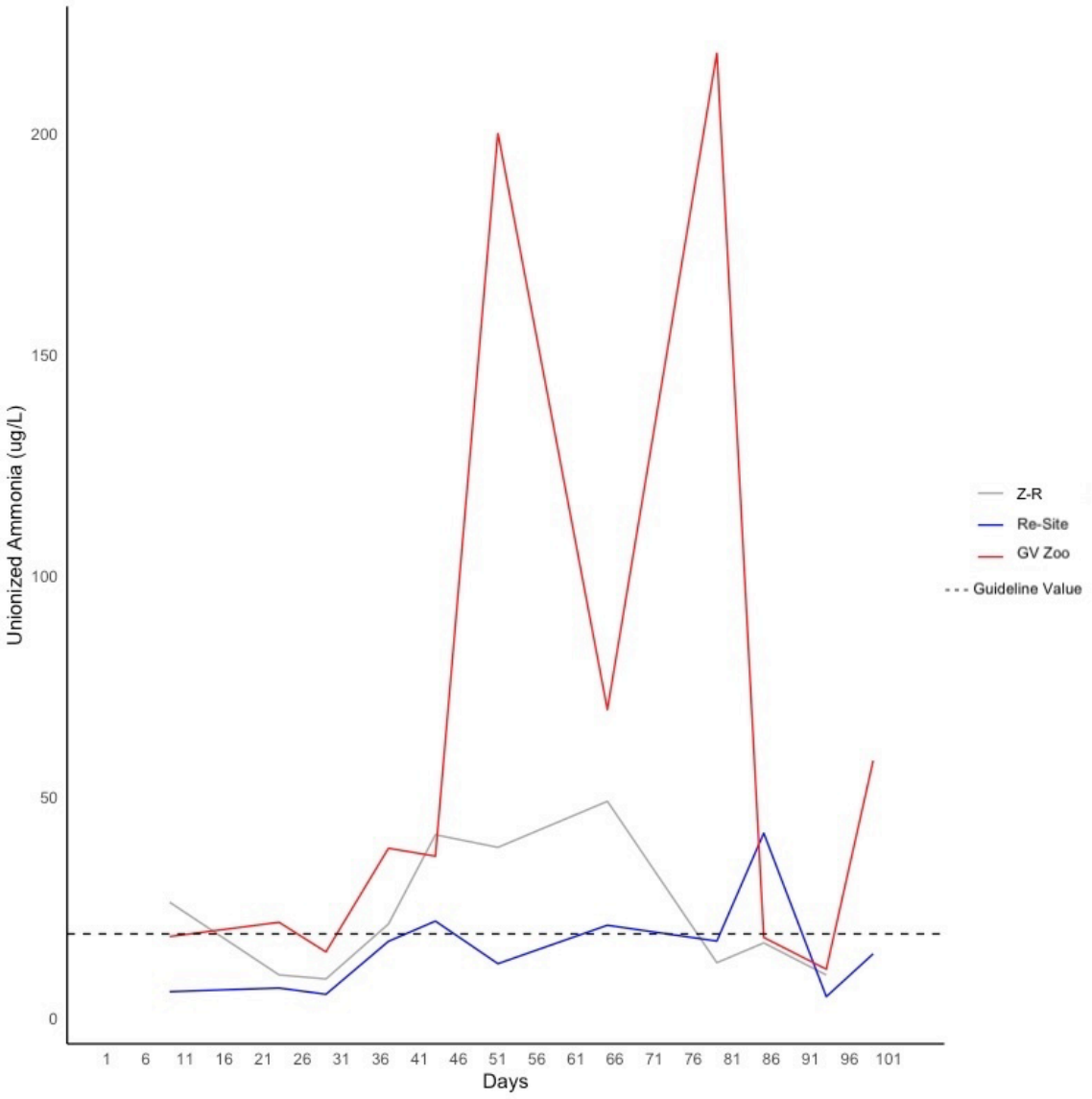

Figure 3.2. **Unionized ammonia concentration (µg/L) during Oregon Spotted Frog (*Rana pretiosa*) experiment exposed to three different water types: Greater Vancouver Zoo (GVZ); Zoo to Reintorduction Site (Z-R); and reintroduction site. Five replicates per each treatment and unionized ammonia was measured in one replicate per treatment once per week over this 103-day exposure. The black dashed line indicates the unionized ammonia long-term freshwater CCME water quality guideline for the protection of aquatic life of 19 µg/L.**

Table 3.2 Water quality parameters and corresponding air temperature at the Oregon Spotted Frog (*Rana pretiosa*) reintroduction site on April 24, 2023 and May 31, 2023.

Date	Time	Water Temp (°C)	pH	Dissolved oxygen (mg/L)	Conductivity (µS/cm)	Hardness (CaCo3 mg/L)	Air Temp (°C)
April 24, 2023	11:00–12:30	12.4	7.14	9.79	147.6	71	14
May 31, 2023	11:30-13:30	17	7.26	8.83	195.5	97.6	18

Table 3.3 Disolved metal concentrations measured in reintroduction water for the Oregon Spotted Frog (*Rana pretiosa*) in British Columbia on April 24 and May 31, 2023 that exceeded water quality guidelines. The Canadian Council of Ministers of the Environment (CCME) Water Quality Guidelines (WQG) and British Columbia (BC) Ambient Water Quality Guidelines (WQG) for protection of aquatic life are presented for comparison

Metal	Sample Collection Date**	Detection Limit	Measured (mg/L)	CCME WQG (mg/L)	BC Ambient WQG (mg/L)
Copper	31-May-23	0.0005	0.00112	0.00232	0.000814
	24-Apr-23		0.00086	0.002	0.000653
Iron	31-May-23	0.01	0.586	0.3	1
Magnesium	31-May-23	0.005	6.23	-	0.647E
	24-Apr-23		5.55	-	

[E] US EPA surface water screening benchmarks

Table 3.4 Disolved metal concentrations measured in reintroduction water for the Oregon Spotted Frog (*Rana pretiosa*) in British Columbia on April 24 and May 31, 2023 that exceeded either the Canadian Council of Ministers of the Environment (CCME) Water Quality Guidelines (WQG) or British Columbia (BC) Ambient Water Quality Guidelines (WQG) for protection of aquatic life.

Metal	Sample Collection Date**	Detection Limit	Measured (mg/L)	CCME WQG (mg/L)	BC Ambient WQG (mg/L)
Magnesium	31-May-23	0.005	6.37		0.647 [E]
	24-Apr-23		5.91		

Table 3.5 Sediment metal concentrations measured at the reintroduction site of the Oregon Spotted Frog (*Rana pretiosa*) on April 24 and May 31, 2023 that exceeded the established soil and sediment quality guidelines established by the Canadian Council of Ministers of the Environment (CCME) probable effect levels (mg/kg), the Canadian Soil Quality Guidelines (CSR) generic numerical sediment standards (µg/g), and British Columbia (BC) Ambient Soil Quality Guidelines (SQG; mg/kg).

Metal	Sample Collection Date	Detection Limit	Measured (mg/kg)	CCME probable effect levees (mg/kg)	CSR generic numerical sediment standards (ug/g)	BC Ambient SQG (mg/kg)
Iron	31-May-23	50	22700	20000[E]		43800
	24-Apr-23		5.91			
	24-Apr-23		260			
Nickel	24-Apr-23	0.5	55.4	37.9[R]		
Selenium	24-Apr-23	0.2	3.76	1		1
Uranium	31-May-23	0.05	0.542	0.015		0.015
	24-Apr-23		1.5			0.015

[E] US EPA surface water screening benchmarks; [R]ARCS PEC sediment screening benchmark

Table 3.6 Water quality parameters and copper concentration measurements across different treatments during Oregon Spotted Frog (Rana pretiosa) 96-h of exposure subjected to different rearing conditions: Greater Vancouver Zoo and reintroduction site. Parameters include temperature, pH, dissolved oxygen, conductivity, hardness, dissolved organic carbon, and copper concentrations. Measurements are presented as mean ± standard deviation (SD), where SD quantifies the variation or dispersion from the mean. Sample size (N) indicates the number of observations used for each parameter.

Parameters	*N*	Mean	Min	Max	SD
Temp (°C)	50	13.68	11.30	15.50	1.45
pH	50	7.63	7.14	7.99	0.21
Dissolved oxygen (mg/L)	50	10.05	9.60	10.81	0.35
Conductivity (µS/cm)	50	53.35	40.90	122.70	10.99
Hardness (CaCo3, mg/L)	10	21.17	20	22	0.79
Dissolved Organic Carbon (mg/L)	10	1.85	1.48	2.28	0.15
Copper concentrations					
Nominal (ug/L)		1	10	100	1000
Measured Dissolved (ug/L)		1	5	50	620

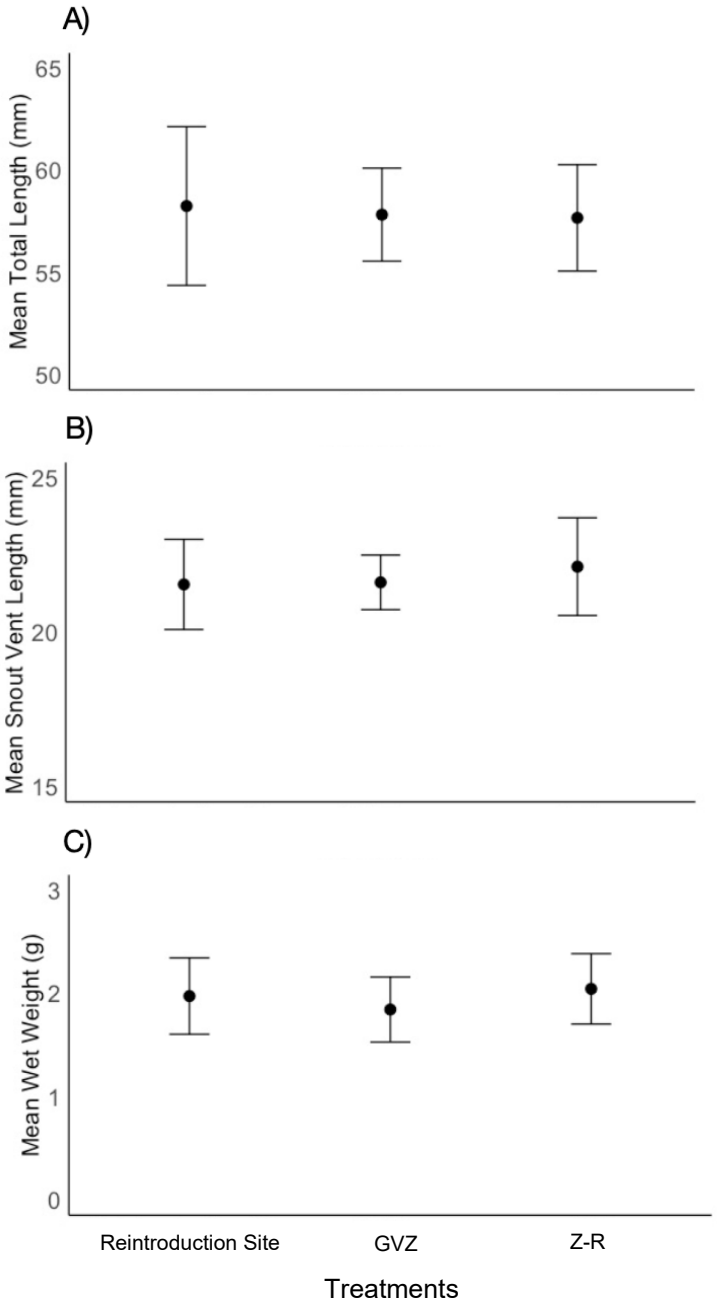

Figure 3.3 **Mean (A) total length, (B) snout-to-vent length and (C) wet weight of Oregon spotted frog (*Rana pretiosa*) at metamorphosis (GS 42) after 103-day exposure to various rearing conditions: Greater Vancouver Zoo (GVZ); Zoo to Reintorduction Site (Z-R); and Rreintroduction Ssite. Means and 95% confident intervals are presented. No signficant differences were observed (one-way ANOVA, p>0.05).**

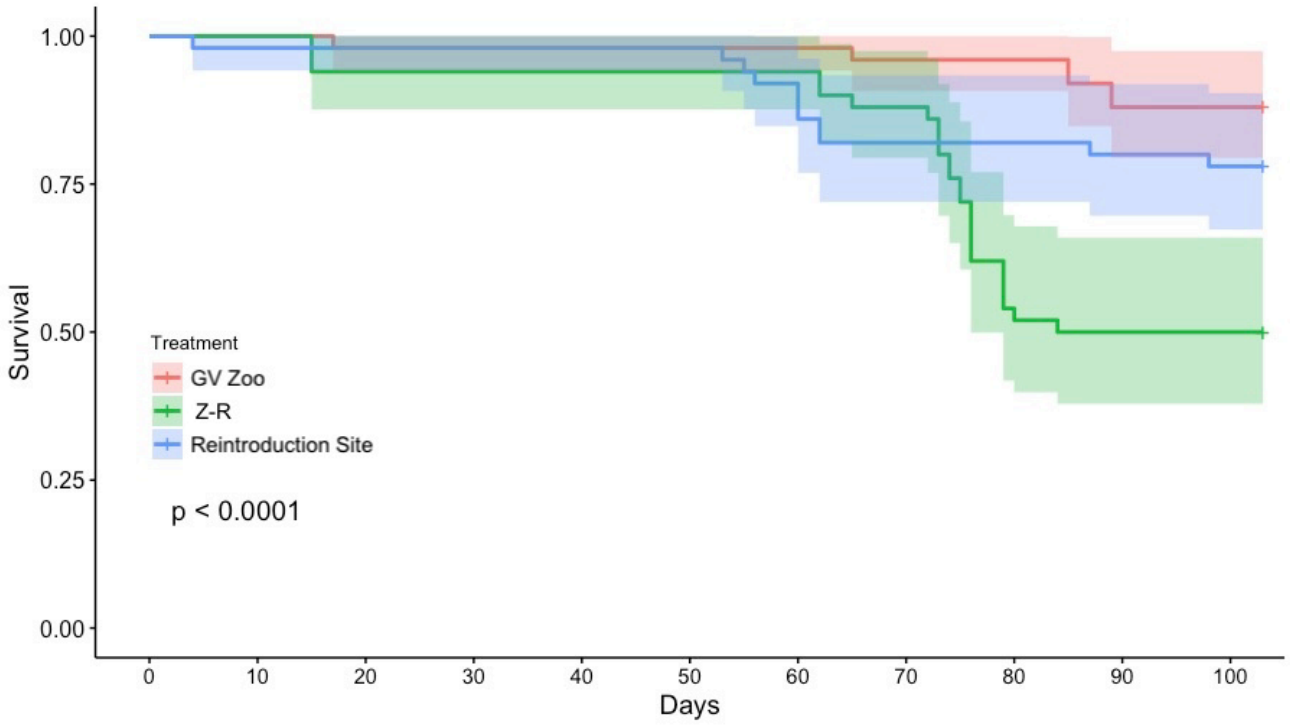

Figure 3.4 **Kaplan Meier survival curves representing the survival probability (%) of Oregon spotted frog (*Rana pretiosa*) exposed to various rearing water treatments: Greater Vancouver Zoo (GVZ); Zoo to Reintorduction Site (Z-R); and reintroduction site. Solid lines represents mean survival of 5 replicates per treatment, and shaded areas show 95% confident intervals. At day 15 tadpoles reach GS 25-26 and water of the Z-R treatment was switched from zoo to reintoduction site.**

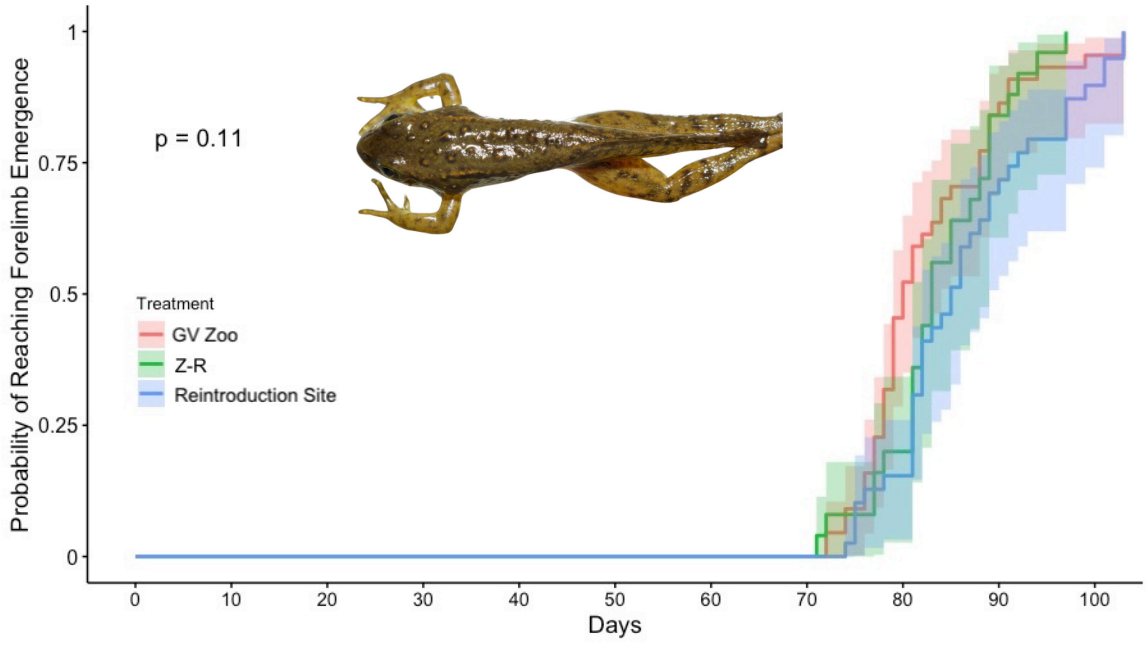

Figure 3.5 **Probability of Oregon spotted frog (*Rana pretiosa*) to reach metamorphosis during a 103-day exposure to different treatmnets: Greater Vancouver Zoo (GVZ); Zoo to Reintroduction Site (Z-R); and reintroduction site. Metamorphosis was defined by the emergence of both forelimbs (Gosner stage 42). Solid lines show treatment means of 5 replicates per treatment and shaded areas show 95% confident intervals. The photo shows a tadpole at GS 42.**

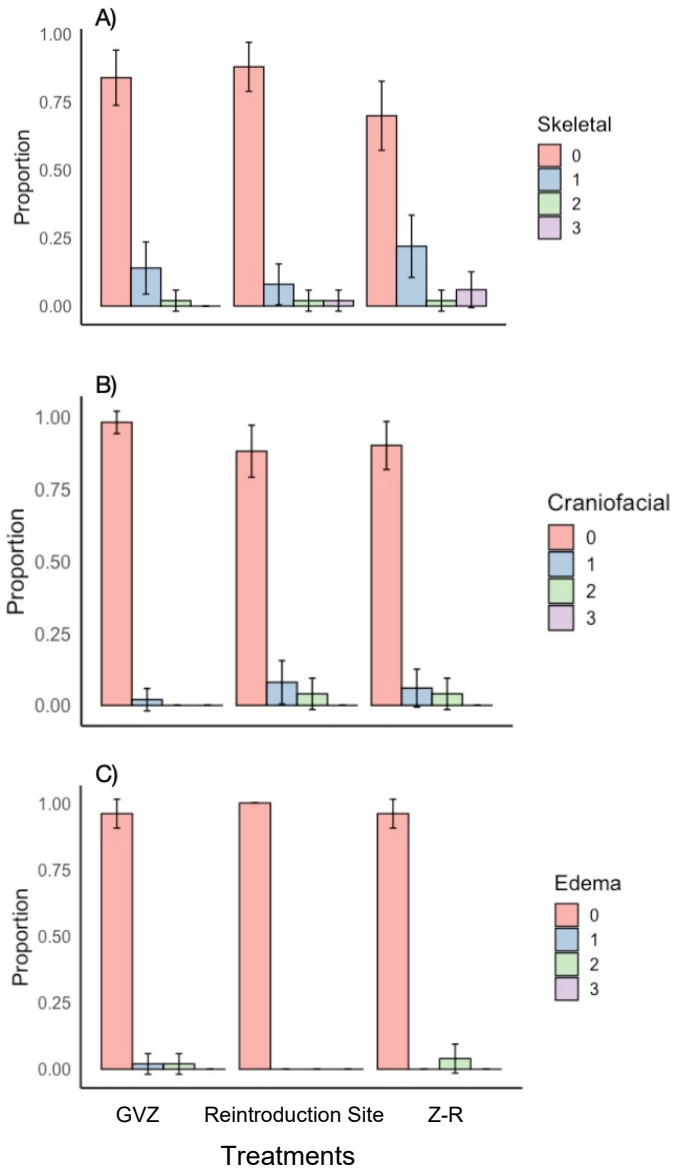

Figure 3.6 **Frequency of (A) skeletal, (B) carniofacial and (C) edema, of Oregon spotted frog (*Rana pretiosa*) at metamorphosis (GS 42) exposed to various rearing conditions: Greater Vancouver Zoo (GVZ); Zoo to Reintroduction Site (Z-R); and reintroduction site. Bars show mean of 5 replicates per treatment and error bars represend 95% confident intervals. There were no significant differences between treatments for skeletal, craniofacial, and edema presence (pairwise Fisher's exact test with Bonferroni correction on the categorical data ranging from 0 (no observation of deformities) and 3 (observation of severe deformities).**

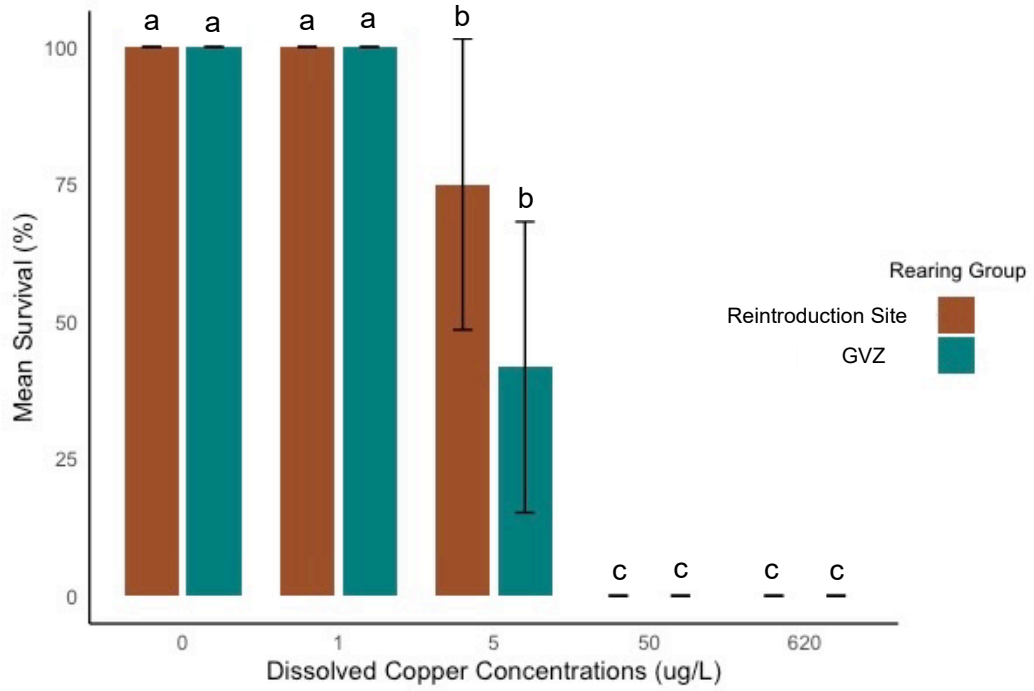

Figure 3.7 **The effects of 96-hour continuous exposure to copper on survival of Oregon Spotted Frog (*Rana pretiosa*) tadpoles at Gosner stage 25-26 for two background rearing conditions (Greater Vancouver Zoo water and reintroduction site water). Means ± 95% confidence intervals are presented. Letters denote Tukey HSD groups.**

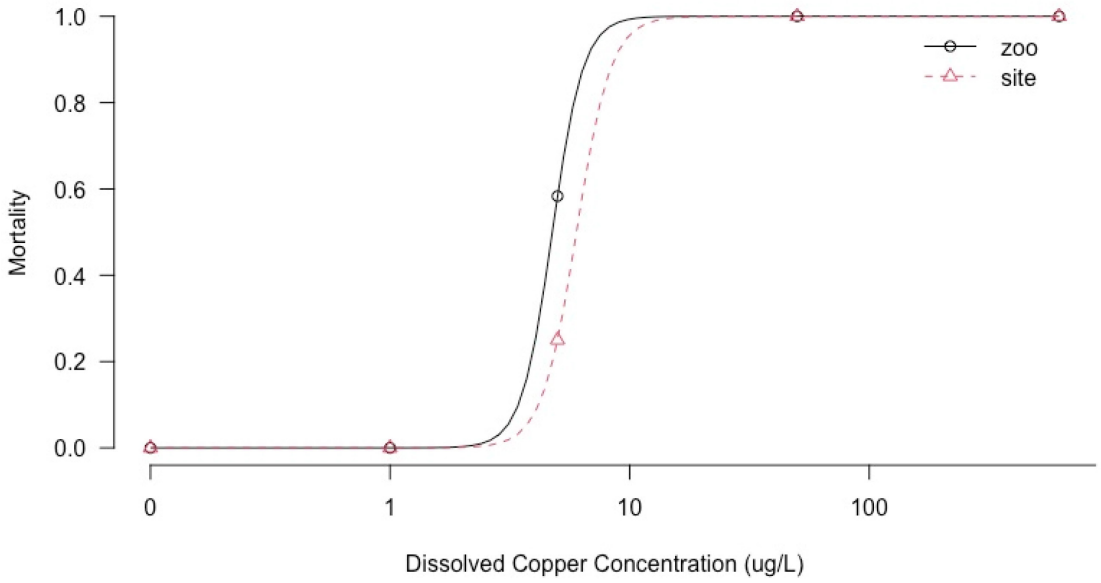

Figure 3.8 **Concentration-response relationship for dissolved copper concentration (measured) and Oregon spotted frog (*Rana pretiosa*) tadpole mortality for tadpoles reared in Greater Vancouver Zoo (GVZ) water and reintroduction site water. The LC50 for GVZ reared tadpoles was 4.76 µg/L and for reintroduction site was 5.93 µg/L (two-parameter logistic model to guided by the lowest Akaike Information Criterion values).**

3.5. Discussion

3.5.1. Transfer Between Zoo Water and Reintroduction Site Water Affects Larval OSF Health

A key finding of the present study was that tadpole survival was significantly decreased in the Z-R treatment that simulated the typical captive breeding program protocol, whereby egg masses are reared in GVZ water followed by release at the reintroduction site at GS 25-26. However, no reductions in survival were observed when OSF egg masses were solely reared through to metamorphosis in GVZ water (GVZ treatment) or solely in the reintroduction site water. Interestingly, several metals in the reintroduction site water (magnesium, copper and iron) and sediments (nickel, selenium, uranium) exceeded environmental quality guidelines, but did not appear to elicit adverse effects on survival, growth or development from late embryo stages through to metamorphic climax. Together, these data indicate that the transfer of tadpoles at GS 25-26 from GVZ water into reintroduction site water was a stressful event that caused significant mortality; whether this is related to pollutants at above guideline concentration in reintroduction site water is unknown but possible. Based on the findings of the present study, it is recommended that captive OSF should be reared in reintroduction site water rather than GVZ water prior to release to increase survival of released tadpoles. Follow-up studies examining the timing of transfer from zoo water to reintroduction site water with respect to developmental stages and acclimation may reveal alternative protocols that also result in optimal OSF larval health and survival.

The onset of increased OSF mortality starting mainly on day 72 in the Z-R treatment group coincided with the commencement of metamorphosis, a critical and vulnerable period in amphibian development. This phase is characterized by extensive physiological and morphological transformations as tadpoles' transition to adult frogs, necessitating significant energetic investments and extensive reorganization of tissues and organs (Orlofske & Hopkins, 2009; Zhu et al, 2020). The energetic demands of this process are substantial, with studies indicating that the physiological stress associated with these rapid changes can deplete energy reserves significantly, thereby elevating susceptibility to additional stressors like toxicants (Denver, 1997; Wassersug & Sperry, 1977). Metamorphosis induces immunological restructuring, during which the adaptive immune capabilities of tadpoles are temporarily reduced (Zhu et al, 2020; Humphries et

al, 2022; Rollins-Smith, 1998). This reduction in immune function during a crucial developmental window can exacerbate the impacts of environmental toxicants, potentially leading to increased mortality rates (Rollins-Smith & Conlon, 2005). Furthermore, environmental conditions such as various water quality parameters (i.e., hardness, pH, dissolved organic carbon, temperature) are known to modify toxicological stressors (i.e., metals and chemicals), thereby influencing health outcomes of exposed amphibians (Alford and Richards, 1999; Croteau et al., 2008; Peluso et al., 2021), and perhaps amplifying the effects of the stressors (Hill et al, 2022). Moreover, hormonal regulation, particularly by thyroid hormones, is pivotal during metamorphosis since these hormones orchestrate complex developmental processes (Das et al., 2006; Laudet, 2011). Several studies have demonstrated that exposure to environmental toxicants during this period can disrupt thyroid hormone pathways, leading to developmental abnormalities and increased mortality (Denver, 2009; Hayes et al., 2010; Thambirajah et al, 2019). In light of the high survival of OSF when reared solely in reintroduction site water despite exceedances of sediment and water quality guidelines for various metals, it is unlikely that water quality alone was the main driver of the reduced survival in the present study. It is likely that that changes in water quality, which included higher metals, nutrients, etc., with no acclimation between the transfer from GVZ water to reintroduction site was a key stressor and driver of mortality. The heightened vulnerability of OSF tadpoles during metamorphosis observed in the present study underscores the need for careful evaluation of environmental contaminants and changes in water quality parameters during sensitive early life stages of amphibians.

The basic water quality parameters at the reintroduction site treatment, including temperature, pH, dissolved oxygen, hardness, and conductivity, were within acceptable ranges for amphibian survival and development, and were similar to those of the GVZ water treatment (McKibbin et al., 2008; Wassersug and Seibert, 1975). However, the levels of unionized ammonia exceeded the long-term freshwater water quality guideline of 0.019 mg/L set by the Canadian Council of Ministers of the Environment (CCME) in both the GVZ and Z-R treatments, indicating potential risks to aquatic life. Despite this, the absence of mortality or adverse effects during periods of elevated ammonia levels (from 50 to 200 ug/L) in the present study suggests that OSF tadpoles possess a relatively higher tolerance to unionized ammonia. This appears to be similar to the resilience observed in wood frog tadpoles exposed to comparable conditions (Stenner,

2024). Specifically, Stenner (2024) reported that despite unionized ammonia levels reaching 25 to 28 ug/L in a control treatment (with a harness of 109 mg/L $CaCO_3$) and 42 to 44 ug/L in the 40% oil sands process water (OSPW) treatments (with a harness of 113 mg/L $CaCO_3$), no adverse effects were observed. The ability of OSF tadpoles to survive in high levels of unionized ammonia indicates a potential for short-term tolerance, as has been noted in other amphibian species (Hecnar, 1995; Marco et al., 1999; Stenner, 2024). Nevertheless, further research is necessary to determine the tolerance limits of OSF regarding the maximum duration and magnitude of exposure to unionized ammonia levels beyond CCME water quality guidelines.

We hypothesize that the unionized ammonia concentrations differed among the GVZ, Z-R, and reintroduction site water treatments is due to the presence or absence of nitrifying beneficial bacteria in respective treatment systems. The well-pumped water source of the GVZ is likely devoid of many beneficial bacteria that are found in natural settings. These bacteria are essential for nitrification, the process by which ammonia is converted into less toxic forms (Reyes Alvin et al. 2019; Anjali et al, 2022). In contrast, the reintroduction site water was derived from a natural environment with beneficial bacteria present. These bacteria will convert unionized ammonia to nitrite and then nitrate very efficiently, which reduces the concentration of unionized ammonia in the water (Barik et al, 2018).

There was higher mortality in OSF transferred to from GVZ water to reintroduction site water in the present study, but for those that survived the transition no effects on growth (total body length, snout-vent length, or wet weight) and development were observed. Also, there was no significant differences in growth and development between the GVZ water and reintroduction site water treatment. This aligns with the findings of Pollard et al. (2017), who reported that golden bell frog (*Litoria aurea*) tadpoles raised in both breeding and non-breeding ponds in Sydney Olympic Park, Australia with different water parameters (i.e., temperature, dissolved oxygen, conductivity and salinity) experienced similar survival rates, growth rates, and development. Similarly, Ruiz et al. (2010) found that bullfrog (*Lithobates catesbeianus*) tadpoles raised in constructed wetlands with treated wastewater with significant differences in water quality (i.e., pH, total phosphorus and ammonium-nitrogen) were comparable in size at metamorphosis to those from natural ponds concluding that the treated wastewater provided a habitat of sufficient quality to support normal growth and

development. In the present study, despite several metals in the reintroduction site water (magnesium, copper, and iron) and sediments (nickel, selenium, uranium) exceeding environmental quality guidelines, the combination of these contaminants did not impact the survival, growth, and development of OSF embryos and tadpoles. Remarkably, over 70% survival was observed for tadpoles in the ex-situ 103-day exposure, and all embryos hatched in this treatment. This outcome is likely due in part to the protective effect of high hardness reducing the toxicity of metals in the reintroduction site water, but it ultimately demonstrates that the reintroduction site water was of sufficient quality to support OSF larval growth and development at levels similar to those observed in the controlled zoo environment. The 70% survival rate of tadpoles and 100% hatching success in the present study are approximately similar to the embryonic survivorship of OSF observed at the reintroduction site (77-84%; McKibbin et al., 2008), suggesting that the reintroduction site water quality is suitable for early life stages of OSF.

The contaminants present at the reintroduction site did not impact the frequency of abnormalities in OSF tadpoles. In the context of existing literature, background rates of deformities in amphibians generally do not exceed 2-5% in natural settings (Wagner et al., 2014; Borkin et al., 2012; Sparling et al., 2010; Siammawii et al., 2022). This is in stark contrast to the high deformity rates reported in environments with anthropogenic influence, such as the oil sands operations where Pollet & Bendell-Young (2000) recorded scoliosis rates substantially higher (20%) than those found in more pristine environments (3%) in Western toad (*Bufo boreas*) and wood frog (*Rana sylvatica*) tadpoles. The findings of the present study demonstrate a low background rate of deformities in British Columbia OSF larvae of approximately 0 to 5%, that was unaffected by elevated water concentrations of magnesium, copper and iron.

3.5.2. Reintroduction Site Water Quality and Chemistry

In the analysis of the water and sediment samples collected at the reintroduction site on April 24 and May 31, 2023, variations in physicochemical parameters and contaminant levels were observed. There was increase in water temperature, pH, conductivity, and hardness between the two sampling events performed in April and in May 31 2023 suggesting natural fluctuations in water quality that could influence the bioavailability and toxicity of contaminants (Lathouri & Korre; Danis and Marlatt, 2019). The exceedance of guideline values for total copper, iron, and magnesium in surface

water samples is of concern, as these metals can impact the metabolic processes and survival of aquatic organisms (CCME, 1999; Budzik et al., 2014; Bakker et al, 2016). It is noteworthy that all surface water concentrations of organochlorines, organophosphates, and neonicotinoids were below detection limits, suggesting a lower risk of pesticide-related toxicity at the reintroduction site at that sampling time. However, the analytical detection limits for some pesticides were above the established guideline values, indicating the need for more sensitive detection methods to accurately assess pesticide contamination (Danis and Marlatt, 2019; Table A1).

In the present study, sediment samples from the OSF reintroduction site contained uranium levels of 0.542 mg/kg and 1.5 mg/kg, exceeding the safety threshold of 0.015 mg/kg. This is of concerns based on other studies examining amphibian responses to uranium exposure. For example, Marques et al. (2008) found that the Iberian green frog showed significant developmental disturbances at uranium concentrations similar to those observed in the present study, including decreased body length, increased mortality, and developmental abnormalities in larvae. Additionally, in the present study nickel was found at levels of 55.4 mg/kg in sediment, exceeding the guideline value of 37.9 mg/kg, indicating potential risks for OSF. Park et al., (2017) demonstrated that high nickel concentrations caused developmental abnormalities and embryonic death in Oriental Fire-Bellied Toad (*Bombina orientalis*) embryos. Given that OSF tadpoles bury themselves for protection in the sediment and adults do so for both protection and overwintering, prolonged exposure to high levels of nickel, selenium, and uranium poses increased risks of adverse effects. Although no impacts on growth, development or survival from late embryonic stages through to metamorphic climax in OSF were observed in the present 103-day controlled laboratory study, many of these metals have been shown to be toxic to amphibians and other aquatic life. Future studies including sampling events with a more robust study design (i.e., replicate samples, in multiple seasons, lower detection limits for pesticides, etc.) to provide a more comprehensive analysis of surface water and sediment contamination at the reintroduction site are warranted. Thus, ongoing monitoring of this reintroduction site for contaminants, and ideally, OSF health and survival are recommended to verify no unacceptable long-term adverse effects are occurring due to the contaminants present at this site.

3.5.3. 96-hour Acute Exposure to Copper

In the present study, the 96-hour exposure experiment on OSF tadpoles to copper revealed no effects on survival at 1 µg/L of copper, while survival dropped to approximately 25-50% at 5 µg/L and 0% at 50 and 620 µg/L of copper, with the water hardness at ~21 mg/L $CaCO_3$. The LC50s from this acute toxicity experiment were 5.93 µg/L and 4.76 µg/L for OSF tadpoles at GS 25-26 exposed in dechlorinated municipal water that were previously reared in reintroduction site and zoo water, respectively. With the higher LC50 obtained for the OSF previously reared in reintroduction site water and the elevated copper in the reintroduction site water, this may indicate that these tadpoles were acclimated to copper prior to the exposure due to some copper concentration in the reintroduction site water. However, additional studies are needed to further investigate this phenomenon.

There are a few studies of acute toxicity in anurans that were recently reviewed by Azizishirazi et al. (2021), but due to water hardness, pH and dissolved organic carbon identified as toxicity modifying factors for copper direct comparisons can only be made to one study. Specifically, Azizishirazi et al. (2021) reported that a temperate species, Iberian Green Frogs (*Pelophylax perezi*) exhibited a 96-hour LC50 of 970 µg/L at a water hardness of 18.2 ± 2.0 mg/L $CaCO_3$.These findings indicate that OSF tadpoles are more sensitive to copper compared to the Iberian green frog. Furthermore, Azizishirazi et al. (2021) described an acute species sensitivity distribution (SSD) comprising 19 amphibian species, with acute LC50 concentrations ranging from 17 to 2996 µg/L of copper. The most sensitive species were the Argentine Toad tadpoles (*Rhinella arenarum*) and Eastern Narrow-Mouthed Toad tadpoles (*Gastrophryne carolinensis)*, exhibiting 24-hour and 96-hour LC50 values of 17 µg/L (at hardness of 45-50 mg/L $CaCO_3$) and 40 µg/L, respectively. Both of these LC50s are approximately 3 to 8 fold higher than the LC50s observed in the present study for OSF tadpoles conducted at a water hardness approximately 2-fold lower, which may suggest OSF tadpoles may be more sensitive. Additionally, Azizishirazi et al. (2021) found that Cope's Gray Tree Frogs (*Hyla chrysoscelis*; 96-hour LC50 = 44.7 µg/L at a hardness of 160 to 180 mg/L $CaCO_3$) and Natterjack Toads (*Epidalea calamita*; 96-hour LC50 = 80 µg/L at a hardness of 170 to 250 mg/L $CaCO_3$) were also among the most sensitive to acute copper exposure even though the acute toxicity studies were conducted in relatively hard water. In contrast, Bullfrog (*Lithobates catesbeianus*) tadpoles appeared to be the least sensitive with a

geometric mean of three 96-hour LC50s of 2996 µg/L (no water hardness was reported), approximately 500-fold less sensitive than the OSF tadpoles examined in this study. Dwyer et al. (2005) reported that Western Toads (*Anaxyrus boreas*) in the tadpole stage had a 96-hour LC50 of 120 µg/L for copper in water hardness of 160 to 180 mg/L $CaCO_3$, which is higher than the LC50 values observed for OSF tadpoles but with the protective effect of the higher hardness in the western toad exposure it is difficult to make a direct comparison. Interestingly, the western toad ranked as the ninth most sensitive species among 17 larval fish species in Dwyer et al. (2005), suggesting these toads fall within the range of sensitivity to copper of several larval fishes. Ultimately, additional studies testing OSF and other amphibians at a range of toxicity modifying factors is necessary to ascertain the acutely lethal concentrations of copper in OSF and in other amphibians. Given the conservation status of numerous amphibians, lacking this critical data on the chemical sensitivity of amphibians required for setting water quality guidelines protective of most freshwater species is of grave concern.

Azizishirazi et al. (2021) concluded that although most amphibians included in the derived acute copper Species Sensitivity Distribution (SSD) are tolerant to acute copper exposure, they were more sensitive to chronic copper exposure under natural environmental conditions. For OSF, the chronic effects of copper exposure were explored to some degree in the present study during the reintroduction site 103 day exposure from late stage embryos to metamorphic climax. The reintroduction site water copper concentrations were measured twice during this aspect of the present study and were 1.12 and 0.86 ug/L with water hardness of 71.0 and 91.6 mg/L $CaCO_3$, both of which exceeded BC Water Quality Guidelines. Although the hardness of the reintroduction site water is considerably higher than the water used in the acute toxicity study, this aligns with the acute toxicity study whereby no adverse effects occurred at 1 ug/L of copper. However, if adverse effects on growth, development and survival occur beyond 1 ug/L copper, this was not investigated in the present study. Future chronic toxicity studies of the adverse effects of copper on the OSF are needed to understand the sensitivity of this species to this common environmental pollutant. In addition, to obtain more environmentally relevant acute and chronic toxicity thresholds for OSF, it is crucial to conduct toxicity tests at water hardness levels closer to their natural environment (i.e., between 71 to 97 mg/L $CaCO_3$). Testing at these hardness levels

would provide a more accurate assessment of OSF sensitivity to copper, reflecting the conditions these amphibians would experience in the wild.

3.5.4. Conclusion

The present study shows the significant impacts of water transfer on the survival of OSF larvae, particularly highlighting the mortality associated with the transition from zoo water (GVZ) to reintroduction site water at GS 25-26. Despite the presence of metals in reintroduction site water that exceeded environmental quality guidelines, no adverse effects were observed on the survival, growth, or development of OSF larvae reared entirely in reintroduction site water. This suggests that the sudden change in water quality, without proper acclimation, is a key stressor. The onset of increased mortality coinciding with metamorphosis underscores the critical need for careful management of water quality during this phase (Denver, 1997; Hayes et al., 2010). The findings emphasize the importance of rearing OSF larvae in reintroduction site water prior to release and suggest that future research should focus on optimal timing and methods for acclimation to enhance survival rates. Additionally, the present study underscores the necessity of ongoing monitoring and robust study designs to better understand the impacts of environmental contaminants and ensure the success of OSF reintroduction programs. The 96-hour acute exposure experiment revealed high sensitivity of OSF tadpoles to copper, with LC50 values of 5.93 µg/L and 4.76 µg/L for tadpoles reared in reintroduction site and zoo water, respectively. The low LC50 values for OSF tadpoles may demonstrate a particular vulnerability to copper, especially compared to one temperate species, Iberian green frogs, also examined under soft water conditions (Azizishirazi et al., 2021; Chen et al., 2007). Water hardness is well known to influence copper toxicity, and higher hardness mitigates its effects. The present study, conducted at a water hardness of approximately 21 mg/L $CaCO_3$, highlights the need for toxicity tests at hardness levels closer to the natural environment of OSF tadpoles (71 to 97 mg/L $CaCO_3$) to obtain a realistic assessment of their sensitivity. Standardizing water hardness in toxicity testing protocols is essential for accurate evaluation and comparison of copper sensitivity across different species, aiding in the development of effective conservation strategies.

Chapter 4.

Recommendations for OSF Conservation Breeding Programs

The reproductive hormone profiles obtained via non-invasive methods in the present study are useful for understanding the reproductive status of OSF and optimizing captive breeding strategies, and ongoing monitoring in the ex situ setting is recommended. Based on the present study, further experiments are needed to verify that E2 levels in OSF females differ from those observed in other anurans, but the present findings suggest suboptimal female reproductive health in captive OSF at both GVZ and VA that merits further investigation. Interestingly, testosterone profiles in males in the present study suggests male reproductive health is good at both facilities and may not be the culprit associated with the low reproductive success at the VA. Indeed, the observed differences between the GVZ and VA in terms of historical reproductive success suggest that environments that more closely mimic natural conditions result in higher numbers of egg masses and viable offspring, as indicated by the higher reproductive success at the GVZ outdoor breeding ponds compared to the indoor VA enclosures. Ultimately, I recommend combining non-invasive monitoring of reproductive hormones with other reproductive health measures (i.e., ovarian ultrasounds, egg counts, sperm motility and counts) to aid in better characterizing the reproductive physiology associated with high reproductive success at the GVZ and to further optimize OSF captive breeding strategies (see Lynch and Wilczynski, 2005; Germano et al., 2009; Narayan et al., 2010).

This thesis demonstrated that late stage embryonic and larval OSF survival was significantly compromised when transferring from GVZ water to the reintroduction site water at Gosner stage 25-26. This indicates that the transition to a different source of water during these early life stages is a significant stressor that can lead to increased mortality (Carpenter et al., 1998; Diaz and Rosenberg, 2008). Although additional experiments examining the effects of different water sources and which water quality parameters may have caused this reduced survival, it is possible that the mixtures of metal contaminants present in the reintroduction site water contributed to some mortality. Therefore, to improve the survival rates of released tadpoles, it is

recommended to release egg masses directly into reintroduction sites, allowing the embryos and larvae to develop in their native environment from the earliest life stages. Furthermore, it is recommended to measure contaminant concentrations in water and sediments at reintroduction sites to characterize the magnitude and number of anthropogenic contaminant stressors exceeding local environmental quality guidelines that may impede long term amphibian health. With virtually no studies examining the effects of environmental contaminants on the endangered OSF and the findings of the present study indicating this species is sensitive to a common pollutant, copper, it is also of paramount importance to improve our understanding of the susceptibility of OSF to pollutants.

In conclusion, reproductive health of OSF is critically important for understanding and enhancing captive breeding efforts, making non-invasive hormone monitoring extremely useful. The findings of this study reveal decreased estradiol levels during the breeding season in females which requires future research to determine the cause. Our finding that the transfer of OSF tadpoles from GVZ to reintroduction site water may affect survival due to contaminants is important because the recovery teams' protocol does not allow sufficient time for acclimation. Rearing OSF under more natural conditions, or releasing eggs directly into reintroduction sites, will likely increase survival in the long term, while measuring contaminant levels may be used to mitigate stressors.

Future Work

Future studies should focus on improving hormone monitoring and reintroduction programs for the OSF. Increasing the sample size for the hormone analysis by including more individuals from various facilities will improve statistical power and reliability of the analyses. Moreover, improved hormone quantification assays will help better detect changes in stress and reproductive hormones of OSF. This will facilitate optimal captive breeding programs while providing new knowledge on the physiological status of frogs, helping to monitor and manage their health and reproductive success better. Additionally, continued monitoring of reproductive hormones is crucial to understand why female OSF exhibit unique hormone profiles compared to other frog species. Histopathological studies in experimental settings may be useful to better investigate the high mortality rates associated with Z-R treatment. Also, DNA and RNA analysis can aid in exploring genetic factors that may reveal potential vulnerabilities in tadpoles after

being exposed to copper or reintroduction site water. Lastly, exposing OSF tadpoles to copper using water parameters (pH, dissolved organic carbon and hardness) similar to the reintroduction site sites will provide a more realistic LC50 value, enhancing our understanding of their environmental tolerance.

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Appendix A. Measured Metals and Pesticides

Table A.1. Metals and pesticides measured at Oregon spotted frog reintroduction site.

Total Metals

Metal	Detection Limit	Detection Limit (ALS)	Measured (mg/L)	CCME WQG (mg/L)	BC Ambient WQG (mg/L)	Sample Collection Date**
Aluminum		0.003	0.0169	0.1	0.178	31-May-23
			0.0144	0.1	0.154	24-Apr-23
Antimony		0.0001	<0.00010	-	0.006	31-May-23
			0.00016	-	0.006	24-Apr-23
Arsenic		0.0001	0.00096	0.005	0.005	31-May-23
			0.00075	0.005	0.005	24-Apr-23
Barium		0.0001	0.0278	-	1	31-May-23
			0.023	-	1	24-Apr-23
Beryllium		0.0001	<0.000100	-	0.00013	31-May-23
			<0.000100	-	0.00013	24-Apr-23
Bismuth		0.00005	<0.000050	-	-	31-May-23
			<0.000050	-	-	24-Apr-23
Boron		0.01	0.013	1.5	1.2	31-May-23
			0.019	1.5	1.2	24-Apr-23
Cadmium		0.000005	0.0000078	0.00016	0.000208	31-May-23
			0.0000058	0.00012	0.000164	24-Apr-23
Calcium		0.05	28.8	-	1000	31-May-23
			19.3	-	1000	24-Apr-23
Cesium		0.00001	<0.000010	-	-	31-May-23
			<0.000010	-	-	24-Apr-23
Chromium		0.0005	<0.00050	-	0.0373	31-May-23

		<0.00050	-	0.0373	24-Apr-23
Cobalt	0.0001	0.00038	-	0.004	31-May-23
		0.00022	-	0.004	24-Apr-23
Copper	0.0005	0.00112	0.00232	0.000814	31-May-23
		0.00086	0.002	0.000653	24-Apr-23
Iron	0.01	0.586	0.3	1	31-May-23
		0.226	0.3	1	24-Apr-23
Lead	0.00005	<0.000050	0.00308	0.0064	31-May-23
		0.000077	0.00206	0.00537	24-Apr-23
Lithium	0.001	<0.0010	-	-	31-May-23
		<0.0010	-	-	24-Apr-23
Magnesium	0.005	6.23	-	0.647[E]	31-May-23
		5.55	-		24-Apr-23
Manganese	0.0001	0.104		1.03	31-May-23
		0.108		0.917	24-Apr-23
Mercury	0.000005	<0.0000050	0.0000026		31-May-23
		<0.0000050	0.0000026		24-Apr-23
Molybdenum	0.00005	0.000644	0.073	7.6	31-May-23
		0.00118	0.073	7.6	24-Apr-23
Nickel	0.0005	0.00122	0.09383	0.0938	31-May-23
		0.00224	0.07367	0.0737	24-Apr-23
Phosphorus	0.05	<0.050	-	0.015	31-May-23
		<0.050	-	0.015	24-Apr-23
Potassium	0.05	1.56	-	-	31-May-23
		0.587	-	-	24-Apr-23
Rubidium	0.0002	0.00089	-	-	31-May-23
		0.0004	-	-	24-Apr-23

Selenium	0.00005	0.000295	0.001	0.002	31-May-23
		0.000375	0.001	0.002	24-Apr-23
Silicon	0.1	5.87	-	-	31-May-23
		1.04	-	-	24-Apr-23
Silver	0.00001	<0.000010	0.000025	5.00E-05	31-May-23
		<0.000010	0.000025	5.00E-05	24-Apr-23
Sodium	0.05	2.87	-	-	31-May-23
		1.86	-	-	24-Apr-23
Strontium	0.0002	0.104	-	7	31-May-23
		0.0681	-	7	24-Apr-23
Sulfur	0.5	2.65	-	-	31-May-23
		3.21	-	-	24-Apr-23
Tellurium	0.0002	<0.00020	-	-	31-May-23
		<0.00020	-	-	24-Apr-23
Thallium	0.00001	<0.000010	0.0008	0.0008	31-May-23
		<0.000010	0.0008	0.0008	24-Apr-23
Thorium	0.0001	<0.00010	-	-	31-May-23
		<0.00010	-	-	24-Apr-23
Tin	0.0001	<0.00010	-	-	31-May-23
		<0.00010	-	-	24-Apr-23
Titanium	0.0003	0.00056	-	-	31-May-23
		0.00066	-	-	24-Apr-23
Tungsten	0.0001	<0.00010	-	-	31-May-23
		<0.00010	-	-	24-Apr-23
Uranium	0.00001	0.000074	0.015	0.0085	31-May-23
		0.000068	0.015	0.0085	24-Apr-23
Vanadium	0.0005	0.00058	-	0.05	31-May-23

		0.00054	-	0.05	24-Apr-23
Zinc	0.003	<0.0030	0.035	0.0173	31-May-23
		<0.0030	0.028	0.0142	24-Apr-23
Zirconium	0.0002	<0.00020	-	-	31-May-23
		<0.00020	-	-	24-Apr-23

Dissolved Metals

Metal	Detection Limit	Measured (mg/L)	CCME WQG (mg/L)	BC Ambient WQG (mg/L)	Sample Collection Date**
Aluminum	0.001	0.0034	0.1	0.1	31-May-23
		0.0013	0.1		24-Apr-23
Antimony	0.0001	<0.00010	-	2.31[S]	31-May-23
		0.00016	-		24-Apr-23
Arsenic	0.0001	0.00074	0.005	0.005	31-May-23
		0.0006	0.005		24-Apr-23
Barium	0.0001	0.0252	-	0.22	31-May-23
		0.0243	-		24-Apr-23
Beryllium	0.0001	<0.000100	-	0.148[S]	31-May-23
		<0.000100	-		24-Apr-23
Bismuth	0.00005	<0.000050	-	-	31-May-23
		<0.000050	-		24-Apr-23
Boron	0.01	0.012	1.5	1.5	31-May-23
		0.019	1.5	1.5	24-Apr-23
Cadmium	0.000005	<0.0000050	0.00016	0.00009	31-May-23
		<0.0000050	0.00012	0.00009	24-Apr-23
Calcium	0.05	30		116[E]	31-May-23
		20.1		-	24-Apr-23
Cesium	0.00001	<0.000010		-	31-May-23

		<0.000010		-	24-Apr-23
Chromium	0.0005	<0.00050		Cr (VI) 0.001	31-May-23
				Cr (III) 0.008	
		<0.00050		-	24-Apr-23
Cobalt	0.0001	0.00021		0.024[E]	31-May-23
		0.0016		-	24-Apr-23
Copper	0.0002	0.00057	0.00232	0.000814	31-May-23
		0.00059	0.002	0.000653	24-Apr-23
Iron	0.01	0.138	0.3	0.35	31-May-23
		0.067	0.3		24-Apr-23
Lead	0.00005	<0.000050	0.00308	0.002	31-May-23
		<0.000050	0.00206		24-Apr-23
Lithium	0.001	<0.0010		0.014 [E]	31-May-23
		<0.0010			24-Apr-23
Magnesium	0.005	6.37		0.647 [E]	31-May-23
		5.91			24-Apr-23
Manganese	0.0001	0.0596		1.27 [S]	31-May-23
		0.0878			24-Apr-23
Mercury	0.000005	<0.0000050	0.0000026	0.000026	31-May-23
		<0.0000050	0.0000026		24-Apr-23
Molybdenum	0.00005	0.000686	0.073	0.073	31-May-23
		0.0011	0.073		24-Apr-23
Nickel	0.0005	0.00108	0.09383	0.65	31-May-23
		0.00214	0.07367		24-Apr-23
Phosphorus	0.05	<0.050		>0.1 mg/L hyper-eutrophic	31-May-23
		<0.050			24-Apr-23

Potassium	0.05	1.46		373	31-May-23
		0.608			24-Apr-23
Rubidium	0.0002	0.00085			31-May-23
		0.00038			24-Apr-23
Selenium	0.00005	0.000271	0.001	0.001	31-May-23
		0.000276	0.001		24-Apr-23
Silicon	0.05	5.71			31-May-23
		1.14			24-Apr-23
Silver	0.00001	<0.000010	0.000025	0.25	31-May-23
		<0.000010	0.000025		24-Apr-23
Sodium	0.05	2.73		680[S]	31-May-23
		1.9			24-Apr-23
Strontium	0.0002	0.107		1.5[E]	31-May-23
		0.0724			24-Apr-23
Sulfur	0.5	2.28			31-May-23
		2.88			24-Apr-23
Tellurium	0.0002	<0.00020			31-May-23
		<0.00020			24-Apr-23
Thallium	0.00001	<0.000010	0.0008	0.0008	31-May-23
		<0.000010	0.0008		24-Apr-23
Thorium	0.0001	<0.00010			31-May-23
		<0.00010			24-Apr-23
Tin	0.0001	<0.00010		0.073[E]	31-May-23
		<0.00010			24-Apr-23
Titanium	0.0003	<0.00030		2	31-May-23
		<0.00030			24-Apr-23
Tungsten	0.0001	<0.00010			31-May-23

		<0.00010			24-Apr-23
Uranium	0.00001	0.000072	0.015	0.015	31-May-23
		0.000064	0.015		24-Apr-23
Vanadium	0.0005	<0.00050		0.041[S]	31-May-23
		<0.00050			24-Apr-23
Zinc	0.001	0.0016	0.035	0.007	31-May-23
		<0.0010	0.028		24-Apr-23
Zirconium	0.0002	<0.00020		2.4[S]	31-May-23
		<0.00020			24-Apr-23
Pesticides and herbicides					
	Detection Limit (ug/L)	Result	CCME WQG (mg/L)	BC Ambient WQG (mg/L)	Sample Collection Date**
DDE, 2,4’	0.004	Not detected			31-May-23
DDE, 4,4’	0.004	Not detected			31-May-23
DDE total	0.006	Not detected			31-May-23
DDT,2,4’	0.004	Not detected			31-May-23
DDT, 4,4’	0.004	Not detected			31-May-23
DDT, total	0.006	Not detected			31-May-23
AMPA	0.5	Not detected	800*	800*	31-May-23
Diquat (ion)	1	Not detected			31-May-23
Glufosinate	0.5	Not detected			31-May-23
Glyphosate	0.2	Not detected	800	800	31-May-23
Linuron	0.1	Not detected	7	7	31-May-23

Acetamiprid	0.005	Not detected			31-May-23
Atrazine	0.1	Not detected	1.8	1.8	31-May-23
Chloronicotinic acid, 6-	0.1	Not detected			31-May-23
Chlorothalonil	0.05	Not detected	0.18	0.18	31-May-23
Chlorothalonil-4-hydroxy	0.05	Not detected			31-May-23
Clothianidin	0.005	Not detected			31-May-23
Flonicamid	0.01	Not detected			31-May-23
Imidacloprid	0.005	Not detected	0.23	0.23	31-May-23
Metolachlor	10	Not detected	7.8	7.8	31-May-23
Nitenpyram	0.005	Not detected			31-May-23
Sulfoxaflor	0.005	Not detected			31-May-23
Thiacloprid	0.005	Not detected			31-May-23
Thiamethoxam	0.005	Not detected			31-May-23
Anions and Nutrients					
	Detection Limit (ug/L)	Result	CCME WQG (mg/L)	BC Ambient WQG (mg/L)	Date**
Nitrate (as N)	0.0050	0.577	13	3	31-May-23
Sulfate (as SO4)	0.30	6.31	-	309	31-May-23

A Australian and New Zealand Surface water screening benchmark

E US EPA Surface water screening benchmark (chronic/long-term)

S Effect Concentration 20% (EC20) Sensitive species surface water

* Glyphosate value used for AMPA as it is a metabolite

**May 31, 2023: Hardness 97.6 (mg/L), pH 7.26, DOC 3.51 (mg/L), April 24, 2023: Hardness 71.0 (mg/L), pH 7.14, DOC 3.57 (mg/L)