Assessments of genetic and reproductive health in Canada's endangered Oregon Spotted Frog (*Rana pretiosa*)

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Biology

The Office of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Office of Graduate Studies/Bureau des études supérieures

Title of Thesis Titre de la thèse (Rana pretiosa)	Assessments of genetic and reproductive health in Canada's endangered Oregon Spotted Frog (Rana pretiosa)
Name of Candidate Nom du candidat	Hunter, Briar
Degree Diplôme	Master of Science
Department/Program Département/Programme	Biology
Date of Defence Date de la soutenance	August 04, 2023

APPROVED/APPROUVÉ

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Abstract

Zoological institutions are increasingly relied upon for *ex situ* management of species at risk, with conservation breeding and reintroduction programs providing both assurance against extinction and a reliable source of offspring to reinforce wild population sizes. *Ex situ* efforts face many challenges, however. One often overlooked challenge is balancing genetic priorities, like retaining genetic diversity and reducing inbreeding, with reproductive priorities, like mate compatibility and reliable breeding, both of which are required for a successful program. Like many amphibians, the Oregon Spotted Frog (*Rana pretiosa*) is highly threatened in its native habitat and ongoing conservation breeding and reintroduction programs are experiencing limited success; the genetic sustainability of these populations remains unassessed. In this study, I evaluated the genetic health of both zoo and wild populations of *R. pretiosa* in Canada and investigated some potential causes of the ongoing *ex situ* reproductive failures associated with egg binding. I found that zoos have maintained stable genetic metrics relative to their wild sources, but ongoing collections from wild populations should be reassessed due to low genetic diversity available therein. No clear causes of egg binding were elucidated but I found older female frogs (> 3 years old) who became egg bound generally had a higher body mass than conspecifics, while body mass did not differ in females becoming egg bound in their first breeding season (2-3 years old). This suggests frogs should be monitored for egg binding and changes in body condition differently depending on their age. Overall, there were no significant differences in genetic or reproductive health across the three zoo conservation breeding populations, but scaled mass index was significantly lower in the zoo with larger holding tanks and less genetic management: a reminder of the importance of husbandry and environment in conservation breeding program outcomes. The costs and benefits of strict genetic management *vs.* a more communal breeding approach should be carefully considered in light of these results. Along with more cohesion, communication is required between all involved institutions to have an effective impact on the conservation of Oregon Spotted Frogs in Canada, the recommendations discussed here have applicability to amphibian *ex situ* programs worldwide.

Acknowledgements

From the get-go, this project was filled with seemingly endless peaks and valleys, yet equally endless was the support I received every step of the way. Despite being on rollercoasters of their own, my co-supervisors Dr. David Lesbarrères and Dr. Gabriela Mastromonaco were the safety bars holding me steady and on track through it all. Thank you for all your advice, knowledge, insight, and encouragement; I left every meeting feeling cheered and re-infused with your passion for these topics. Thank you for reigning in my "flowery" language while simultaneously encouraging every science communication outlet I showed interest in. I would do it all again every time.

Thank you to my committee members, Kendra Morgan and Dr. Arne Mooers, who went above and beyond typical committee duties, stepping in as my BC support system. Thank you for providing me with bikes, waders, helping hands, roommates, community, and of course, your unmatched expertise. You are part of the reason I cannot get BC out of my veins and were absolutely instrumental in the completion of this project. Thank you.

A special thanks to everyone who helped me in the field, the lab, the depths of R Studio, and everywhere in between, from Ontario to British Columbia. Thank you Pourya Sardari for being my right-hand-OSF-man, helping me measure and swab and ultrasound and clean tanks and watch frogs for hours on end. I would be here another two years if I hadn't had your help. Thank you Megan Winand for letting me sleep on your couch on day one, for snowshoeing adventures, and for your episodes of "Love is Blind: Frog Edition"; may our friendship last as long as Bob and Steve's love for one another! Thank you to Paula Mackie and Allison Julien for putting up with all my ultrasound questions and pointing out to me over and over what a follicle looks like. Thank you to Michelle Hill and Ori Urquhart for flying to BC just to spend hours on hours silently watching frogs do mostly nothing – your enthusiasm for every one of my bizarre ideas is unmatched and so appreciated. Thank you to my co-authors Anne-Laure Ferchaud and Éric Normandeau from Institut de Biologie Intégrative et des Systèmes (IBIS) at Université Laval for all your sequencing and genomics expertise. Thank you to my external reviewer, Dr. Natalie Calatayud, whose own research I must have referenced at minimum daily while writing my second chapter.

Throughout this project I was continuously impressed by the dedication and passion within the Oregon Spotted Frog Recovery Team – you are the reason I think there is still hope for this endangered species. To everyone at the Fraser Valley Conservancy, thank you for helping me swab frogs and for collecting and storing miscellaneous frog parts in your own freezers until I could collect them. Thank you to Kris Rossing and Darren Smy from Vancouver Aquarium for all your help and for trusting me with your frogs even when things went awry. Thank you to Andrea Gielens and Wildlife Preservation Canada for not only trusting us novices to ultrasound your frogs but also for letting me show up at the Greater Vancouver Zoo whenever I wanted to hang-out with some frogs or turtles. A big thanks to Toronto Zoo for hosting my ReNewZoo internship, giving me ample opportunities to talk and write about frogs while simultaneously getting to witness and participate in the inspiring conservation work you do. Thanks to the whole Repro

Lab at Toronto Zoo for your help in the lab and to Rick Vos for letting me shadow you and get a taste for being an amphibian curator.

Finally, I would not be where I am today without my friends and family. You are my most loyal cheering squad, my eager audience, boosting me up but also keeping me humble. Thank you for your smiles and laughter whenever I monopolize the conversation with frog-talk, despite hearing the same stories hundreds of times over. To my parents in particular, thank you for nurturing a love of God's creation within me and giving me so many childhood memories full of swamps and frogs and joy – I feel blessed to be making the same kind of memories now in adulthood. Shoutout to the 6th floor crew – peace and love to you all. And to Beau and Vanilla I owe much of my sanity and mental wellbeing through the final stages of writing and analysis somehow providing both freedom and routine – I trust our adventures have only just begun.

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General Introduction

Zoological Conservation

Human actions have driven more species towards extinction now than ever before (IPBES, 2019), with many requiring active management both *in situ* (in the wild) and *ex situ* (*i.e.* in zoos and aquaria; hereafter "zoos"). Yet, while conservation science has often prioritized *in situ* efforts, deeming methods such as captive breeding as a last resort (Snyder *et al.*, 1996), the role of zoos has become increasingly more important and more accepted (Conde *et al.*, 2011). Zoos provide a unique setting for research and conservation of wildlife (Lewis *et al.*, 2019), hosting captive populations both as assurance against extinction and for conservation breeding. Breeding programs strive to increase reproductive output by releasing captive-born offspring to reinforce wild census population size (Palm *et al.*, 2003; Osborne *et al.*, 2006) and provide time for threats in the species' native habitat to be reduced or mitigated.

In-situ and *ex-situ* actions complement and rely upon one another, and thus, should be planned and managed collaboratively (Byers *et al.*, 2013; Johnson *et al.*, 2020). Maintaining *ex situ* populations invariably has many challenges, particularly for endangered species where populations are founded with few individuals, putting them at higher risk of inbreeding and the concomitant loss of fitness (Kraaijeveld-Smit *et al.*, 2006). Animals can also become adapted to captivity (Gilligan and Frankham, 2003; Heath *et al.*, 2003), with *ex situ* conditions often causing changes in behaviour, physiology, and morphology (O'Regan and Kitchener, 2005; Schulte-Hostedde and Mastromonaco, 2015). Due to the inherent genetic risks of breeding small groups of animals, many conservation breeding programs are intentionally designed for genetic sustainability, with standards around genetic diversity, inbreeding and effective population sizes (Ne) being well outlined if not always achievable (Lees and Wilcken, 2009). These species management goals focus on retaining sufficient genetic potential within current and future generations, but often neglect the necessity of reliable breeding for optimal genetic goals to be realized (Asa *et al.*, 2011). Many *ex situ* programs are initiated when knowledge of the species' life history remains scarce and suitability to *ex situ* breeding has not been evaluated, which can greatly impede program outcomes (Michaels *et al.*, 2014; Bradfield *et al.*, 2022) as it leaves caretakers to discern any necessary reproductive cues or conditions after the fact (Kouba *et al.*, 2009). Yet, even where cues can be determined, close genetic management often comes at the expense of more natural mating systems, removing mate-selection and altering or obscuring many behavioural and environmental stimuli (Asa *et al.*, 2011). Allowing more natural mate selection can improve reproductive success by avoiding mate incompatibility (Asa *et al.*, 2011), which can improve offspring fitness but may incidentally decrease Ne due to increased variability in family sizes (Wedekind, 2002). Furthermore, even if breeding is successful over multiple generations (Carrillo *et al.*, 2015), the evolutionary context (Schulte-Hostedde and Mastromonaco, 2015) and physiological consequences of an *ex situ* existence (O'Regan and Kitchener, 2005) often go unassessed.

Amphibians *ex situ*

The number of *ex situ* amphibian collections has increased significantly over the past two decades (Conde *et al.*, 2011; Dawson *et al.*, 2016) in response to the global extinction crisis

facing amphibians (Stuart *et al.*, 2004). These precipitous declines are driven by complex, often synergistic factors predominantly anthropogenic in nature (Gascon *et al.*, 2007; Green *et al.*, 2020). While many of these threats can and are being addressed *in situ*, others (e.g. infectious diseases) remain irreversible (Smith and Sutherland, 2014). The suitability of amphibians for *ex situ* conservation, however, has recently been called into question (Tapley *et al.*, 2015; Bradfield *et al.*, 2022). While amphibians have relatively low holding costs, high reproductive output, and high conservation impact (Bloxam and Tonge, 1995), they also have unique life cycles, often requiring species-specific protocols in human care (Kouba *et al.*, 2009). Amphibians rely on a variety of behavioural and environmental cues to trigger reproduction (Ulloa *et al.*, 2019), are often managed in large groups, and breed communally, making both genetic management and reproductive success challenging (Kouba *et al.*, 2009; Carrillo *et al.*, 2015). Reproductive failures (ex. egg retention) are increasingly common in amphibian breeding programs (Kouba *et al.*, 2009), but the causes of these failures are rarely known. Assisted reproductive technologies (ART) are increasingly relied upon for individual wellbeing, to treat or prevent reproductive dysfunction, and for genetic management (Kouba *et al.*, 2009; Graham *et al.*, 2018); however, they should not be used as an alternative to the replication of natural environments (Calatayud *et al.*, 2018; Silla *et al.*, 2021).

The Oregon Spotted Frog, *Rana pretiosa*

Conservation breeding and reintroduction programs were established for the Oregon Spotted Frog (OSF) in 2010 in response to its emergency designation as Endangered in Canada in 1999; a status re-examined and confirmed in 2000 and 2011 (COSEWIC, 2011). These aptly named anurans rarely leave the water and often select the same shallow, thermally stable oviposition sites year after year for their communally laid egg masses (McAllister and Leonard, 1997; Phillipsen *et al.*, 2010). Males typically arrive at breeding sites first and call to attract females for 1-3 weeks after spring ice-out. The number of eggs laid is highly variable and the number of egg masses per communal cluster can range from 5 to 75 (Licht, 1969; McAllister and Leonard, 1997). OSF have disappeared from up to 90% of their extrapolated historical range (McAllister and Leonard, 1997), with only six populations remaining in Canada, with fewer than 200 breeding pairs at each (Kendra Morgan, pers. comm.; Kissel *et al.*, 2017). The continuous loss of suitable wetlands, human intrusion and disturbance, invasive species, and pollution are only a few of the threats faced by this species across its limited range within British Columbia (COSEWIC, 2011; Environment Canada, 2015). Two zoos and one aquarium now operate independent but coordinated conservation breeding and reintroduction programs for OSF, with tadpoles successfully bred and released since 2010. Despite this, all three facilities have historically experienced low reproductive performance, a high incidence of reproductive dysfunction (particularly egg binding) and have no knowledge of their current or long-term genetic sustainability.

Objectives

The production of genetically healthy offspring is critical not only for demographic stability in *ex situ* populations but also for effective reintroduction efforts. Retention of genetic diversity and avoidance of inbreeding are important for reproductive success and overall fitness, yet neither

zoo nor wild OSF populations have been assessed in that respect. My first objective was to assess the genetic make-up of both *ex situ* and *in situ* populations to determine their sustainability and evaluate the management of wild populations as genetic sources for the zoo populations over the past decade. To this end, I measured genetic variability, inbreeding coefficients, and genetic structure from DNA by buccal swabbing frogs from both zoo and wild populations of OSF. Genetic variability has implications not only for the adaptive potential of captive-bred frogs being reintroduced to the wild, but also for the long-term sustainability of these programs and the species as a whole.

For such an endangered species, individual wellbeing within *ex situ* populations is also critical since wild sources are limited and should not be relied upon too heavily for supplementation of zoo populations. Mortalities resulting from female reproductive dysfunction (*i.e.* egg binding) have become common in amphibian breeding programs (Kouba *et al.*, 2009), but the causes of egg binding in particular remain unclear and not yet investigated. Given the high incidence of egg binding in OSF zoo populations, my second objective was to explore some of its potential causes, focusing on female body condition, follicular development and amplexus behaviours. I aimed to inform and improve current husbandry practices in OSF *ex situ* populations, provide cohesiveness to current Oregon Spotted Frog recovery efforts across Canada, and ultimately shed much-needed light on a reproductive issue impacting amphibian breeding programs worldwide.

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Chapter 1:

Ex situ management stabilizes the genetic health of Canada's most endangered amphibian, the Oregon Spotted Frog (*Rana pretiosa*); but is it enough?

Abstract

Retaining sufficient genetic variability for both short and long-term sustainability is a chief aim of *ex situ* programs for threatened species. Conservation breeding and reintroduction programs exist but oftentimes little is known about the genetic health of *in situ* or *ex situ* populations. We collected genetic samples from both wild and zoo populations of Canada's most endangered anuran, the Oregon Spotted Frog (*Rana pretiosa*), and compared their genetic diversity (observed heterozygosity (Ho), inbreeding coefficients (F), and effective population sizes (Ne)) using single-nucleotide polymorphisms (SNPs). We also assessed population structure to inform current breeding strategies. We found zoos have retained stable genetic variability despite the low diversity available in wild populations (maximum Ho = 0.155), but inbreeding levels remain high in both zoos and the wild and genetic diversity will be depleted from wild populations within 50 to 100 generations. Zoo populations were less differentiated from their wild source populations than the latter among themselves, indicating sufficient representation of wild populations in zoo populations. The patterns we uncover support continued collaboration of *ex situ* and *in situ* endeavours as supplementation will likely be required for the long-term viability of the very wild populations the zoos rely on for genetic sustainability.

Introduction

Human actions have placed more species at risk of extinction now than ever before (IPBES, 2019), with more and more species requiring active management both *in situ* (hereafter "wild") and *ex situ* (ex. zoos and aquaria; hereafter "zoos"). Yet, while conservation science has often prioritized *in situ* efforts, deeming methods such as captive breeding a last resort (Snyder *et al.*, 1996), the role of zoos in conservation has become increasingly more important and more accepted (Conde *et al.*, 2011). Zoos provide a unique setting for research and conservation of wildlife, hosting captive populations both as an assurance against extinction and for the purpose of conservation breeding. The latter programs strive to breed offspring *ex situ* for release *in situ,* thus boosting wild census population sizes and standing genetic diversity (Palm *et al.*, 2003; Osborne *et al.*, 2006); providing time for threats in the species' native habitat to be reduced or mitigated. As these actions complement and rely upon one another, they should be planned and managed collaboratively (Byers *et al.*, 2013; Johnson *et al.*, 2020).

Genetics in zoological conservation

While an explicit goal of breeding and reintroduction programs is the conservation of genetic diversity, there are inherent genetic risks associated with breeding small groups of animals *ex situ* and releasing them into the wild. Animals held *ex situ* may become adapted to captivity (Heath *et al.*, 2003; Frankham, 2008), experience reductions in effective population size through increased variance in family size (Ryman and Laikre, 1991), and face the concomitant increased risk of inbreeding depression (Falconer, 1981; Frankham, 1995). The release of zoo-sourced individuals might then depress rather than improve the mean fitness of the population(s) they supplement and reduce the probability of a species' long-term persistence (Osborne *et al.*, 2006). Therefore, a critical question for all conservation breeding programs is whether they are improving, or at least maintaining, the genetic health of wild populations. To this end, zoo populations must be demographically robust and genetically representative of the wild populations they are derived from, and both must be managed for long term sustainability (Lees and Wilcken, 2009). It is generally accepted that *ex situ* populations should maintain 90% of wild genetic diversity over 100 years and keep inbreeding levels below 0.125 to be considered sustainable (Soulé *et al.*, 1986; Ballou *et al.*, 2010). Intentional management of breeding populations can also bring *ex situ* programs closer to a sustainable effective population size (Lees and Wilcken, 2009) (generally, Ne of at least 500), which is the number of individuals from the total population size (N) contributing to the next generation (Wright, 1931). These three goals (rate of genetic diversity loss, $F < 0.125$, and $Ne > 500$) aim to retain sufficient adaptive potential in captive populations to achieve long-term *ex situ* persistence and produce offspring suitable for supplementation or reintroduction efforts (Lees and Wilcken, 2009). Measuring and tracking genetic metrics such as individual pedigrees, genetic diversity, inbreeding, and effective population size in *ex situ* populations as well as in wild populations affected by *ex situ* practices is thus key. Indeed, a recent study found that the genetic diversity retained in translocation efforts for small populations could be significantly improved by optimizing the composition of individuals (Bragg *et al.*, 2021). Such optimization not only requires basic genotyping of source populations but is dependent on the genetic variability available within, as well as the level of genetic differentiation between populations (Bragg *et al.*, 2021). Understanding the genetic

variability available in all wild populations is also crucial in identifying sustainable source populations and ensuring these genetic lineages are maintained *ex situ* if their survival in the wild is at risk (Shaffer *et al.*, 2015). Thus, assessing genetic health in both zoo and wild populations is essential for informed and effective conservation breeding and reintroduction programs.

Amphibian extinction crisis

Globally, amphibians are more at risk of extinction than reptiles, birds, or mammals, facing a suite of threats including habitat loss, invasive species, and several infectious diseases (Stuart *et al.*, 2004; Green *et al.*, 2020). Amphibian species are also particularly prone to loss of genetic diversity (Allentoft and O'Brien, 2010); they often have naturally low effective population sizes (Funk *et al.*, 1999; Rowe and Beebee, 2004; Beebee and Griffiths, 2005) which, coupled with their overall population declines, leaves them more vulnerable to genetic drift and inbreeding (Allentoft and O'Brien, 2010). Habitat fragmentation, one of the most severe threats to amphibians, reduces gene flow between populations, producing small sub-populations in which genetic differentiation increases but genetic diversity erodes much faster (Hitchings and Beebee, 1997; Lesbarrères *et al.*, 2006). Even in unfragmented habitat, local extinctions are common (Cushman, 2006) and naturally low dispersal rates (common to many amphibians) increase the risks of genetic drift (Allentoft and O'Brien, 2010).

In the face of these threats, amphibians are prime candidates for *ex situ* breeding, due to their relatively low holding costs, high reproductive output, and high conservation impact (Bloxam and Tonge, 1995; Tapley *et al.*, 2015). The number of amphibian collections in zoos has particularly increased in response to chytridiomycosis, a deadly disease devastating wild populations of amphibians worldwide (Conde *et al.*, 2011; Dawson *et al.*, 2016; Estrada *et al.*, 2022). Yet, tracking *ex situ* amphibian pedigrees, necessary for genetic management, has proven challenging because they are often managed in large groups, breed communally, and show high inter-individual variability in reproductive output, making the retention of genetic lineages difficult (Carrillo *et al.*, 2015). One highly vulnerable species under *ex situ* management is the Oregon Spotted Frog (*Rana pretiosa*; OSF), Canada's most endangered anuran (COSEWIC, 2011).

The Oregon Spotted Frog

The Oregon Spotted Frog had already been lost from as much as 90% of its extrapolated historical range by 1997 (McAllister and Leonard, 1997; Environment Canada, 2015) and the 6 remaining Canadian populations are estimated to have fewer than 200 breeding pairs each (Kissel *et al.*, 2017). The continuous loss of suitable wetlands, human intrusion and disturbance, invasive species, and pollution are only a few of the threats faced by this species across its limited range within British Columbia (COSEWIC, 2011; Environment Canada, 2015). Moreover, habitat fragmentation has left all extant populations genetically isolated and susceptible to inbreeding depression, further loss of genetic diversity, and to stochastic events (Allendorf and Luikart, 2007; Environment Canada, 2015). Due to their highly threatened status, OSF thus

require *ex situ* conservation in addition to ongoing mitigation of *in situ* threats (Conde *et al.*, 2011).

Two zoos and one aquarium (hereafter "zoos") established independent but coordinated conservation breeding and reintroduction programs for OSF in the 2000s, but the genetic sustainability of these *ex situ* populations has not been assessed. The limited work on OSF genetics focused on populations from its American range, finding low genetic diversity in the wild (Blouin *et al.*, 2010; Phillipsen *et al.*, 2010; COSEWIC, 2011). Data collected in 2009 suggested that Canadian populations were distinct from American ones, displayed small effective population sizes, and were likely experiencing inbreeding (Blouin pers. comm. 2009 in COSEWIC 2011). Since collecting individuals from already genetically impoverished populations into captivity can depress population sizes, increasing the risk of inbreeding and genetic drift (Willoughby *et al.*, 2015), it is imperative to assess the current level of genetic diversity in both zoo and wild OSF populations.

Using Genotyping-By-Sequencing (GBS), we investigated patterns of genetic variability and structure in both *ex situ* and *in situ* populations with the goals of (i) assessing whether this measured variability might be sufficient for long term genetic sustainability of the species in Canada and (ii) informing reintroduction efforts based on current population structure. We compared genetic diversity between and among three zoo and five wild OSF populations, predicting that it could be either lower in zoos due to founding effects or higher due to cross-breeding of differentiated wild sources. We also estimated the effective population sizes (Ne) of both wild and zoo populations and calculated their inbreeding coefficients (F). We predicted zoos would have relatively high Ne due to management of population demographics, but higher F than wild populations due to fewer mating options between non-related individuals in the zoos. Finally, we assessed wild OSF population structure to inform *ex situ* demographics and breeding strategies, predicting each population would be highly differentiated due to their isolated locations, limited dispersion capabilities, and small Ne-induced drift.

Results

Genetic Diversity

Based on total DNA extracted from 322 individuals we constructed a GBS library (using *PstI/MspI restriction enzymes)*, performed Illumina Novaseq sequencing, filtered the raw data, and called single nucleotide polymorphisms (SNPs) with STACKS v.2.62 (Catchen *et al.*, 2013) (*denovo* mode). This resulted in a total of 22,230 SNPs genotyped across 321 samples from three zoo and five wild populations (Fig. 1.1). Fewer than 95 SNPs had alleles unique to the zoo or wild categories. The mean observed heterozygosity by population ranged from 0.124 (MV) to 0.155 (ST) with the three zoo's Ho ranging from 0.129-0.130 (Table 1.1). Zoo (n=239) and wild (n=82) samples did not differ in mean Ho (Kruskal-Wallis test p=0.45). Pairwise comparisons of populations identified EK as having significantly greater Ho than MS (one-way ANOVA with post-hoc Tukey HSD, p=0.01), MV (p=0.002), GVZ (p=0.03), and VA (p=0.01), although the small sample size for ST (n=3) limited the statistical power (Fig. 1.2a).

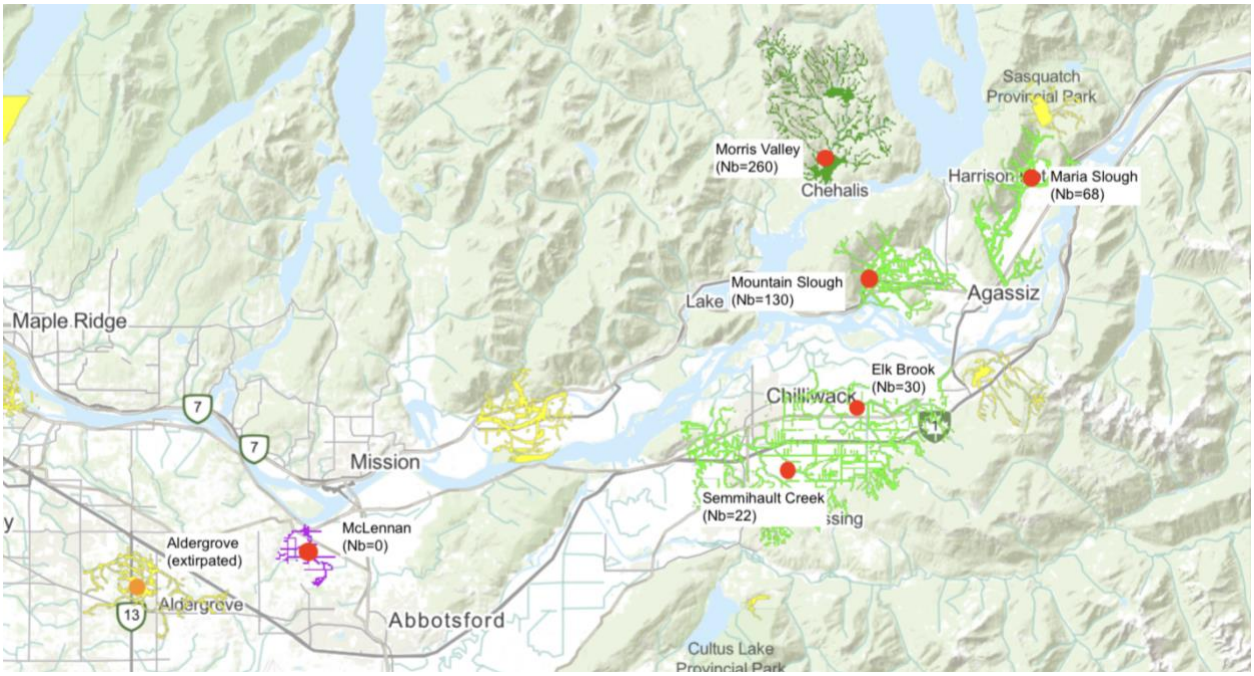

Figure 1.1. The remaining wild populations of Oregon Spotted Frog in British Columbia, Canada. Each population is indicated by a red circle within the known watershed (coloured green), with the estimated breeding population size (Nb) from egg surveys in 2022. McLennan is still considered extant but no egg masses or frogs have been seen since 2017.

Inbreeding coefficients (F) ranged from 0.083 (ST) to 0.268 (MV; Table 1.1). EK's (F) was significantly lower than that of all other populations except MT, ST, and TZ (one-way ANOVA, p=0.0005; significant post-hoc Tukey HSD comparisons: EK:MS p=0.01, EK:MV p=0.002, EK:GVZ p=0.03, EK:VA p=0.01). ST's F was lower than EK's but no significance was found due to low statistical power (n=3; Fig. 1.2b). Zoo and wild category inbreeding coefficients did not significantly differ (Kruskal-Wallis p=0.45) with values of 0.24 and 0.23, respectively. Effective population sizes (Ne) for populations with at least 15 individuals ranged from 4.4 (MS) to 19 (MV) in the wild (Ne was not calculated for MT and ST due to low statistical power), and were 16.1, 31.8, and 34.8 for TZ, GVZ, and VA, respectively (Table 1.1). Interestingly, the breeding population sizes (Nb) estimated from egg mass surveys were much larger than the estimated Ne values (Table 1.1). Using estimated Ne values, we projected the loss of heterozygosity over time and observed that MS is expected to lose all genetic diversity in 50 generations (one generation = 2 years) while it will take 150 generations for EK and 200 for MV (Fig. 1.3).

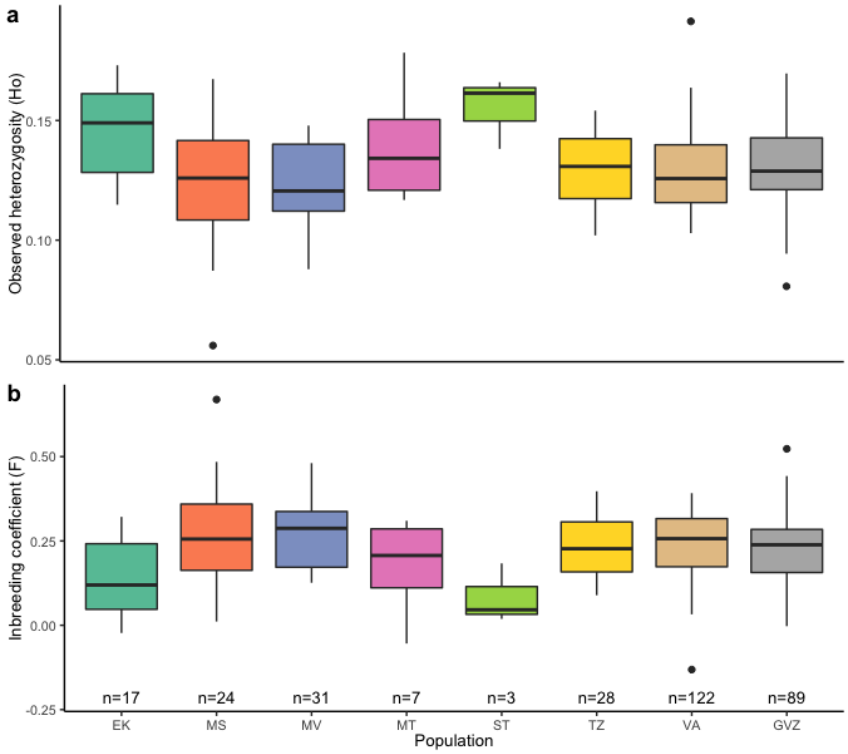

*Figure 1.2. Genetic diversity estimates for all Oregon Spotted Frog populations sampled in Canada. Mean estimates of **a)** observed heterozygosity (Ho), and **b)** inbreeding coefficients (F) at five wild (EK, MS, MV, MT, ST) and three zoo (TZ, VA, GVZ) populations.*

Table 1.1. Mean genetic diversity indices with standard error for Oregon Spotted Frog populations calculated from a total of 22,230 SNPs. The first two rows display average values for combined zoo (from populations TZ, VA, GVZ) and wild (from populations EK, MS, MV, MT, ST) individuals respectively. N = sample size, Ho = observed heterozygosity, F = inbreeding coefficient, Ne = effective population size. The number of breeding adults (Nb) was determined by egg mass surveys for wild populations and represented the total number of adults for zoo populations.

POPULATION	N	H_o	F	N_e	N_b
ZOOS (n=3)	239	0.129 ± 0.001	0.235 ± 0.006		
WILD (n=5)	82	0.131 ± 0.002	0.226 ± 0.015		
Elk Brook (EK)	17	0.145 ± 0.005	0.144 ± 0.027	12.5 (7.6 - 22.3)	112
Maria Slough (MS)	24	0.125 ± 0.005	0.259 ± 0.031	4.4 (2.5 - 8.5)	154
Morris Valley (MV)	31	0.124 ± 0.003	0.268 ± 0.018	19 (12.1 - 32.1)	348
Mountain Slough (MT)	7	0.139 ± 0.008	0.179 ± 0.050	*	102
Semmihault Creek (ST)	3	0.155 ± 0.009	0.083 ± 0.051	*	22
Toronto Zoo (TZ)	28	0.129 ± 0.003	0.237 ± 0.018	16.1 (11.4 - 23.9)	19
Vancouver Aquarium (VA)	122	0.129 ± 0.001	0.238 ± 0.009	34.8 (29.4 - 41.5)	111
Greater Vancouver Zoo (GVZ)	87	0.130 ± 0.002	0.231 ± 0.010	31.8 (26.7 - 38.2)	98

**values could not be computed accurately due to low sample size*

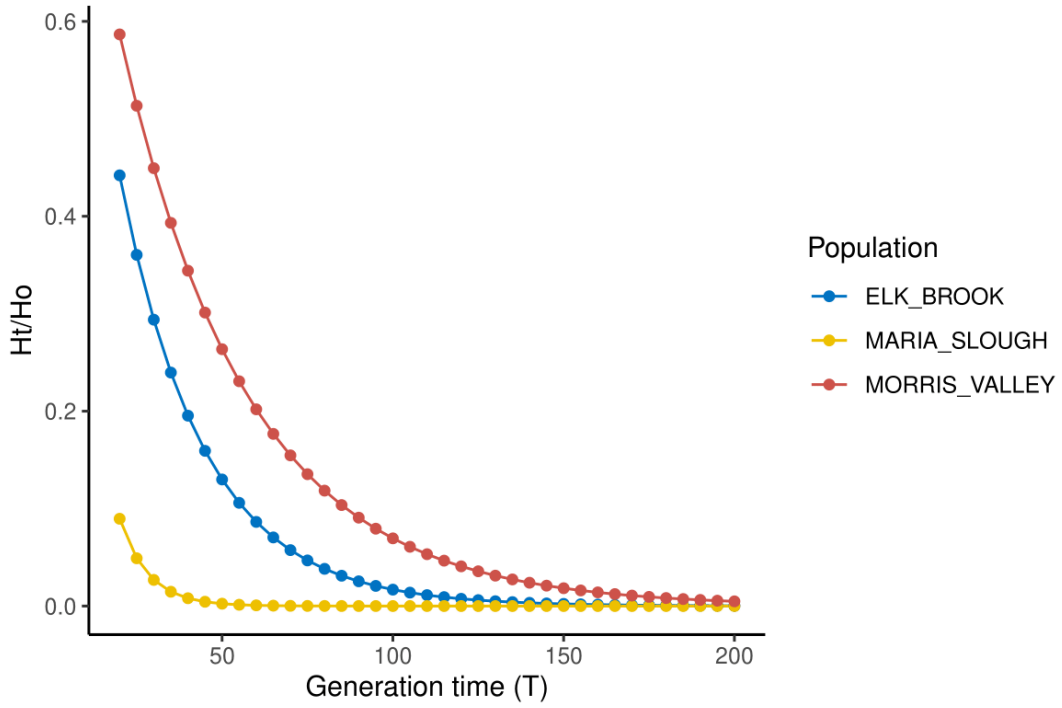

Figure 1.3. Heterozygosity loss through time in three wild populations of Oregon Spotted Frog. The proportion of remaining heterozygosity (Ht/Ho) was inferred from effective population size estimates (Ne) for T = 20 to T = 200. Generation time (T) for OSF is 2 years.

Population Structure

A principal component analysis (PCA) conducted on genotypes of all individuals revealed an absence of clustering according to zoo *vs.* wild populations but rather exhibited clustering according to individual genetic source, with zoo frogs clustering according to their respective genetic sources (*i.e.* their tracked lineage). Three main clusters, with a less distinct fourth, were differentiated by the first and second PC axes (PC1: 23.17%, PC2: 16.96%; Fig. 1.4a). Among the wild populations, EK and ST clustered together, while MS, MV, and MT form unique clusters, although MT is barely distinguished from the MS cluster (Fig. 1.4a). The third PC axis (11.39%) differentiated an MT cluster from a cluster consisting of MS + MV, while the fourth PC axis (6.79%) delineated a cluster of EK + ST from zoo cross-breeds of EK x ML (Fig. 1.4b). Furthermore, using a PCA with just the main wild populations, EK, MS, and MV formed distinct clusters when considering the first and second PC axes (Fig. 1.4c), while the third and fourth PC axes differentiated an MS + MV cluster from EK (Fig. 1.4d).

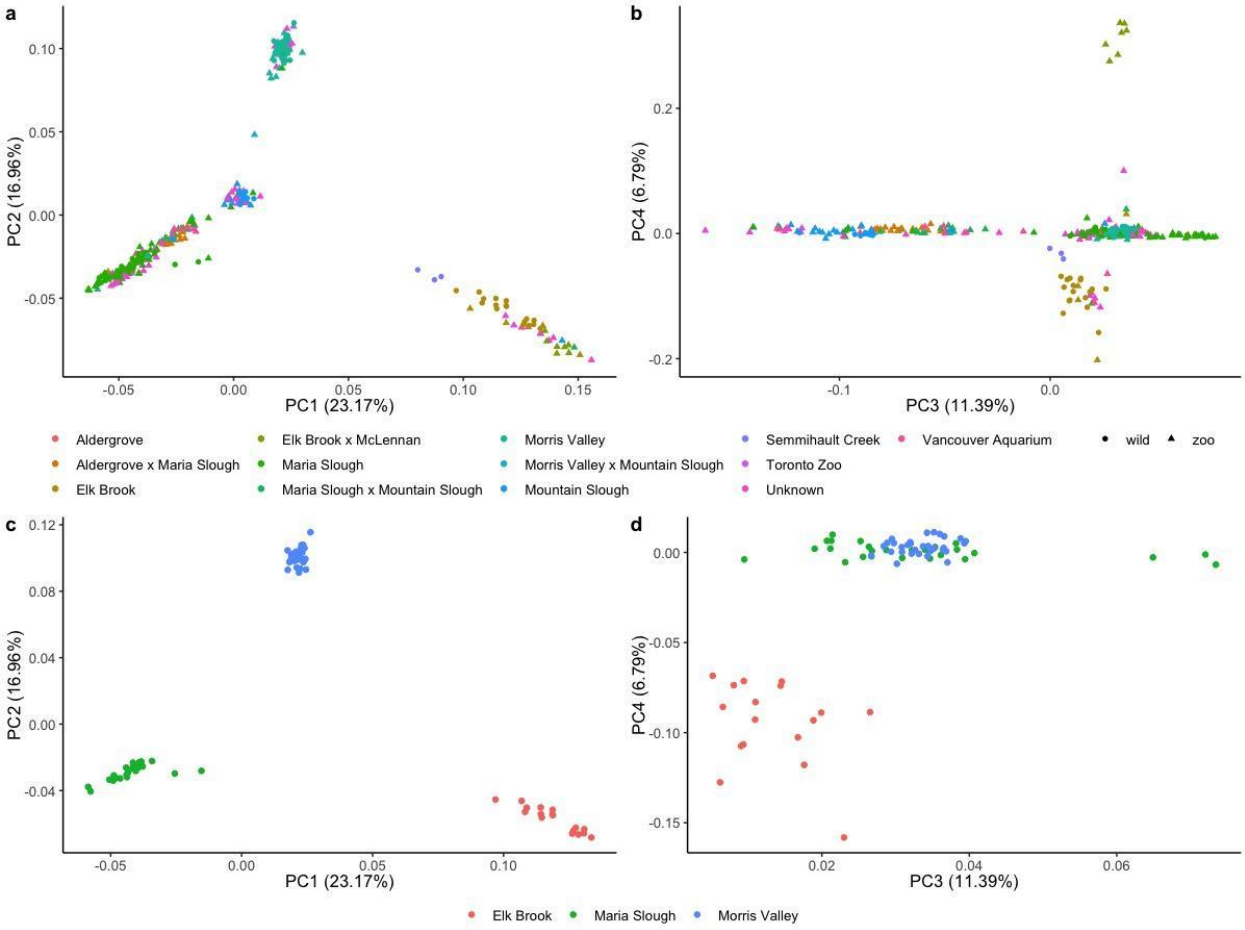

Figure 1.4. Principal component analyses of Oregon Spotted Frog populations. Each axis indicates the amount of variation respectively explained in the data. ***a****) and* ***b****) contain all sampled populations coloured by their genetic source, with wild-caught frogs indicated by circles and frogs sampled in zoos indicated by triangles. Cross-bred zoo frogs are indicated by an 'x' in the name (e.g. Aldergrove x Maria Slough). Zoo frogs with unknown parentage are indicated as "unknown" or according to their respective zoo (if many-generations zoo-born).* ***c****) and* ***d****) contain only the three wild populations with ≥ 15 samples.*

We also investigated genetic structure for K ranging from 2 to 15. Cross-validation indices (Appendix I.I, Fig. A1.1) and population history suggested the best statistical K to be within K=8-10 (Fig. 1.5a). When grouping ADMIXTURE results by population, wild populations formed distinct clusters in comparison to the very mixed zoo populations. EK formed a distinct cluster at K=8-10 (Fig. 1.5a) and remained distinct all through K=15 (Appendix I.I, Fig. A1.2). MV was a distinct cluster until K=7 but became mixed at K=8, sharing some of its ancestry here with MT; for K=9-10 however, MV was still mixed but no longer clustering with MT (Fig. 1.5a). MT remained a relatively distinct cluster until K=13, with the aforementioned grouping with MV occurring at K=8 only. MS showed more variation than the other wild populations and had 24% mixed ancestry by K=5 (Appendix I.I, Fig. A1.2). Zoo populations were all highly mixed from K=2 onwards, displaying all the wild population ancestry and showing some unique ancestry as well (Fig. 1.5a). When grouping individuals by genetic source, zoo cross-breeds (EK x ML) clustered

with EK until K=10, after which they formed a distinct cluster (Appendix I.I, Fig. A1.3). Aldergrove x MS did not cluster with MS but showed distinct ancestry through K=8-10 aligning primarily with MS x MT crosses for K=8 but forming its own distinct cluster for K=9-10. Individuals with EK in their genetic source presented distinct (≥95% unmixed) bars. Since MS itself was mixed when K=8-10, its main ancestral sources (indicated by different colours in Fig. 1.5a) were all present in zoo populations, appearing there as both mixed and distinct bands. Where MV appeared in zoo populations, it almost always formed distinct bands. When analysing the five wild populations alone, the best statistical K was 3 (Appendix I.I, Fig. A1.4), with EK, MS, and MV representing distinct clusters, while MT and ST were mixtures of the other three (Fig. 1.5b). The ST individuals grouped primarily (82%) with EK while the MT bands grouped primarily (54%) with MS. For K=4 however, MT formed a distinct cluster while ST remained grouped with EK (Appendix I.I, Fig. A1.4).

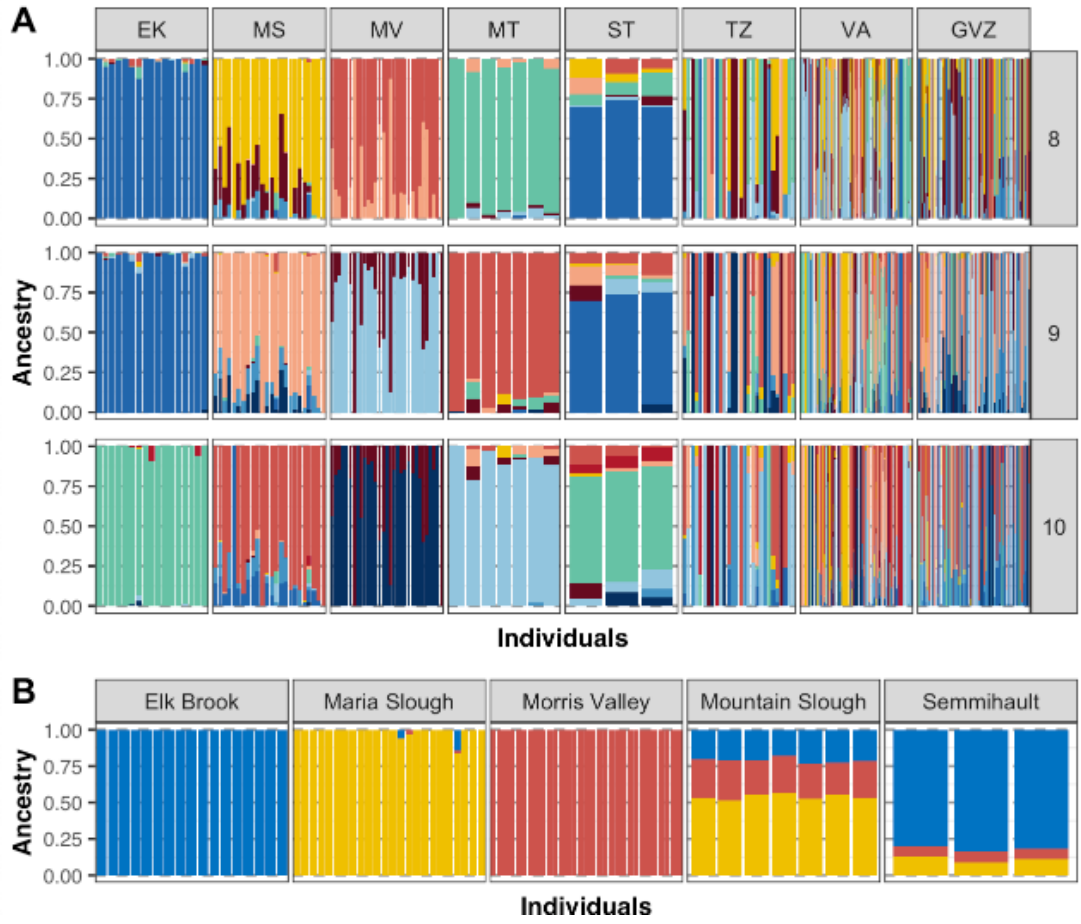

*Figure 1.5. ADMIXTURE population structure analysis with individuals grouped by population for **a)** wild (EK, MS, MV, MT, ST) and zoo populations (TZ, VZ, GVZ) from K=8 to K=10, and **b)** wild populations (MS, MV, EK, MT, ST) for K=3 where K is the inferred number of ancestral populations.*

Weir and Cockerham's Fst-values were calculated for the zoo and wild categories as well as individual populations. Comparing the combined zoo population samples and the combined wild population samples resulted in Fst = 0.012 (ranging from 0 to 0.232). All zoo populations

were <1% differentiated from one another (Table 1.2) while the average pairwise Fst between wild populations was 0.105, ranging from 0.088 (between MS and MV) to 0.116 (between EK and MV; Table 1.2). Pairwise Fst between individual wild and zoo populations were lower than between wild populations themselves (Table 1.2), with EK the most differentiated from all zoo populations (mean Fst [0.068-0.082]) and MS the least differentiated (mean Fst [0.012 – 0.024]). Furthermore, while average Fst values remained below 0.12 among all three wild populations, some genomic regions were highly differentiated (e.g. 0.975, 0.979, and 0.963 maximum range values; Table 1.2).

Table 1.2. Mean Fst estimates (range in brackets) across Oregon Spotted Frog SNPs. Zoo populations are: Vancouver Aquarium (VA), Greater Vancouver Zoo (GVZ), and Toronto Zoo (TZ). Wild populations are: Elk Brook (EK), Maria Slough (MS) and Morris Valley (MV). Two wild populations were not included due to low sample size (ST and MT).

Population	VA	GVZ	TZ	EK	MS	MV
GVZ	0.01 [0; 0.29]					
TZ	0.003 [0; 0.28]	0.008 [0; 0.32]				
EK	0.068 [0; 0.835]	0.082 [0; 0.84]	0.068 [0; 0.826]			
MS	0.022 [0; 0.499]	0.012 [0; 0.504]	0.024 [0; 0.568]	0.111 [0; 0.975]		
MV	0.041 [0; 0.729]	0.053 [0; 0.70]	0.053 [0; 0.812]	0.116 [0; 0.979]	0.088 [0; 0.963]	

Discussion

Conservation breeding programs are often established to “buy time” for species on the brink of extinction (Byers *et al.*, 2013). Such programs have played a critical role in reducing the threat level in 16 of the 64 species down-listed by the IUCN (Traylor-Holzer *et al.*, 2018). In amphibians, the number of species managed *ex situ* for conservation breeding and reintroduction has greatly increased since 2007 (Dawson *et al.*, 2016; Harding *et al.*, 2016) but not all of these species are in fact suitable for *ex situ* actions (Bradfield *et al.*, 2022). Endangered species with existing conservation breeding programs should be assessed to ensure *ex situ* actions are warranted (Bradfield *et al.*, 2022) as successful breeding *ex situ* is not sufficient in itself (Carrillo *et al.*, 2015) because offspring must have sufficient genetic diversity for short and long-term population adaptation to the wild as well (Lees and Wilcken, 2009). In comparing genetic diversity and population structure indices among both *ex situ* and *in situ* populations of Oregon Spotted Frog in Canada, our study underscores the encouraging implementation of recovery strategies for this species and provides the basis for practical recommendations for conservation breeding and reintroduction efforts more broadly.

Although the genetic diversity of American populations of OSF is low compared to other ranids (Blouin *et al.*, 2010; Phillipsen *et al.*, 2010; COSEWIC, 2011), the three zoos hosting breeding populations of OSF have maintained sufficient genetic health *ex situ*. The combined zoo genetic diversity surpasses the 90% threshold relative to the combined wild populations, although EK, ST, and MT showed greater genetic diversity individually than any of the three zoos. On a less encouraging note, the zoo populations all exceed the recommended inbreeding coefficient (0.125; WAZA, 2011) and the only wild population below this threshold is ST (an estimate which may be inflated by its small sample size). All three zoos have a lower F than MS and MV though. Inbreeding itself may not severely impact OSF as other vulnerable species have been found to persist with high inbreeding levels (Wang *et al.*, 2015); regardless, all OSF populations should be monitored for signs of inbreeding depression (e.g. decreasing fertility and offspring survivorship). Founding zoo populations with fewer than 200 individuals increases susceptibility to genetic drift, inbreeding depression, and demographic stochasticity, and leads to decreases in population size and genetic diversity (Lacy, 1997; Ballou *et al.*, 2010). The apparent lack of these genetic declines here points to the effectiveness of ongoing *ex situ* management. Other zoos have likewise been found to maintain stable genetic diversity and inbreeding levels despite few founders and small population sizes (Che-Castaldo *et al.*, 2021; Humble *et al.*, 2023). Many zoo populations rely on cooperative management to effectively prevent demographic and genetic declines and Che-Castaldo et al. (2021) argue that such stability is an achievement in itself under the current biodiversity crisis. Relatively low Ne has been observed in amphibian populations in the wild (Funk *et al.*, 1999; Rowe and Beebee, 2004; Beebee and Griffiths, 2005), as Ne is affected by many factors including variance in reproductive success (Palstra and Ruzzante, 2008) and limited dispersal ability (Nunney, 2016). Through careful management of these factors, such as increasing the number of breeding individuals, stabilizing population sizes across generations, and equalizing operational sex ratios *ex situ*, zoos can increase their Ne (Lees and Wilcken, 2009). Such effective management is taking place at all three zoos where we estimated higher Ne-values relative to wild populations despite the latter harbouring more than twice the number of breeding adults (Nb) according to ongoing population monitoring. While Ne in all three zoo populations remains well below the conservative goal for short term persistence (Ne=50; Franklin, 1980)) and the threshold to avoid inbreeding depression (Ne ≥ 100; Frankham *et al.*, 2014)), increasing Ne in zoos relative to the wild will help reduce genetic drift and improve retention of genetic diversity.

The Oregon Spotted Frog may have had low Ne and genetic diversity in the wild for many generations. Rapid reduction in genetic diversity due to anthropogenic effects (*i.e.* habitat fragmentation) enhances the risk of inbreeding depression (Allentoft *et al.*, 2009), but if a species has existed for generations with low genetic diversity, many deleterious alleles may have already been purged and the risk of inbreeding depression is thus reduced relative to species with historically large Ne (Allentoft and O'Brien, 2010). Although OSF have experienced continued habitat fragmentation over the last 150 years (COSEWIC, 2011), it is possible that subsequent sub-populations persisted in their fragmented landscape with low genetic diversity due to their naturally low dispersal rates and highly aquatic nature. Cushman (2006) has suggested that those species with low dispersal rates have higher-than-expected survivorship in small and fragmented habitats due to decreased mortality risks associated with dispersal. For

example, OSF do not experience the road mortality rates of other amphibians (Watson *et al.*, 2003), perhaps contributing to their persistence. Alternatively, it is possible that allelic diversity has decreased while heterozygosity remains stable in the zoo populations (Säisä *et al.*, 2003; Osborne *et al.*, 2006). Kraaijeveld-Smit et al. (2006) found allelic diversity decreased in Mallorcan toad breeding programs after 3-8 generations while heterozygosity took 12 generations to decrease in comparison to wild populations. This discrepancy may be due to allelic richness being more sensitive than heterozygosity to bottlenecks (Kraaijeveld-Smit *et al.*, 2006), so the former should also be assessed in light of potential local selection which would inform breeding strategies and recovery actions.

Our genetic structure analysis revealed that average Fst values were moderate (Wright, 1978) among wild populations of OSF (Fst [0.09-0.12]) and low among zoos (Fst [0.003-0.01]) and between zoos *vs*. wild populations (Fst [0.012-0.082]). In particular, biologically relevant differentiation was observed between EK and MS, and EK and MV, with mean pairwise Fst > 0.1. In their study on OSF genetics, Blouin et al. (2010) sampled three Canadian populations and found they were all well differentiated from one another but suggested this finding was inflated by small sample sizes. Yet, while average Fst values are only moderate among wild populations, some genomic regions were highly differentiated (maximum Fst = [0.96-0.98]). Further research should be conducted with a reference genome to investigate whether these regions of fixation are a result of local adaptation or random genetic drift, as is prevalent in isolated populations (Frankham, 1996; Wang *et al.*, 2014). Several studies have reported small, isolated populations of amphibians in fragmented landscapes to be highly differentiated (Hitchings and Beebee, 1997; Lesbarrères *et al.*, 2006), especially species with low dispersal capability and high site fidelity (Shaffer *et al.*, 2000; Spear *et al.*, 2005). In this context, the low to moderate average Fst values may be a result of the Fraser River. Rivers have mixed effects on amphibian dispersal (Ficetola and Bernardi, 2004; Mikulíček and Pišút, 2012) and the Fraser River may have historically improved gene flow between populations while extensive dyking to prevent flooding in the area may now decrease gene flow (Environment Canada, 2015). Both the ADMIXTURE and PCA results also indicate historical mixing of populations in the Fraser River floodplain (MS, MV, MT) suggesting that the latter acted as a water corridor for OSF as they travel almost exclusively by water (Watson *et al.*, 2003; Pearl and Hayes, 2004).

Maria Slough (MS) shows the most admixture among the extant wild populations, and this admixture is also visible in zoo frogs with MS as their genetic source. The lack of clustering with other extant populations, however, suggests MS was once connected to ancestral sources, perhaps from further upriver. The low genetic diversity and high inbreeding observed in MS may indicate these sources, if still extant, are no longer connected to MS (Allendorf and Luikart, 2007). Interestingly, some of these ancestral genetics have been carried into the zoo populations, evidenced by zoo frogs with distinct ADMIXTURE ancestry (*i.e.* unique colours) but MS parentage. More recently extirpated populations (*i.e.* Aldergrove) are also represented in zoo populations, which explains why the optimal K for all populations (zoo and wild; K=8-10) is more than 2x larger than that estimated for only the five wild populations (K=3). It will be critical to identify these unique individuals and ensure persistence of these ancestral lines in the breeding programs to carry on this impressive level of genetic representation. Of the remaining

extant OSF populations, EK and ST show a separate and perhaps ongoing connectivity. These two populations lie in a watershed network of ditches but known frog occurrences are separated by more than 3 km, their maximum travel distance (Pearl and Hayes, 2004). Importantly, the hydraulic connectivity of the ditch networks is currently not well understood. These ditches generally lack the stability of shallow shelf oviposition habitat which occurs more readily in slough and wetland habitat types (Environment Canada, 2015), and may be prone to more frequent, high velocity flows. It is possible the continuous corridor of deep water in this ditch habitat increases movement and connectivity as larger OSF movements (>1 km) have been documented along analogous linear riparian systems in the past (Pearl and Hayes, 2004). Human-modified environments can sometimes unintentionally increase connectivity or create additional habitat for amphibians (Wang *et al.*, 2014) and it is thus possible that this human-made ditch system has increased connectivity between EK and ST. Such a connection should be thoroughly investigated and protected for the role these populations might play in the long-term sustainability of OSF in Canada.

The retention of comparable genetic variability among zoos and wild populations may indicate encouraging stability in the face of potential genetic decline (Che-Castaldo *et al.*, 2021), but is stability enough for the persistence of OSF in Canada? One of the primary source populations for the conservation breeding programs (MS) is projected to lose all genetic diversity within 50 generations. This not only highlights the necessity of conservation breeding and reintroduction programs to ensure the persistence of OSF wild populations, but also suggests some changes should be made to their implementation. The frequent collections from MS have likely contributed to its genetic decline, in turn jeopardizing the sustainability of the zoo populations which depend on both their internal population genetics and that of their source populations (Lees and Wilcken, 2009). Thus, external supplementation of zoos should be adjusted both to protect MS and to diversify zoo population demographics so as not to rely too heavily on one source. Optimizing the individual composition of zoo populations can also significantly improve the genetic diversity they harbour (Bragg *et al.*, 2021). Together, this suggests that incorporation of more EK and ST frogs in zoo populations might increase *ex situ* genetic diversity as these populations have the best genetic health of all wild populations. Optimization of zoo genetics also depends on the level of differentiation among sources (Bragg *et al.*, 2021) so all offspring should be monitored for signs of both inbreeding and outbreeding depression. While concerns for the latter are increasingly hailed as overstated, it may take generations to manifest (Bell *et al.*, 2019), so monitoring is warranted. Such monitoring will also inform potential supplementation of MS which should be strongly considered lest we monitor this population to the point of extirpation rather than risk taking *in situ* action alongside ongoing *ex situ* actions (Ralls *et al.*, 2018). The collaboration of both *ex situ* and *in situ* partners in the OSF recovery team has already contributed much to the persistence of the Oregon Spotted Frog in Canada and this study suggests continued, unified efforts between these two arms of conservation may prove an essential strategy for the genetic sustainability of endangered species in general.

Methods / Analysis

Sampling

All extant wild populations of OSF in Canada – Morris Valley (MV), Mountain Slough (MT), Maria Slough (MS), Elk Brook (EK), Semmihault Creek (ST), and McLennan (ML) – were sampled in the spring of 2021. These populations are between 3 and 55 kms apart, occurring in floodplain marshes, sloughs, or channelized watercourses in the Fraser River Lowlands of British Columbia with low to moderate amounts of emergent vegetation and silty substrate (Environment Canada, 2015; Fig. 1.1). Egg mass surveys are conducted annually (March-May) at each of these sites and a more extensive capture-mark-recapture program is ongoing at both MS and MV. The Aldergrove (AD) population was recently extirpated but remains represented *ex situ* by a few frogs of Aldergrove descent.

Two zoos and one aquarium currently run conservation breeding and reintroduction programs as part of a combined recovery effort for this endangered species. Due to differing facilities, resources, and location, each zoo has a unique program and approach. The Vancouver Aquarium (VA) began its breeding program in 2010 and regularly holds 100-200 OSF. The Toronto Zoo's (TZ) program, established as both a breeding program and assurance population, started in 2010 as well and holds an average of 20 OSF at a time. While the Greater Vancouver Zoo (GVZ) has helped headstart OSF since 2003, their year-long breeding program did not begin until 2017 but now holds roughly 80-100 OSF.

Genetic samples were collected primarily by buccal swabbing (Broquet *et al.*, 2007), an effective and less harmful alternative to toe clipping. Each swab was collected by gently prying open the frog's mouth with a flat tip (*i.e.* guitar pick) and rolling a sterile cotton swab around the inside of the mouth (Pidancier *et al.*, 2003). Swabs were dried and stored at -20°C until DNA extraction. When dead OSF were found in the wild or captivity, tissue samples were collected (approximately 5x5 mm sample of skin or muscle) and stored in ethanol at -20°C. A total of 7-30 unique swabs were collected from each of the four wild populations (MS, MV, MT, EK) during the breeding season (March-April), when frogs were most active. In addition, five eggs each from different egg masses were collected and stored in ethanol at -20°C for the wild populations EK and ST, as no frogs were captured at the latter site. No eggs or frogs were found at ML in 2021 but this lineage remains represented in zoo frogs. Buccal swabs were also collected from all mature (≥ 2 years old) OSF at the Vancouver Aquarium (n=129), Greater Vancouver Zoo (n=91), and Toronto Zoo (n=19).

All samples were collected in accordance with approved Animal Care protocols from Laurentian University (2019-02-01, File No.6020970), Toronto Zoo (Project #2021-02-01), Vancouver Aquarium (#2021-01), Greater Vancouver Zoo (Approval #2021-02-18), and the Ministry of Forests, Lands, Natural Resource Operations and Rural Development (Wildlife Act Permit: SU21-618374).

Extraction and Sequencing

DNA was extracted using a modified protocol from Qiagen DNEasy Blood and Tissue kits adapted to swabs. We evaluated DNA quality on a 1% agarose gel and quantified yield on a Qubit dsDNA HS Assay Kit. Due to low DNA yield, individual samples were then modified to 10 ng/uL for construction of Genotyping by Sequencing (GBS) libraries. All samples below this concentration were considered unsuitable for sequencing (n=5). Laval University's genomic analysis platform constructed the GBS libraries (*PstI/MspI*) and Génome Québec performed Illumina Novaseq sequencing. Data preparation, genotyping, and filtration were done by Laval University's genomic analysis platform using STACKS v.2.62 (Catchen *et al.*, 2013; *denovo* mode), using only forward reads. During bioinformatics treatment, 84 samples were dropped as they were missing more than 15% of genotype calls, were too similar, or had extremely low heterozygosity. We used a minimum genotype coverage of 4, and excluded SNPs which were missing for more than 20% of samples. A total of 5.54 billion reads were demultiplexed and cleaned, translating to an average of 17.2 million reads per sample. Further in the analysis, one MS sample was removed from the dataset due to questionable origins, leaving 321 samples for final analysis.

Bioinformatics

Putative bias due to missing data was investigated by performing an identity-by-missingness analysis on the filtered SNPs using PLINK version 1.90b5.3 (Purcell *et al.*, 2007). The resulting multidimensional scaling was represented graphically using sequencing plate number, sample type, population type (zoo *vs.* wild), and source information. No clustering by missingness (a signature of bias) was found (Appendix I.I, Fig. A1.5).

Genetic Diversity

Genetic diversity was assessed using four different indices within each sampled population as well as within the two following two categories: wild *vs.* zoo individuals. First, the minor allele frequency (MAF) of each SNP was estimated using the -freq argument in PLINK, and the number of polymorphic SNPs (MAF different from 0 or 1) was reported. Then, the individual observed heterozygosity (Ho) and inbreeding coefficient (F) were assessed using *vcftools* (Danecek *et al.*, 2011) and averaged within each population and category described above. Comparisons between populations and categories were investigated by performing a one-way analysis of variance (ANOVA) or a Kruskal-Wallis test (in the case of non-normal data) with the R package "stats" (v4.1.1; R Core Team, 2021)). Finally, effective population size was estimated for populations with at least n=15 using NeEstimator 2.0.1 (Do *et al.*, 2014) and the LDNe algorithm (Waples and Do, 2008) with a lowest allele frequency of 0.1. For comparison, breeding adult population sizes (Nb) were estimated for all wild populations from egg mass surveys in 2021 as 2 x the number of egg masses. This method was determined to be an effective method of estimating Nb for OSF (Phillipsen *et al.*, 2010) and is the primary means by which population size is monitored by the OSF recovery team. The Nb for all zoo populations was determined by a count of all sexually mature (≥ 2 years-old) adults in 2021. Loss of heterozygosity through the next 200 generations was inferred from the Ne estimates using the following equation:

Proportion of remaining heterozygosity = $(1 - \left(\frac{1}{2Ne}\right))^t$, where t is the number of generations.

Genomic Differentiation

We investigated the genetic structure among all sampled populations using three approaches. First, a principal component analysis (PCA) was conducted on the individual genotype data using the -pca argument in PLINK. The R package "ggplot2" (Wickham, 2009; R Core Team, 2021) was used to plot PCA results to investigate any clustering according to 1) population type (zoo *vs.* wild individuals) and 2) genetic source, where genetic source refers to population of origin: a frog caught at MS has a genetic source of MS but a frog sampled within a zoo (population of either TZ, VA, or GVZ) would have a genetic source according to where it had been born in the wild or, if zoo-born, its tracked parentage, corresponding to a single wild population or a cross of both parents (ex. MS or MS x EK). Another PCA was performed using only individuals belonging to MS, MV, and EK since global results indicated a clustering pattern mainly driven by those three main wild populations (see results section).

Second, genetic structure among all individuals was investigated with ADMIXTURE 1.3.0 (Alexander *et al.*, 2009) for K ranging from 2 to 15. Cross-validation indices were used to discuss the best values of K across all populations to allow interpretation of the best K in combination with population history (noting a minimum of 11 subpopulations have been recorded historically in Canada (Environment Canada, 2015)). Genetic structure among the wild populations was also investigated running the same ADMIXTURE analysis including only individuals collected from the five wild populations for K ranging from 2 to 7.

Finally, the extent of genomic differentiation was estimated by computing Weir and Cockerham's Fst a) between zoo and wild categories, and b) among each of the three zoos and the three main wild populations (MS, MV, EK), all using weir-Fst-pop in *vcftools* version 0.1.17 (Danecek *et al.*, 2011).

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Tables, Figures, and Appendices

Tables

Table 1.1. Mean genetic diversity indices with standard error for Oregon Spotted Frog populations calculated from a total of 22,230 SNPs. The first two rows display average values for combined zoo (from populations TZ, VA, GVZ) and wild (from populations EK, MS, MV, MT, ST) individuals respectively. N = sample size, Ho = observed heterozygosity, F = inbreeding coefficient, Ne = effective population size. The number of breeding adults (Nb) was determined by egg mass surveys for wild populations and represented the total number of adults for zoo populations.

Table 1.2. Mean Fst estimates (range in brackets) across Oregon Spotted Frog SNPs. Zoo populations are: Vancouver Aquarium (VA), Greater Vancouver Zoo (GVZ), and Toronto Zoo (TZ). Wild populations are: Elk Brook (EK), Maria Slough (MS) and Morris Valley (MV). Two wild populations were not included due to low sample size (ST and MT).

Figures

Figure 1.1. The remaining wild populations of Oregon Spotted Frog in British Columbia, Canada. Each population is indicated by a red circle within the known watershed (coloured green), with the estimated breeding population size (Nb) from egg surveys in 2022. McLennan is still considered extant but no egg masses or frogs have been seen since 2017.

*Figure 1.2. Genetic diversity estimates for all Oregon Spotted Frog populations sampled in Canada. Mean estimates of **a)** observed heterozygosity (Ho), and **b)** inbreeding coefficients (F) at five wild (EK, MS, MV, MT, ST) and three zoo (TZ, VA, GVZ) populations.*

Figure 1.3. Heterozygosity loss through time in three wild populations of Oregon Spotted Frog. The proportion of remaining heterozygosity (Ht/Ho) was inferred from effective population size estimates (Ne) for T = 20 to T = 200. Generation time (T) for OSF is 2 years.

*Figure 1.4. Principal component analyses of Oregon Spotted Frog populations. Each axis indicates the amount of variation respectively explained in the data. **a**) and **b**) contain all sampled populations coloured by their genetic source, with wild-caught frogs indicated by circles and frogs sampled in zoos indicated by triangles. Cross-bred zoo frogs are indicated by an 'x' in the name (e.g. Aldergrove x Maria Slough). Zoo frogs with unknown parentage are indicated as "unknown" or according to their respective zoo (if many-generations zoo-born). **c**) and **d**) contain only the three wild populations with ≥ 17 samples.*

*Figure 1.5. ADMIXTURE population structure analysis with individuals grouped by population for **a)** wild (EK, MS, MV, MT, ST) and zoo populations (TZ, VZ, GVZ) from K=8 to K=10, and **b)** wild populations (MS, MV, EK, MT, ST) for K=3 where K is the inferred number of ancestral populations.*

Appendix I.I: supplementary figures

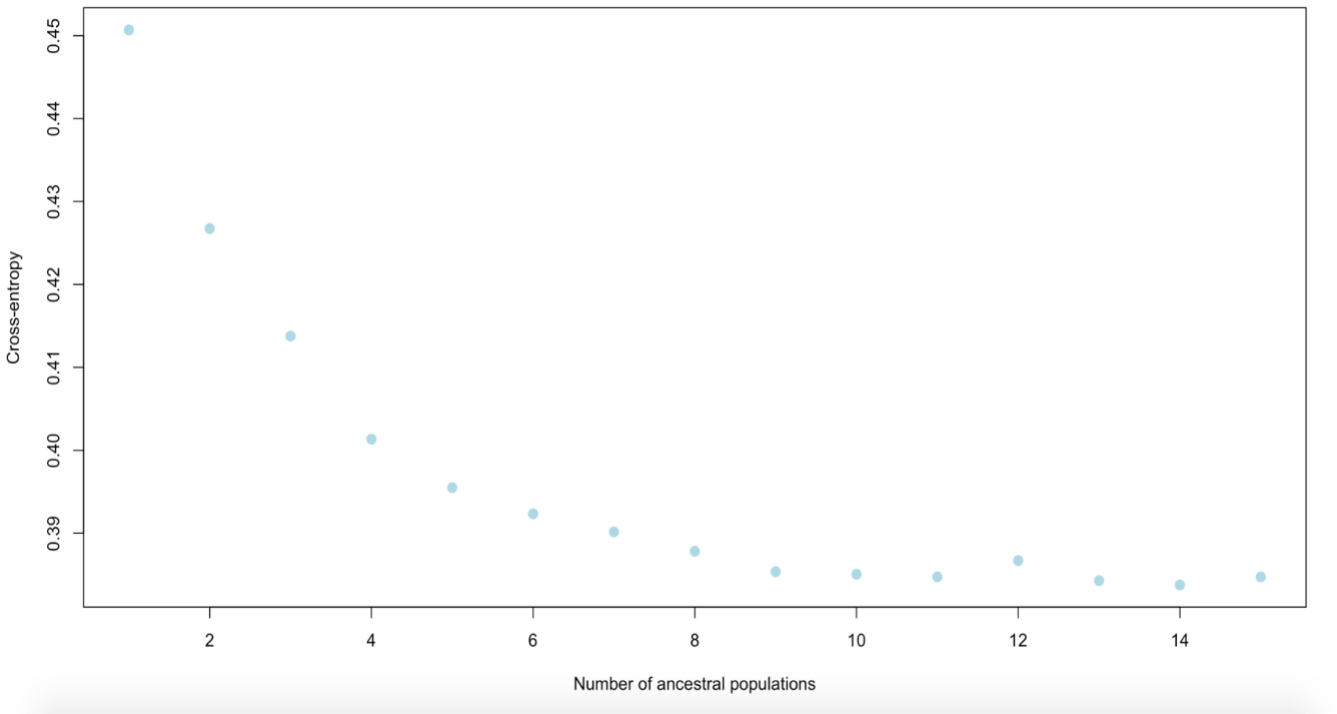


Figure A1.1. Cross validation for all sampled Oregon Spotted Frogs in Canada (5 wild populations, 3 zoo populations). The lowest cross-entropy value indicates the statistically optimal number of ancestral populations (K).

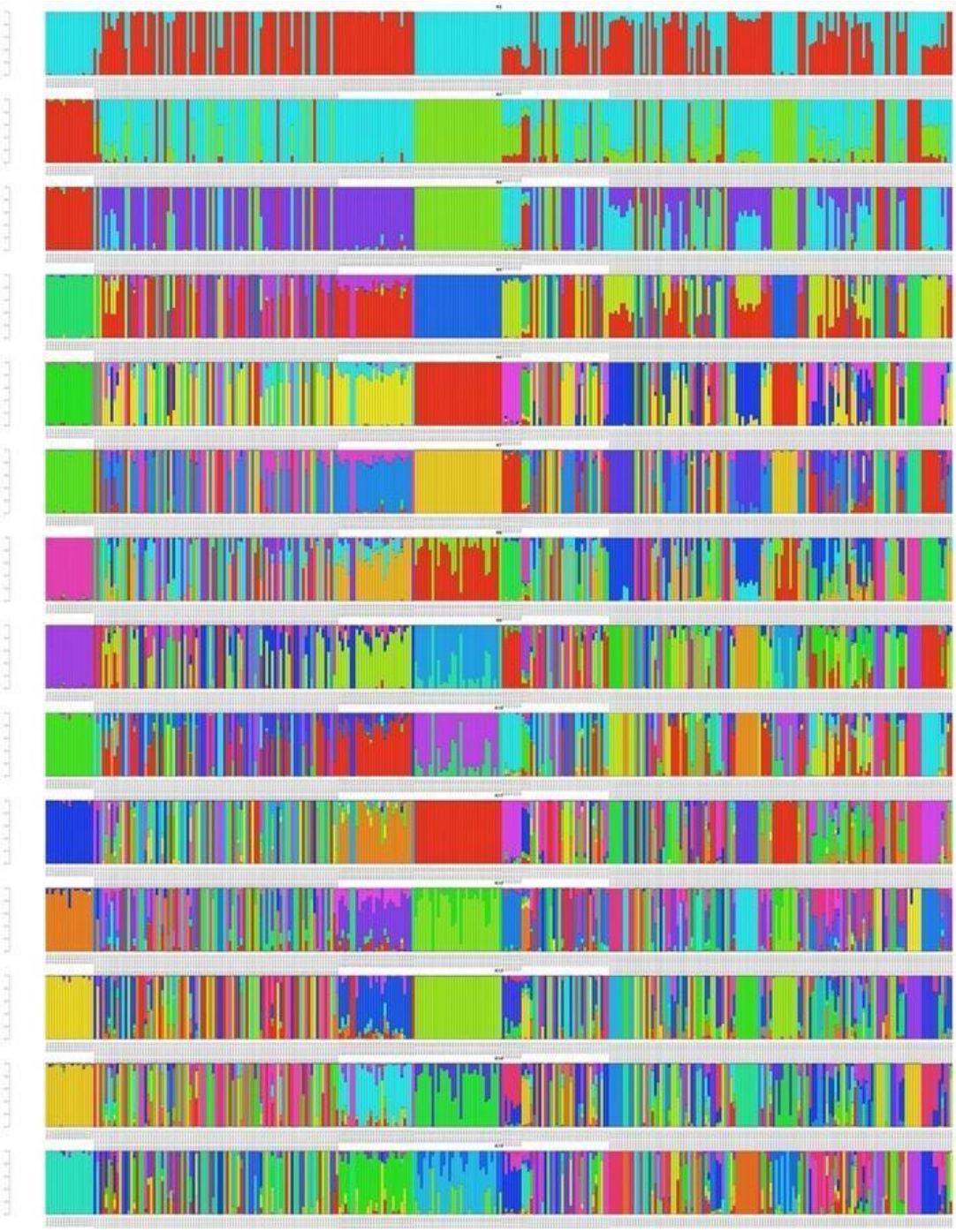


Figure A1.2. ADMIXTURE from K2 to K15 of all sampled populations of Oregon Spotted Frog (5 wild and 3 zoo populations), ordered by population. Each bar represents one individual frog and colours represent K, the postulated number of ancestral populations.

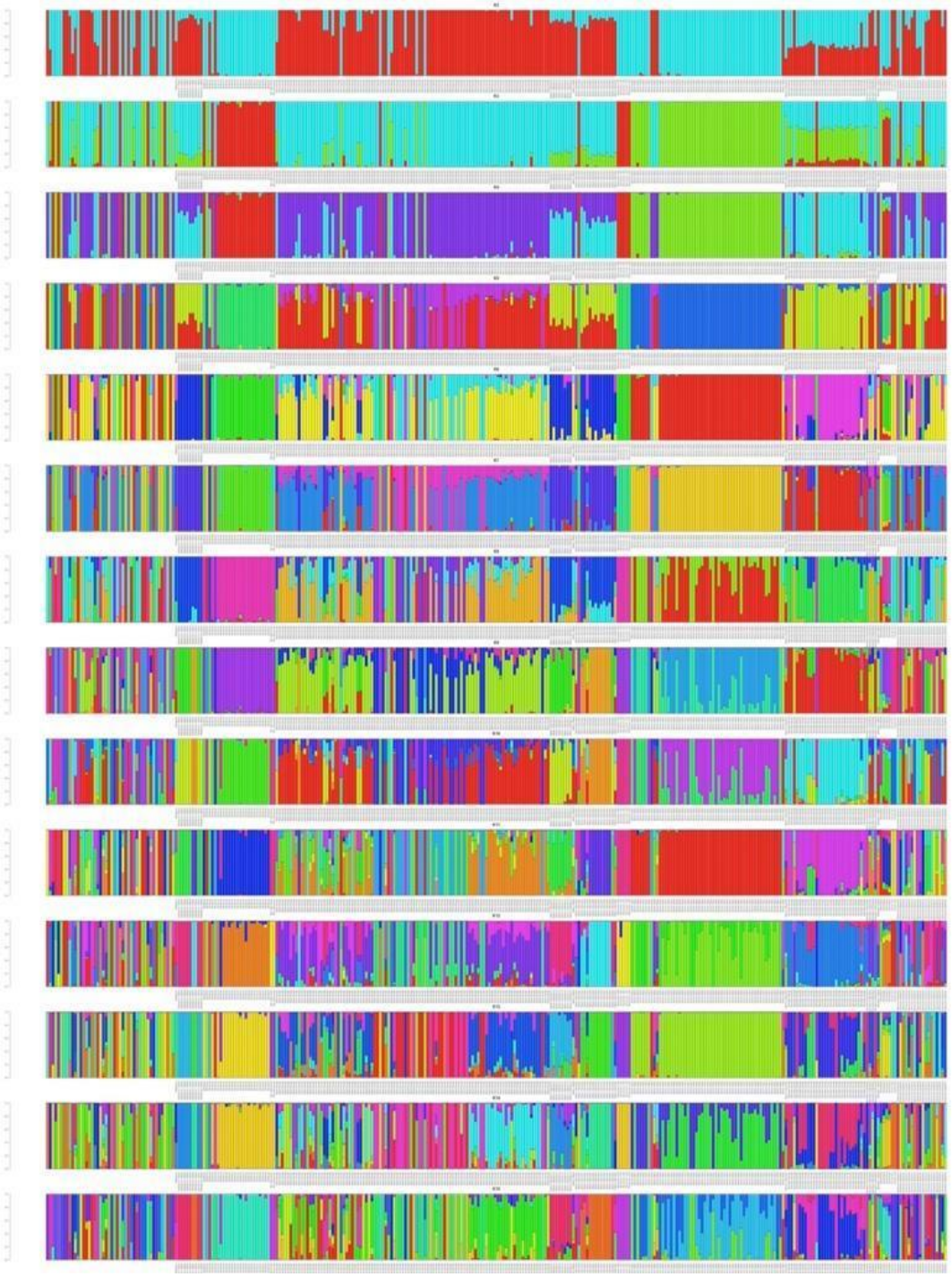


Figure A1.3. ADMIXTURE from K2 to K15 of all sampled populations of Oregon Spotted Frog (5 wild and 3 zoo populations), ordered by genetic source – the tracked or current lineage of each frog. Each bar represents one individual frog and colours represent K, the postulated number of ancestral populations.

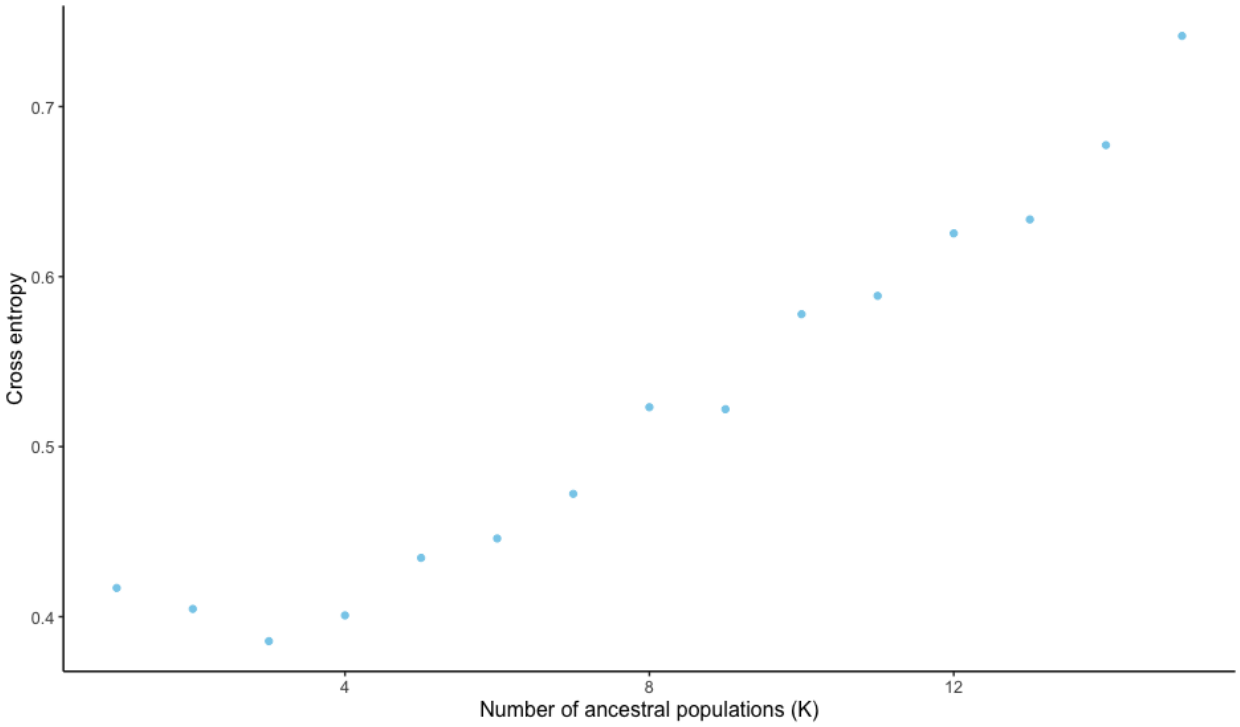


Figure A1.4. Cross validation for the wild populations of Oregon Spotted Frogs in Canada (MS, MV, MT, EK, ST). The lowest cross-entropy value indicates the statistically optimal number of ancestral populations (K).

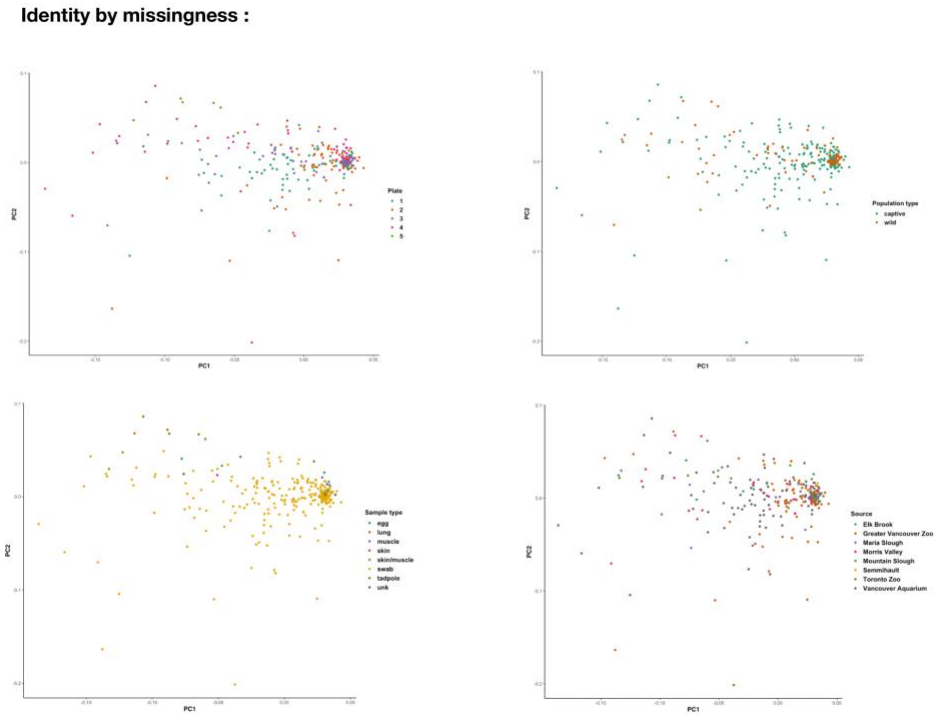


Figure A1.5. Identity by missingness analysis on the filtered Oregon Spotted Frog SNPs. The data is represented using sequencing plate number, sample type, population type (zoo vs. wild), and source information. No clustering by missingness was found.

Chapter 2:

Egg binding displays age-dependent impact on Canada's most endangered anuran, the Oregon Spotted Frog (*Rana pretiosa*)

Abstract

Post-ovulatory egg retention, or dystocia, is a significant risk to amphibians held *ex situ* but its documentation, and thus understanding, is minimal. Potential links to age, body condition, amplexus behaviours, and environmental conditions have been suggested but remain primarily anecdotal. Here, I investigated the causes of egg retention in Canada's most endangered anuran, the Oregon Spotted Frog (*Rana pretiosa*). Using post-mortem reports from conservation breeding programs, I classified mortalities related to egg retention as egg binding and compared body condition of conspecifics in zoo and wild populations. I also looked for links between egg binding and age, follicular development, and amplexus behaviours using video and ultrasound technology. While two out of three zoos had significantly higher scaled mass index (SMI) than wild conspecifics, the SMI of egg bound frogs did not significantly differ from others in their populations. However, frogs who became egg bound later in life (> 3 years old) generally had higher body mass than other females of the same age. Higher follicular grades, indicative of mature ovaries, were predictive of egg binding but may have been conflated by altered environmental conditions. Most amplexus contacts lasted less than 8.4 minutes but were not predictive of egg binding. While the causes of egg binding in *R. pretiosa* and amphibians overall remains indistinct, our study contributes to the documentation of this issue, potentially aiding amphibian breeding programs worldwide. Further, findings from this study will inform husbandry practices for *R. pretiosa* and improve the cohesiveness of recovery actions among partners for this endangered species.

Introduction

Egg Retention and Associated Causes

Post-ovulatory stasis, also known as egg retention, egg binding, or dystocia, is a reproductive disorder observed in numerous animal species (Lloyd, 1990; Frye, 1991; DeNardo, 2006; Calatayud *et al.*, 2018; Abou-Zahr *et al.*, 2019). Unusually long egg retention has been observed in wild individuals, but evidence of reproductive failures as seen *ex situ* (ex. in zoos) is negligible (Kummrow *et al.*, 2010). Domestic animals, like cattle, frequently require assistance in birthing; dystocia in these cases is defined as a difficult, prolonged birth or severe extraction (Mee, 2004). Egg binding in avian medicine – the delay of eggs in the reproductive tract – can be the result of multiple factors, including, but not limited to, mechanical obstruction (termed dystocia), abnormally large eggs, dietary deficiencies, or stress (Abou-Zahr *et al.*, 2019). In non-avian reptiles (hereafter "reptiles"), similar reproductive failure is categorized as either pre-ovulatory egg retention (follicular stasis) or post-ovulatory egg retention (dystocia), where ovulated eggs are retained in the oviduct without being oviposited (DeNardo, 2006). In many reptile species, pre- and post-ovulatory retention can be distinguished using ultrasound or x-ray technologies as ovulated eggs take on a distinct ovoid, calcified form, with both conditions regularly linked to either housing conditions or individual body condition (Kummrow *et al.*, 2010; Laycock, 2015; Pimm *et al.*, 2015). Sexually mature female reptiles are often closely monitored and cases of egg retention well documented, but predictive factors are multifaceted and yet to be clearly identified (Stacy *et al.*, 2008). If untreated in reptiles or birds, retained eggs not only prevent reproductive activity but can cause inflammation, edema, egg rupture, bacterial infections, and even death (Frye, 1991; Cuadrado *et al.*, 2002; Rivera, 2008; Stacy *et al.*, 2008; Abou-Zahr *et al.*, 2019) making it a serious and potentially life-threatening condition.

In amphibians, documentation of similar reproductive failures is increasing but still scarce and often anecdotal (Kouba *et al.*, 2009; Roth *et al.*, 2010). Kouba et al. (2009) cited dystocia as a "major cause of death in amphibians that fail to lay eggs." As in reptiles, it is important to monitor sexually mature female amphibians to ensure they lay mature eggs rather than retain them (Whitaker, 2001). Amphibians, particularly those with external fertilization, can reabsorb retained eggs through apoptosis (cell death; Tokmakov *et al.*, 2020), but side effects of such egg retention (*ex.* lethargy, edema, bacterial infections, death) occur in anurans when retained eggs are not properly reabsorbed (Whitaker, 2001; Roth *et al.*, 2010; Silla *et al.*, 2021). Descriptions of egg retention in amphibians are still limited and language varies across taxa and within amphibian literature itself (Kouba *et al.*, 2009; Calatayud *et al.*, 2018; Silla *et al.*, 2021). For the purposes of this study, mortalities resulting from post-ovulatory egg retention were classified as *egg binding* (see Methods for more details). Egg binding is a major conservation concern as the loss of any individual in tightly managed *ex situ* populations, particularly before they have made a genetic contribution (*i.e.* producing offspring), can greatly impact demographic stability and retention of genetic diversity (Kouba *et al.*, 2009; Asa *et al.*, 2011). Furthermore, the highly threatened status of amphibians (Stuart *et al.*, 2004) and the challenge of breeding species with unique life cycles (Kouba *et al.*, 2009) makes this taxon particularly vulnerable to this threat. Knowledge of life history is often lacking even after establishment of *ex situ* programs (Bradfield *et al.*, 2022) and this, along with the scarcity of literature references, leaves the causes of egg

retention in amphibians equally if not more difficult to elucidate than in other taxa. In attempts to fill in some of these knowledge gaps, this study investigates three areas highlighted in the literature as potentially linked to egg retention in reptiles and amphibians.

a) Body Condition

Animals held *ex situ* often have a higher body mass than wild conspecifics – a result of more consistent, high calorie diets, the elimination of predation, and/or different environmental conditions (Blanco and Sherman, 2005; Kummrow *et al.*, 2010). In anurans and many other species, there is generally a positive allometric relationship between body weight and reproductive output (*i.e.* total ovarian weight; Jørgensen, 1981) with body size often imposing the upper limit on reproductive output (Gibbons and McCarthy, 1986). Reproductive output can also vary with environmental conditions (Beattie, 1987; Cummins and Swan, 1995) since reproductive output depends on energy investment by the female (Berven, 1988), which itself varies with the availability of energy sources. Overweight (*i.e.* over-conditioned) anurans in zoos, therefore, may be able to invest more into reproductive output, potentially exceeding their body's physical capacity to hold and reabsorb unlaid eggs (Kummrow *et al.*, 2010). In fact, obesity (relative to wild conspecifics) has been observed in anurans with egg retention in the past (Roth *et al.*, 2010). Energy reserves, such as those used for egg development, can be estimated by using a body condition index where a greater index indicates greater energy reserves (Schulte-Hostedde *et al.*, 2005). The Scaled Mass Index (SMI; Peig and Green, 2009) was developed as a standardized condition index which accounts for body shape adjustments to better compare different populations of the same species and provides a more accurate measure of reproductive state than other indices (Calatayud *et al.*, 2018).

b) Sexual Maturity

Age or reproductive experience have also been highlighted in cases of egg retention, suggesting female anurans in their first breeding season show increased susceptibility to reproductive dysfunction (Toronto Zoo unpublished records; Roth *et al.*, 2010). Growth is impacted by brumation, and the removal or alteration of this overwintering period can shorten the time it takes amphibians held *ex situ* to reach sexual maturity (Jorgensen, 1992; Calatayud *et al.*, 2018). Brumation is a period of inactivity and reduced metabolic rate for anurans (Pinder *et al.*, 1991) tightly linked to reproduction and often essential for follicular maturation (Sotowska-Brochocka, 1988; Calatayud *et al.*, 2018). Temperate anurans accumulate fuel reserves prior to brumation and most of a female's fat reserves go into egg development over this overwintering period (Pinder *et al.*, 1991). In the wild, the delay of sexual maturity has been linked to changes in reproductive output and egg number as younger anurans frequently produce greater numbers of eggs, but the eggs are of smaller individual size (Berven, 1988). This occurs due to the negative relationship between the number and size of eggs produced (Jørgensen, 1981; Gibbons and McCarthy, 1986). Females with delayed sexual maturation or those in their second year of breeding are no longer devoting so much energy to growth so they can direct more available energy to egg growth, increasing the overall reproductive output while producing fewer eggs (Berven, 1988). As such, the timing of sexual maturity and consequent size or number of eggs produced may be altered in *ex situ* amphibians, presenting novel challenges females would

otherwise not face in the wild. The use of ultrasound technology to monitor follicular development is becoming increasingly popular for visualizing the presence, size, number, and possible retention of eggs in amphibians (Schildger and Triet, 2001; Calatayud *et al.*, 2018; Graham *et al.*, 2018). The grading of follicular development has also proven useful in identifying which females are ready to breed and which should be left until mature eggs have developed or until next season (Graham *et al.*, 2018; Burger *et al.*, 2021). Conducting such ultrasound examinations in consecutive and repeated fashion greatly improves the ability to correctly identify ovarian status using changes in shape and size of follicles to track follicular development (Pimm *et al.*, 2015).

c) Amplexus Behaviours

Despite their best attempts, *ex situ* environments inevitably differ significantly from their wild counterparts, increasing the risk of losing physiological and behavioural responses which can impact reproduction (Schulte-Hostedde and Mastromonaco, 2015; Calatayud *et al.*, 2018). Unsuitability of housing or oviposition conditions is frequently linked to egg retention cases in reptiles (Kummrow *et al.*, 2010; Pimm *et al.*, 2015). Similarly, insufficient environmental stimuli and inconsistent or incompatible amplexus partners have been linked to egg retention in amphibians (Whitaker, 2001; Calatayud *et al.*, 2018). Anuran reproduction can be triggered by many different environmental stimuli such as temperature, rainfall, or mating behaviours of conspecifics (Ulloa *et al.*, 2019). Amplexus, the mating position of anurans, not only aids the synchrony of egg and sperm release but male amplectants may also aid the transition of eggs from ovary to oviduct and release (Calatayud *et al.*, 2018). However, in many conservation breeding programs, genetic management pairs individuals with optimal genetics rather than allowing for more natural mate-selection, often leading to incompatible mates and reproductive failure (Asa *et al.*, 2011). Such behavioural challenges are so commonplace that exogenous hormones are often used to increase reproductive behaviours and/or induce oviposition of the retained eggs (Burger *et al.*, 2021; Silla *et al.*, 2021) rather than allowing for mate selection. Thus, the existence of *ex situ* amplexus behaviours resembling those observed *in situ* may be critical to successful breeding (Schulte-Hostedde and Mastromonaco, 2015). If *ex situ* programs hope to emulate natural mate selection and breeding behaviours, a better understanding of the signals and mechanisms used by the species of concern is essential (Asa *et al.*, 2011).

Egg Retention in the Oregon Spotted Frog

The Oregon Spotted Frog (OSF) is currently held in two zoos and one aquarium (hereafter "zoos") as part of ongoing conservation breeding and reintroduction programs. Despite some of these *ex situ* programs being in place for 10+ years, reproductive output is inconsistent, with low fertility and viability among laid egg masses. Further, this species has a uniquely communal breeding approach, often showing strong oviposition site fidelity and laying egg masses in communal piles (Phillipsen *et al.*, 2010), which is difficult to replicate in captivity. Alongside and perhaps related to these challenges, egg retention has become a consistent cause of mortality for female OSF in all three zoos. The relatively high incidence of egg binding (*i.e.* mortality from egg retention) may be an artefact of how closely OSF health is monitored *ex situ*, but the cause(s) have yet to be elucidated. For instance, OSF held in these *ex situ* populations have

frequently been reported to have a much higher body mass than wild conspecifics, suggesting this may be associated with egg binding, but this has never been qualitatively assessed. In this study, I aim to determine whether female body condition, sexual maturity or amplexus behaviours play a role in the incidence of egg binding in OSF conservation breeding programs across Canada.

Hypotheses and Predictions

This study uses historical and current cases of egg binding in OSF from three zoological institutions to investigate three areas of *ex situ* husbandry previously linked to cases of egg retention in reptiles and amphibians. The purposes of this study were two-fold: 1) to determine whether OSF becoming egg bound display a higher body condition than other females in their population; and 2) to determine if egg binding can be predicted, retroactively, based on physiological attributes such as age and follicular development, or by amplexus behaviours. The predictions accompanying these goals are as follows: 1) if OSF in zoos are over-conditioned, their reproductive output will exceed their body's ability to reabsorb unlaid eggs, leading to egg binding; 2) young OSF with increased follicular development will reach sexual maturity too quickly and produce a greater number of eggs than their body is able to lay or reabsorb, leading to egg binding; 3) a lack of adequate behavioural stimuli, evidenced by inconsistent or scarce amplexus with males, will cause OSF to retain eggs rather than lay them, leading to egg binding. This study could prove crucial to improving the management practices of OSF in captivity by addressing knowledge gaps and revealing patterns that may be present in amphibian breeding programs worldwide.

Methods

Study Area

There are six extant populations of OSF remaining in Canada: Morris Valley (MV), Mountain Slough (MT), Maria Slough (MS), Elk Brook (EK), Semmihault Creek (ST), and McLennan (ML); with one reintroduction site in a restored wetland (Chaplin, CH) within the Maria Slough watershed. These populations are primarily located in floodplain marshes, sloughs or side channels associated with the Fraser River Lowlands in British Columbia with low to moderate amounts of emergent vegetation and silty substrate (Environment Canada, 2015). Egg mass surveys are conducted annually at each of these sites to determine population size estimates, and a more extensive capture-mark-recapture program is ongoing at both MS and MV. Due to differing facilities, resources, and locations, each of the three zoos participating in the conservation breeding and reintroduction program has a unique approach. The Vancouver Aquarium (VA) began its breeding program in 2010 and regularly holds 100-160 OSF in 25-30 gallon tanks housed in a climate-controlled greenhouse system, with frogs generally ranging in ages from 0-11. The Toronto Zoo's (TZ) program, established as both a breeding program and assurance population, started in 2010 as well and holds an average of 20 OSF at a time, all of mature breeding age (> 2 years old). These frogs are housed similarly to those at VA, in 25-30 gallon tanks in a quarantine facility under strict conditions to account for its distance from local British Columbia climate. While the Greater Vancouver Zoo (GVZ) has helped headstart OSF

since 2003, their year-long breeding program did not begin until 2017. They now house roughly 80-100 OSF of all ages, in 100 gallon stock tanks held outdoors; running a more communal, hands-off breeding setup compared to the closely controlled conditions at VA and TZ.

Egg Binding *vs.* Egg Retention

Post-mortem reports on OSF deaths were obtained from both TZ and VA for the years 2010-2022. While egg binding or retention was often a suggested diagnosis, few reports identified it as a definitive cause of death. For the purposes of this study, "egg binding" was defined as any case where a female OSF died due to egg retention or due to otherwise unknown causes while retaining mature eggs. In necropsy reports, such cases often highlighted the presence of largely distended ovisacs, necrotic eggs, viscous masses, or numerous eggs free floating in the body cavity (unpublished data). The diagnosis of egg binding was also applied in cases where OSF suffered common symptoms of egg retention, such as lethargy or edema, but eggs were removed either surgically or by palpation to prevent death. "Egg retention", on the other hand, was applied to cases where mature eggs were retained but no acute adverse symptoms or reactions were noted by husbandry staff. In other words, egg binding refers to cases where frogs suffered acute, severe side effects (including death) from egg retention. Using these definitions, all OSF who had been documented as dying of egg retention since 2010 were classified as egg bound for comparison to ongoing mortalities.

Body Condition

Morphometric data were acquired, including mass (g) and snout-vent length (mm; SVL), from wild, gravid female OSF, primarily from populations MS and MV, over the years 2012-2022. These measurements were all taken in spring (Feb-Apr) when OSF are most active, as part of regular population monitoring. If a gravid female was caught multiple times in a season, the average of her collective mass and SVL measurements was used to account for human error. Similarly, morphometric data were collected from zoo female OSF where available throughout 2010-2022. Due to differing husbandry practices, there was less consistency in these data. Frog mass was regularly recorded in the fall (Sept-Nov) at TZ and VA and in the spring (Feb-April) at TZ and GVZ. VA also recorded lengths in the fall but measured snout-urostyle length (SUL) rather than snout-vent length (SVL). GVZ measured SVL in accordance with protocols for wild populations but did so intermittently. To complement this historical dataset, a series of measurements (mass and SVL) were taken on available female OSF at all three zoos in March 2021 and November 2021. A full list of the available morphometrics is available in Appendix II.I.

Using these data, a Scaled Mass Index (SMI) was calculated for female OSF using a regression of mass by SVL according to Peig and Green (2009). As mentioned, length was recorded differently between different populations, so I performed a regression of SUL by SVL from 24 adult female OSF at VA, with both measurements taken on the same day, in triplicate to improve accuracy (Fig. 2.1). This regression produced the equation $SVL = 1.196x - 10.709$, where x = SUL. This equation was then used to convert any SUL measurements from VA into SVL for more accurate comparison to measurements from other zoo and wild populations.

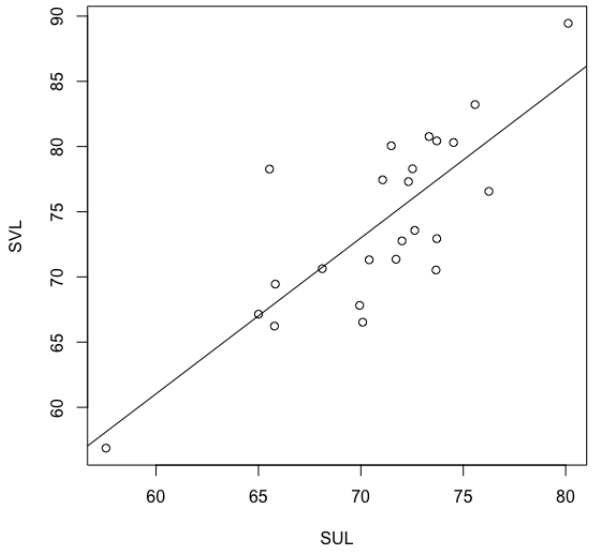


Figure 2.1. Average snout-vent length (SVL; measured ventrally) by snout-urostyle length (SUL; measured dorsally) of 24 female Oregon Spotted Frogs from Vancouver Aquarium. Each point indicates the average of three measurements taken in sequence in October 2022.

An SMI ($\widehat{M}_i$) was then calculated for each female OSF (from all zoo and wild populations) using a mass by SVL regression according to the following equation (Peig and Green, 2009):

$$\widehat{M}_i = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

where M_i and L_i represent the mass and length of individual i, respectively and L_o is the arithmetic mean length for the study population. The scaling exponent (b_{SMA}) was determined from the standardized major axis (SMA) regression of lnM on lnL. A different b_{SMA} was calculated for each zoo population (VA, TZ, GVZ) and one for the combined "wild" category. SMIs calculated from measurements collected over 2020-2021 formed the most complete dataset of all sampled populations and was then divided into pre-brumation (fall) and post-brumation (spring) categories to account for female gravidity in the spring measurements. This SMI dataset was then used to perform the following comparisons: i) between all wild populations, ii) between all zoo populations and a combined "wild" category, and iii) between egg bound (EB) and non-egg bound (non-EB) females in VA and GVZ (TZ did not have any egg bound females over these years). All statistical analyses were conducted using R Statistical Software (v4.1.1; R Core Team, 2021), using ANOVAs, or Kruskal-Wallis tests in the case of non-normal data, performed with the R package "stats".

To investigate potential differences in body condition over a longer duration than the 2020-2021 dataset, available measurements from 2010-2021 were compiled into a dataset which consisted

of pre-brumation mass records from OSF held at VA and TZ. Corresponding lengths were not available for most of these records, so mass was used as the body condition estimate instead of SMI. The corresponding age at the time of weighing was determined using zoo records, with the fall immediately following hatch considered 0 years old. Pre-brumation mass was then plotted over age for each population (VA and TZ) with individuals identified as either EB or non-EB by necropsy reports. Differences in the growth of EB *vs.* non-EB individuals were then analysed via mixed modelling. Generalized linear mixed models (GLMM) were performed via R package *lme4* (R Core Team, 2021) to evaluate whether pre-brumation mass, age or population were significant predictors of egg binding at TZ or VA over the years of 2010-2021. The repeated measure of mass across the lifespan of each frog was accounted for by inclusion of frog ID as a random effect. Mass and age were standardized to aid interpretation using the coefficient of variation method (subtracting the mean and dividing by the standard deviations) through the function *scale()* in R (v4.1.1.; R Core Team, 2021). The full model was fitted as:

*egg binding ~ mass * age + population + mass:population + (1|frog ID)*

and the significance of each variable and interaction was then estimated by its two-tailed p-value using alpha = 0.05. Visualization of this model indicated the relationship between mass and age differed between TZ and VA, so a new GLMM was then fitted to each population separately as:

*egg binding ~ mass * age + (1|frog ID)*

and the significance of each variable and interaction estimated by their p-values at alpha = 0.05.

Egg binding at Vancouver Aquarium in 2022

From fall 2021 to spring 2022 the holding conditions for OSF at VA were altered for the purposes of an experiment assessing overwintering conditions. Frogs were housed in standard tanks, but leaf litter and some cover objects were removed to create a more sterile environment for water analysis. In early March, frogs were placed in breeding groups of 2 females and 4 males, with the exceptions of i) one group where a male died over the winter and was not replaced, and ii) five groups of one-male: one-female pairings. In this 2022 breeding season, a high number of OSF became egg bound and no viable eggs were laid by any of the females. The data collected on these frogs was used to retrospectively predict egg binding. Data collected on all mature (≥ 2 yo) female OSF at VA included: i) age at the start of the breeding season, ii) genetic source (*i.e.* wild population origin and/or origins of parents), iii) provenance (wild or zoo born), iv) post-brumation mass (g; measured in March 2022), v) the number of eggs laid over the course of the breeding period (regardless of viability), and vi) their past breeding history as either 'Yes' (*i.e.* has laid eggs in a previous year), 'No', or 'Unknown'. Breeding behaviours (*i.e.* amplexus) were also monitored for n=30 of the female OSF over the first 59 hrs of the 2022 breeding season, as described below.

A generalized linear model (GLM) was used via R package *nlme* (Lindstrom and Bates, 1990*)* to evaluate the influence of life history characteristics on the incidence of egg binding in the 35 female OSF held at VA in 2022. Specifically, egg binding was fit as a response variable in a GLM with the following potential explanatory variables: i) initial follicular grade (described in Section: *1. Follicular Development*), ii) provenance, iii) age, iv) post-brumation mass, v) previous breeding

history, vi) number of eggs laid, and vii) genetic source. As the number of potential predictors (each level of i-vii counts as a predictor) exceeded the number of responses (n=35 observations), forward selection was performed to avoid overfitting, starting from the null model:

egg binding ~ 1

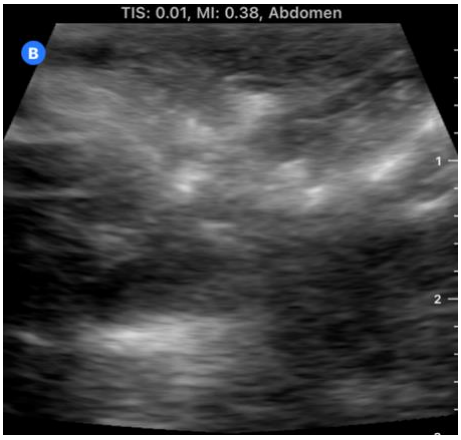
Before proceeding from the null GLM, a null GLMM was fit to the same data using i) tank, ii) treatment (from the concurrent overwintering experiment), and iii) both tank and treatment as potential random effects to account for variability caused by holding conditions. The null GLM had a better fit than any of the listed GLMMs, according to the Akaike Information Criterion (AIC), and proceeding with the GLMM caused overfitting so modelling was continued with the GLM instead. From the above null GLM model, forward model selection was performed by fitting each of the explanatory variables previously listed (i-vii) to the response (ex: *egg binding ~ age*) and the AIC of each resulting model compared. The model with the lowest AIC was selected and an additional explanatory variable or interaction was then added to this model (ex: *egg binding ~ age + provenance*) with the best model selected by lowest AIC again. This pattern was repeated until addition of explanatory variables no longer improved the model fit. The significance of each variable and interaction in the final model was then estimated by their two-tailed p-values at alpha = 0.05.

1. Follicular Development

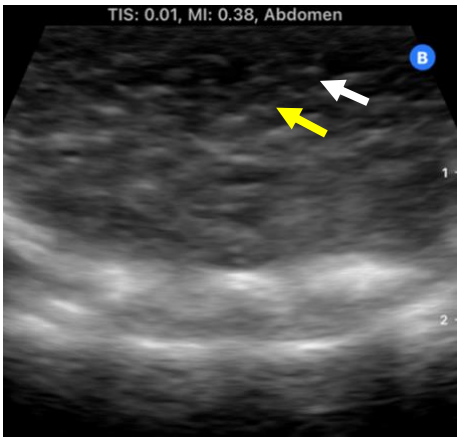
During the 2022 breeding season, ovarian images were obtained via ultrasonography from female OSF at both VA and GVZ to track follicular development. At VA, the first ultrasound image was taken on all adult female OSF the day before frogs were put into their breeding groups (March 7, designated “day 00”). At GVZ, the first ultrasound image was taken immediately prior to the frogs being placed in their breeding groups (March 2, day 01). Two more ultrasounds were collected on the OSF at VA during the breeding season (March 25, day 18, and April 13, day 37), and a final collection of ultrasound images was taken on each surviving female at the end of the breeding season (April 30, day 54). The end of the breeding season at VA was determined by the cessation of breeding in the wild and few VA frogs remaining in amplexus. The OSF at GVZ were ultrasounded only once more on March 29 (day 28), as most had already laid their eggs by this point. Ultrasound images were also collected opportunistically from wild females collected at the wild capture-mark-recapture sites during the active breeding season for comparison to those collected on zoo OSF. A follicular grading scheme for OSF was generated using the images collected following the grading schemes developed by Calatayud et al. (2018), Graham et al. (2018), and Julien et al. (unpublished). Taking species and technology specificity (*i.e.* the use of different ultrasound technology) into account, I used these references and ultrasound images collected from wild frogs as an indicator for the natural appearance to create a grading scale (0-4; Figures 2.2 and 2.3).

Grade 0 delineates a non-gravid ovary: few to no detectable follicles, which occurs in immature females or post-oviposition of all eggs (Fig. 2.2A). Grade 1 is early-gravid: small, sparse follicles are detectable as echogenic (white or grey appearance) dots (Fig. 2.2B). Grade 2 is mid-gravid: pre-ovulatory follicles begin to get larger (*vs.* Grade 1 follicles) and show more evenly spaced patterns of anechoic (dark) follicular fluid between them (Fig. 2.2C). Grade 3 is late-gravid: eggs

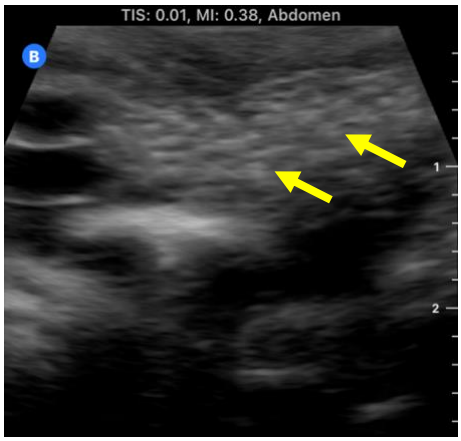
are larger still, yolked, more spread out and surrounded by large hypoechoic (grey-black) areas indicating the accumulating follicular fluid (Fig. 2.2D). Ovulated eggs may appear more caudal. Grade 4 was assigned post-hoc to OSF with mature, ovulated eggs who failed to oviposit a complete egg mass – which could indicate egg binding or egg retention (Fig. 2.3). Ultrasound images assigned a Grade 4 may completely resemble those of a Grade 3, with the potential addition of visibly degenerating eggs and/or elongated, circular eggs (Fig. 2.3A).



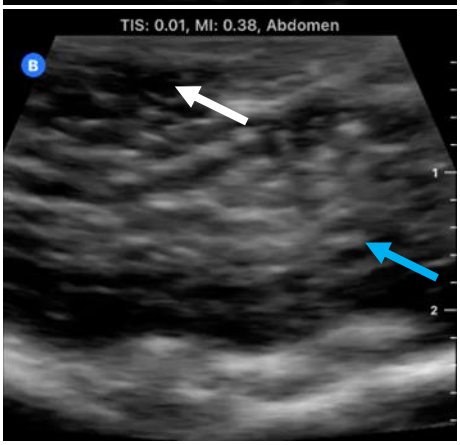
A) Grade 0 – Non-gravid. Few to no detectable follicles. May be from immature female or post-oviposition of all eggs



C) Grade 2 – Mid-gravid. Pre-ovulatory follicles (yellow arrows) are getting larger and elongated, surrounded by even patterns of anechoic (dark) follicular fluid (white arrow)



B) Grade 1 – Early gravid. Small, sparse but clustered follicles detectable as echogenic dots (yellow arrows)

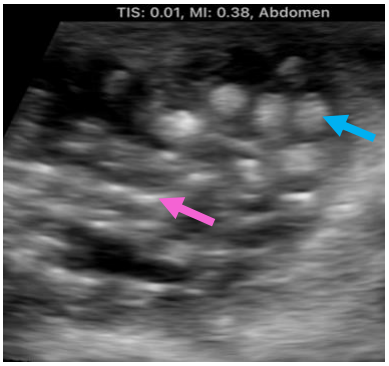


D) Grade 3 – Late-gravid. Still larger, yolked eggs (grey centre; blue arrow) are more spread out and caudal, surrounded by more hyperechoic follicular fluid (white arrow). Eggs are more elongated or circular

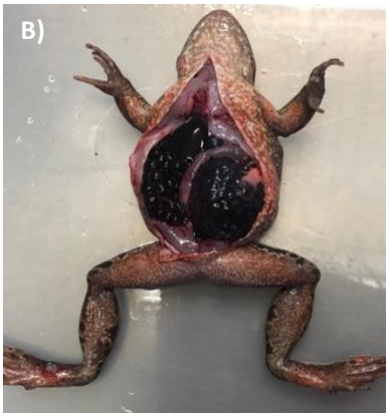
Figure 2.2. Follicular development grading scheme for the Oregon Spotted Frog with four stages of development displayed A) through D). Coloured arrows are explained in each image legend.

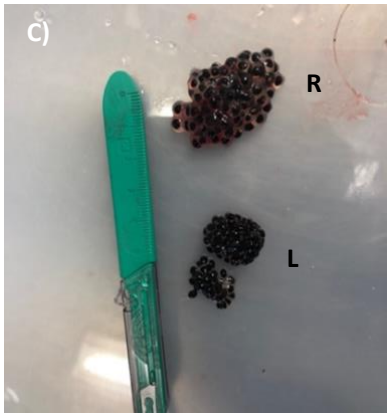
Using the grading scheme in Figure 2.2, each OSF was assigned a follicular grade from 0-3 for every ultrasound event (ex. day 00, day 18). The distribution of VA follicular grades, particularly the first grade (day 00), were then compared across the other measured OSF traits (ex. age, mass, breeding history) and across reproductive outcomes (EB, ER, LA, NF; described below). The initial follicular grade for VA OSF was also used as an explanatory variable in the GLM described in the section above (*Egg binding at Vancouver Aquarium 2022*). Reproductive outcomes were assigned to each OSF at the end of the breeding season. OSF with either a grade 2 or 3 follicular development on their final ultrasound image but who exhibited no adverse side effects were given a reproductive outcome of Egg Retention (ER). Females who laid all their mature eggs prior to day 54, with no mature eggs discernable on ultrasound, were given a

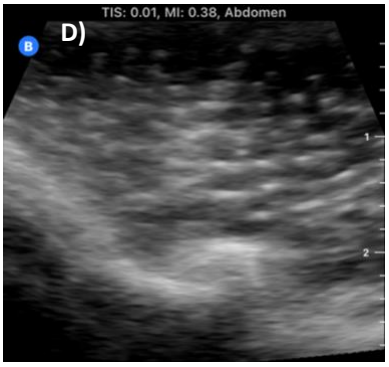
reproductive outcome of Laid All Eggs (LA). Females who never developed mature follicles, remaining at either grade 0 or 1 throughout the breeding season, were given a reproductive outcome of No Mature Follicles (NF). Most NF females had not reached sexual maturity yet. Females who died, or who exhibited other severe side effects, while retaining mature eggs (grade 2 or 3) were Egg Bound (EB). EB and ER were not distinguishable by ultrasound (see Figures 2.2 and 2.3) but only by the presence of symptoms such as lethargy, edema, renal failure, and death.



A) Grade 4 – Egg bound or egg retention
Contain mature, circular, ovulated eggs (blue arrow) and some degenerating eggs (pink arrow) surrounded by fluid (white arrow). Individuals failed to oviposit a complete egg mass.







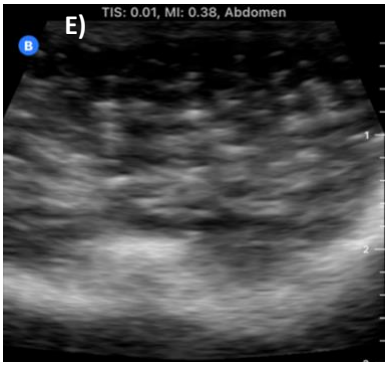


Figure 2.3. Necropsy of an egg bound Oregon Spotted Frog. The ultrasound image (A) was taken immediately prior to death and the necropsy (B-C) performed once death was confirmed. Retained eggs were found loose in the body cavity on the right side (D), and in the left ovisac (E). Eggs are labelled in (C) as being removed from the right side (R) or left side (L).

2. Amplexus behaviour

Starting at 0hr, the breeding groups housed as 2:4 female:male ratio (30 females:60 males total across 15 tanks) were monitored by two observers until 59hr in 6-hour shifts from 7:00-13:00 and 13:00-19:00. Surveillance cameras were used to record behaviour during all hours that

observers were absent. Each observer was responsible for monitoring 7 or 8 tanks, sitting where they could view most, if not all, of their assigned tanks. Monitoring began after a 10 minute acclimation period. Every 15 minutes (or when additional visibility was needed) observers stood to check each tank and ensure no behaviours had been missed. The time for each of the following behaviours was recorded per female OSF: i) time at first amplexus (latency); ii) failed amplexus (male fully releases a female he was amplexing); and iii) new amplexus (initiation of any amplexus after the first amplexus). These same time stamps were recorded for all video footage. Due to significant variation in the recorded amplexus durations, the durations were separated into three distinct phases before analysis: Touch ($x < 0.14$ hrs), Attempt ($0.14 < x < 4$ hrs), and Extended Amplexus ($x > 4$ hrs).

Validity of VA breeding season data

Due to the conditions of the concurrent overwintering experiment, there was a risk of the amplexus behaviour of VA frogs being negatively impacted and resulting in 'abnormal' behaviour during the 59 hr observation period. While behaviour was not recorded at GVZ, follicular development was compared between VA and GVZ to validate the behaviours recorded at VA. It was known that OSF at GVZ entered amplexus within hours of males and females being put together and most females laid eggs within two weeks, prior to their second ultrasound. Therefore, using follicular grade as a measure of female receptivity (Wilczynski and Lynch, 2011), I predicted that if the distribution of VA follicular grades matched that of GVZ frogs, then amplexus would not be hindered by female receptivity more than was typical in the zoo populations. If initial follicular grades were consistently i) higher or ii) lower at VA compared to GVZ then the overwintering conditions had most likely already led to (i) egg binding or (ii) had delayed follicular development, enough to negatively impact female receptivity.

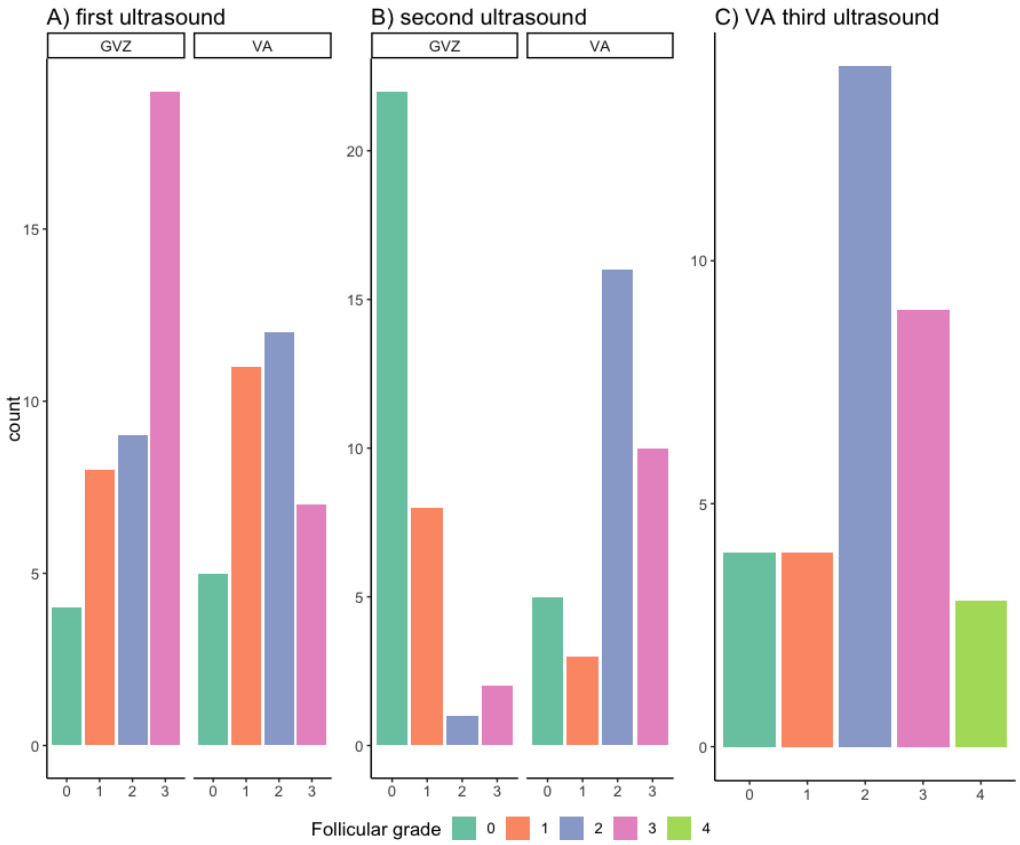
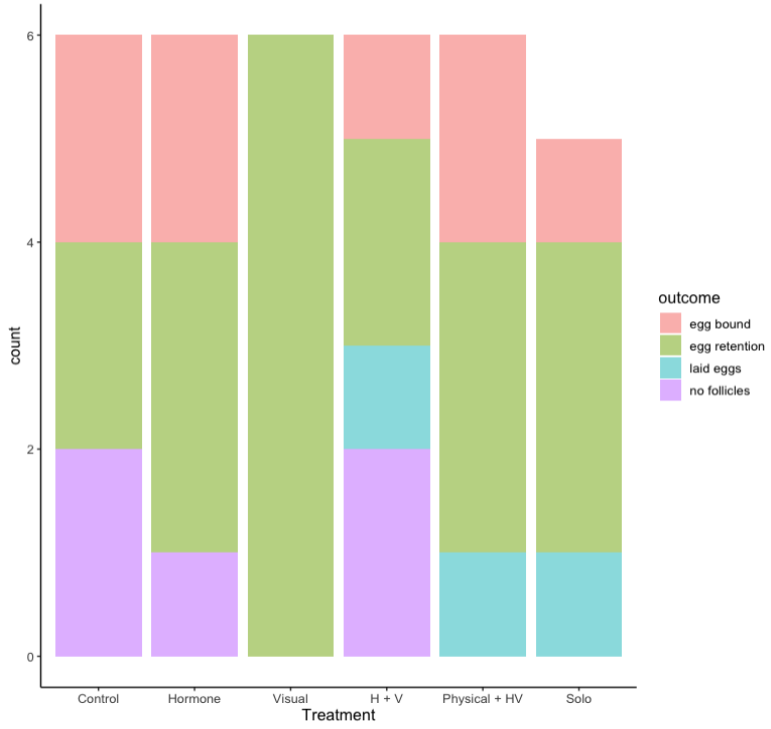


Figure 2.4. Grades of follicular development from ultrasonography on OSF at Vancouver Aquarium (VA) and Greater Vancouver Zoo (GVZ). A) The first ultrasound images were taken on March 2 at GVZ and March 7 at VA. B) The second round of ultrasound images was taken on March 29 at GVZ and March 25 at VA. By the second ultrasound, most GVZ frogs had already laid their eggs, so a third ultrasound was not taken, but no viable eggs had been laid at VA. C) The third round of ultrasound images was taken only at VA, on April 13. Follicular grade 4 was assigned post-hoc to EB OSF once deceased.

A similar trend in the distribution of grades was observed between VA and GVZ on their first ultrasound (Fig. 2.4A), although some VA frogs had delayed follicular development, with fewer frogs at grade 3 than at GVZ. By the second ultrasound (Fig. 2.4B), most GVZ frogs had dropped to grade 0 after laying their eggs. At VA, most grade 1 OSF had now developed to grade 2 or 3 but still no oviposition had occurred. By the third ultrasound at VA (taken on day 37; Fig. 2.4C), some OSF had become egg bound, but the grade distribution was otherwise much the same as for the second ultrasound (Fig. 2.4B), indicating a continued delay in oviposition. While amplexus of males and females usually begins as soon as made possible, it is common for oviposition to occur later at VA relative to wild populations and GVZ (Kris Rossing pers. comm), so the delayed follicular development portrayed in Figure 2.4A is not itself surprising. The major follicular development delays at VA appear to be: i) from grade 2 to grade 3, and ii) from grade 3 to oviposition, suggesting it was not the initial stages of follicular development that differed from GVZ, but rather the lack of egg laying. Amplexus occurred within hours for all GVZ frogs regardless of follicular grade, so the same should have been possible at VA since they followed a similar grade distribution as GVZ. These data seem to validate the assumption that amplexus was not hindered at VA, despite follicular development delays. However, caution should still be taken when extrapolating any results from this recorded amplexus data or when implying any

'normal' versus 'abnormal' behaviours. As a final validation, the overwintering treatment groups were tested as predictors of EB in a generalized linear model. No treatment group was found to be a significant predictor (all $p > 0.05$) and egg binding cases were found in all treatments except Visual (Fig. 2.5), suggesting these overwintering treatment groups were not primarily responsible for the egg binding cases found therein.

Figure 2.5. Distribution of egg binding cases across overwintering treatment groups for Oregon Spotted Frogs at the Vancouver



Aquarium. Treatment groups had varying levels of exposure between male and female OSF. Frogs were separated in Control, had circulating water between separate tanks in Hormone and shared a clear wall in separate tanks in Visual. The H+V had both Hormone and Visual exposure, and in Physical +HV the sexes were together for the whole overwintering period. The Solo treatment had one female per tank. Outcome refers to the reproductive outcome of each OSF at the end of the breeding season (EB, ER, LA, NF).

The influence of amplexus behaviours on the incidence of egg binding in the OSF at VA in 2022 was modeled with a generalized linear model (GLM). This modelling was performed separately from the aforementioned life history GLMs (See section: *Egg binding at Vancouver Aquarium 2022*) to avoid errors due to missing data as only n=30 frogs had their amplexus behaviour recorded during the breeding season. Thus, a GLM was fit to egg binding (the response variable) using the explanatory variables of i) total hours in amplexus, ii) time to first amplexus (latency), iii) total number of amplectants, and the number of iv) touch, v) attempt, or vi) extended contact events. A null model of *egg binding ~ 1* was fit with the data of the 30 OSF with amplexus data. Forward selection was conducted by adding one explanatory variable at a time and selecting the model with the lowest AIC, until the addition of explanatory variables no longer improved model fit. Then the significance of each variable and interaction in the final model was estimated by their two-tailed p-values at alpha = 0.05.

Results

Scaled Mass Index

The mean SMIs (± standard error) for all wild populations measured post-brumation (spring) in 2021 were 46.2 ± 2.3 (MV; n=13), 48.9 ± 3.1 (CH; n=7), and 54.7 ± 1.2 (MS; n=25; Appendix II.II, Fig. A2.1). There were no significant differences among these SMI estimates (Kruskal-Wallis with post-hoc Dunn test: all pairwise $p > 0.05$), so they were combined into one "wild" estimate for all further analyses. Comparing zoo population post-brumation measurements with the "wild" estimate, the mean SMI was highest at VA (75.2 ± 6.1) and lowest at GVZ (40.9 ± 1.8). Both TZ and VA had significantly higher mean SMI than the "wild" (Kruskal-Wallis, Dunn test: both $p < 0.001$), while GVZ had a significantly lower mean SMI than the "wild" (Kruskal-Wallis, Dunn test: $p = 0.0005$). Pre-brumation (fall), the mean SMI did not significantly differ between VA and GVZ (Kruskal-Wallis $p = 0.78$). Interestingly, the mean SMI at GVZ decreased from fall to spring while the opposite trend was observed at VA (Fig. 2.6). In the case of SMI from egg bound (EB) compared to non-EB females within their respective population (VA, GVZ), no significant differences were observed pre- or post-brumation from 2020-2021 (ANOVA $p = 0.167$; Fig. 2.7).

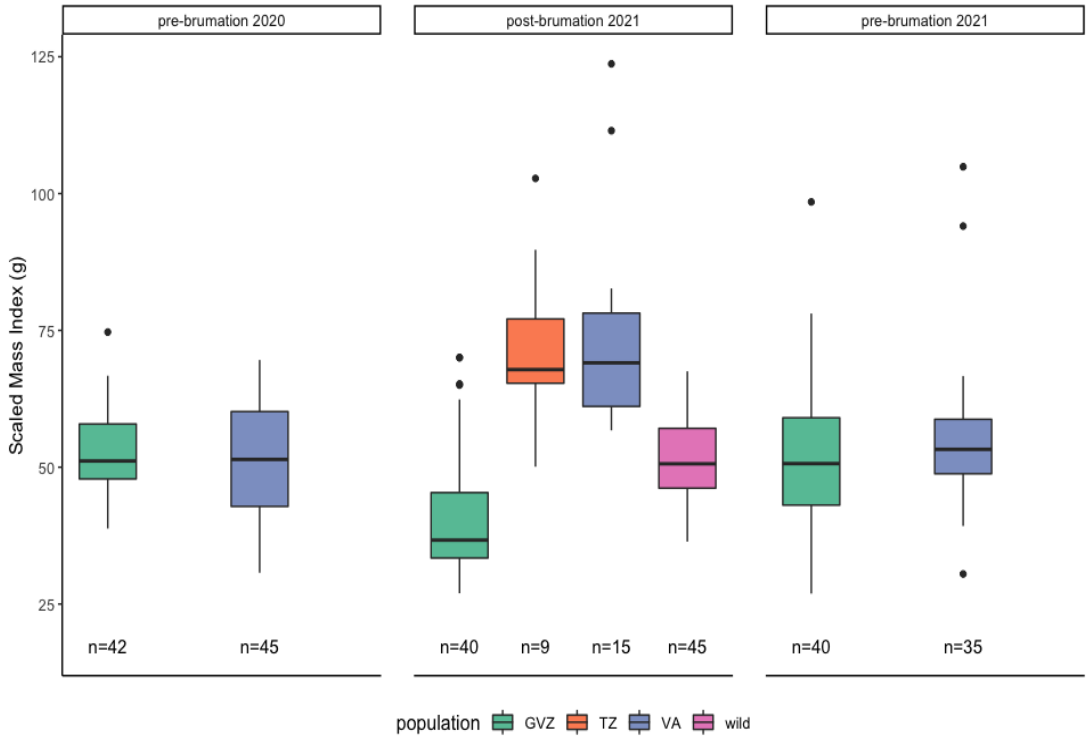


Figure 2.6. Scaled mass index based on mass and snout-vent length for female Oregon Spotted Frogs in 2020-2021. Pre-brumation estimates were measured in the fall (Oct-Dec) while post-brumation estimates were measured in the spring (Feb-May). Zoo sample sizes (GVZ, TZ, VA) do not represent the whole female population but those available and measured in each season respectively. The wild category contains all wild female OSF captured through regular population monitoring in 2021, including individuals from Maria Slough, Morris Valley, and Chaplin.

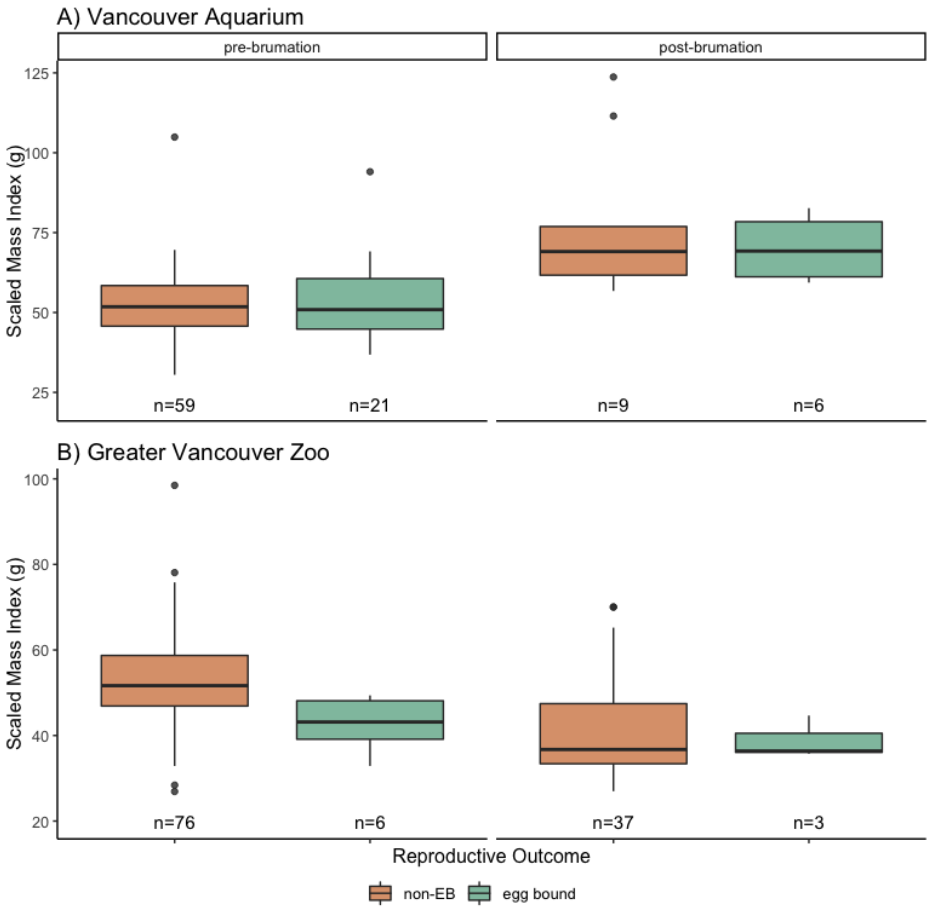


Figure 2.7. Scaled mass index for female OSF the zoo populations A) VA and B) GVZ, measured in 2020-2021. Pre-brumation (fall) includes measurements from 2020 and 2021 while post-brumation (spring) includes measurements from 2021 only. Individual OSF who became egg bound any time from 2020-2022 were counted as egg bound (EB) for all SMI estimates, while all others were considered non-EB.

Growth of Female OSF

The best-fit GLMM to predict EB at VA and TZ included the variables mass, age, and population and all of their interactions. This final model, however, contained no significant predictors of egg binding (all $p > 0.05$), indicating that mass, age, and population did not significantly influence the incidence of egg binding (see Appendix II.III for full model output). Separate analyses per population (VA, TZ) did not reveal any significant influence of mass, age, or their interaction either (all $p > 0.05$). Scaled estimates for the VA GLMM were all low and EB had a negative relationship with both mass (estimate = -0.009) and age (estimate = -0.20), suggesting that individuals with lower mass or age had a higher chance of egg binding. Their interaction (mass*age), however, had a positive estimate (0.05), suggesting that as an individual ages, a higher mass leads to greater risk of egg binding (Fig. 2.8A). Individual estimates were higher in the TZ GLMM, although again not significant. In this case, mass had a positive estimate (3.3), age had a negative estimate (-2.6), and their interaction was close to 0 (0.006). The strength of these estimates may be influenced by small sample sizes per age group (with n decreasing as age increases). Overall, the model indicates individuals with higher mass or of younger age have

a greater risk of egg binding, but the relationship between these factors is less distinct (Fig. 2.8B).

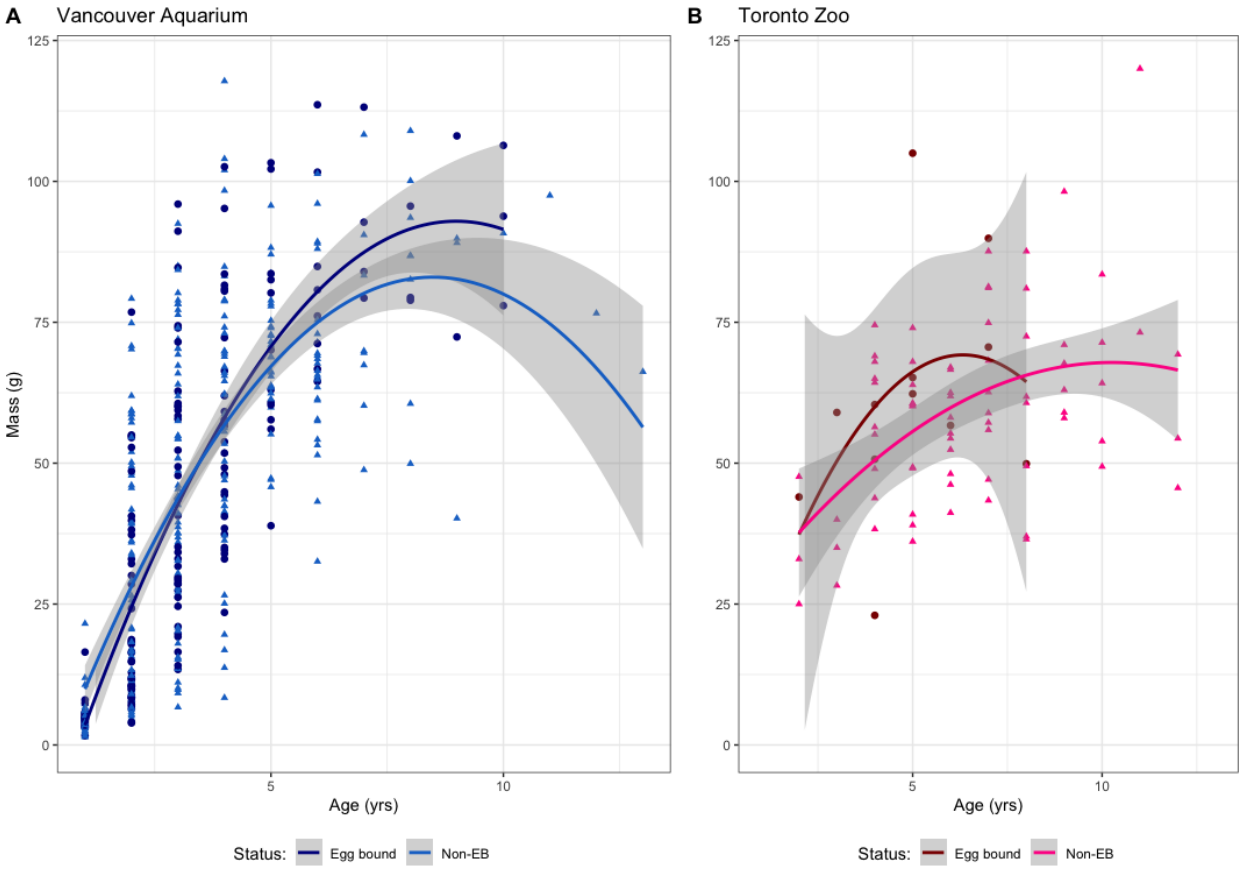


Figure 2.8. Mass changes in female OSF as they age at A) Vancouver Aquarium and B) Toronto Zoo. Individual frogs were identified as egg bound (darker circles) or non-egg bound (lighter triangles) by zoo necropsy reports. A quadratic regression is fit to the individual data points and the shaded area represents each line's respective 95% confidence interval. Group sizes varied with A) 12 EB and 74 non-EB OSF from TZ, and B) 167 EB and 303 non-EB from VA. Frog weights were collected pre-brumation (Oct-Dec) from 2010-2021.

Follicular Grade

Comparing the initial follicular grade (from day 00) by the reproductive outcome of OSF at VA (EB, ER, LA, NF) revealed that females with more developed follicles (higher follicular grade) at the start of the breeding season had a higher chance of experiencing egg binding (Fig. 2.9A). The mean follicular grade (± SE) was 2.38 ± 0.3 for EB, 1.6 ± 0.2 for ER, 1.7 ± 0.9 for LA, and 0.4 ± 0.2 for NF. The majority (63%) of OSF who became egg bound had late-gravid (grade 3) follicular development on day 00 (Fig. 2.9B). In comparison, 89% of females which showed egg retention had either a grade 1 (n=7) or 2 (n=10) on day 00. By the second ultrasound date (day 18; Fig. 2.9C) all EB frogs had developed fully mature follicles (grade 3 or 4). By the final time point (day 54; Fig. 2.9D) 7 out of 8 EB cases had already occurred, leaving the grade 2 and 3 follicular development as 95% ER frogs (Table 2.1).

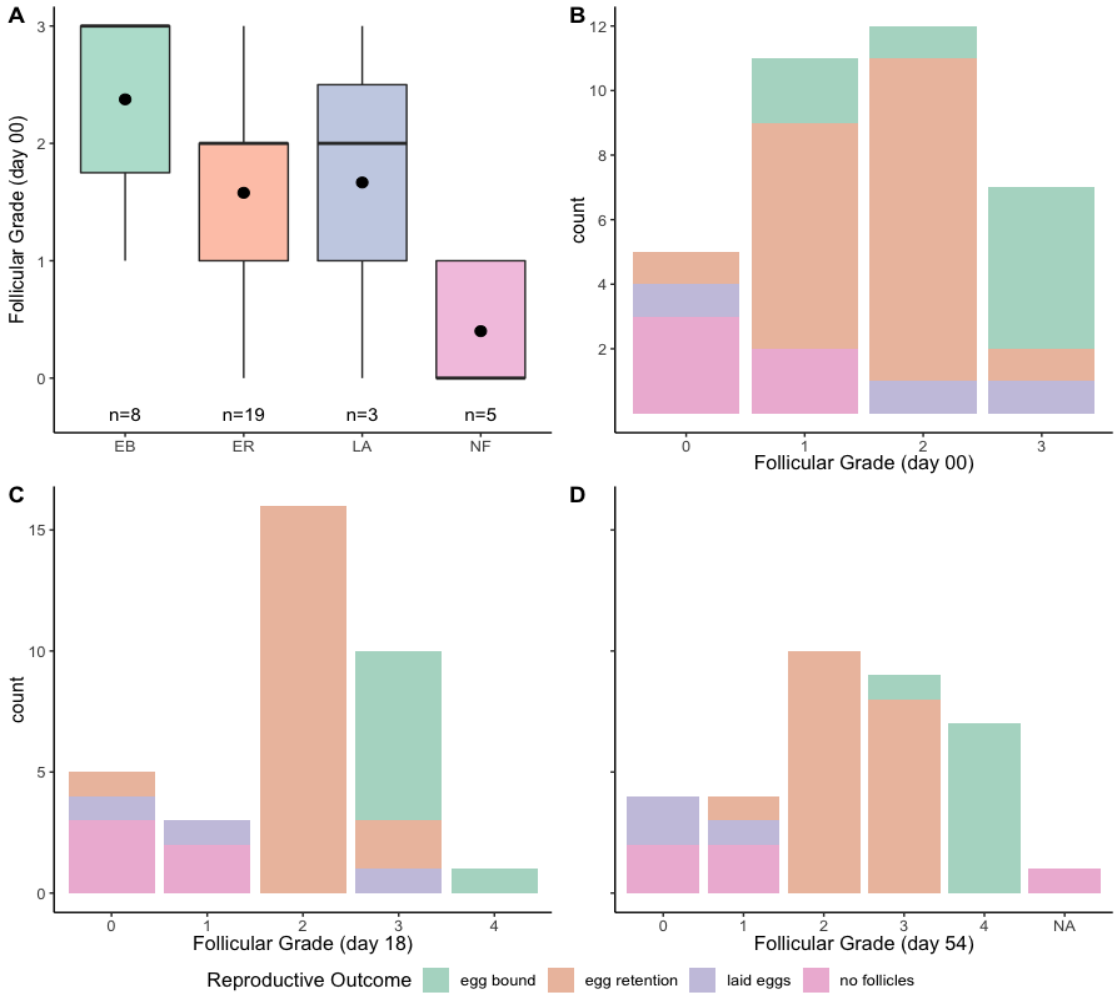


Figure 2.9. Follicular grades by reproductive outcome for female Oregon Spotted Frogs at Vancouver Aquarium. Panels B) through D) show the distribution of reproductive outcomes by follicular grade, determined by ultrasound, on days 00 (B; the day prior to being put with males in breeding groups), 18 (C), and 54 (D; the end of the breeding season). Grades range from 0 (no follicular development) to 3 (mature eggs), with grade 4 assigned post-hoc to EB individuals after their death. The NA in D) indicates an OSF who died due to causes unrelated to egg retention. The black circles in A) indicate the mean follicular grade.

Table 2.1. Oregon Spotted Frog follicular grades over the 2022 breeding period (n=35). Reproductive outcomes were: egg bound (EB), egg retention (ER), laid eggs (LA), or no mature follicles developed (NF).

Day of ultrasound	Grade 0	Grade 1	Grade 2	Grade 3	Deceased
00	*1 ER, 1 LA, 3 NF* *Total = 5*	*2 EB, 7 ER, 2 NF* *Total = 11*	*1 EB, 10 ER, 1 LA* *Total = 12*	*5 EB, 1 ER, 1 LA* *Total = 7*	0
18	*1 ER, 1 LA, 3 NF* *Total = 5*	*1 LA, 2 NF* *Total = 3*	*16 ER* *Total = 16*	*8 EB, 1 ER, 1 LA* *Total = 10*	*1 EB*
37	*2 LA, 2 NF* *Total = 4*	*1 ER, 3 NF* *Total = 4*	*14 ER* *Total = 14*	*6 EB, 3 ER* *Total = 9*	*3 EB, 1 NF* *Total = 4*
54	*2 LA, 2 NF* *Total = 4*	*1 ER, 3 NF* *Total = 4*	*10 ER* *Total = 10*	*1 EB, 8 ER* *Total = 9*	*7 EB, 1 NF* *Total = 8*

Mass and Age

The mean (± SE) post-brumation mass of VA OSF was 31.0 ± 2.5g for 3-year-olds and 59.9 ± 2.6g for 4+ year-old individuals, with older frogs (4+) having a significantly greater mass than the 3 year-olds (ANOVA $p < 0.0001$). Within the 3-year-old group, the mean mass ranged by reproductive outcome from 18.0 ± 1.7g (NF) to 40.7 ± 4.0g (LA; Fig. 2.10). The mass of NF individuals was significantly lower than LA individuals (Tukey HSD p=0.02), but no other reproductive outcomes significantly differed in this age group. For all individuals 4+ years old (combined to accommodate low sample sizes), the mean mass ranged from 48.4 ± 3.5g (NF) to 81.8 ± 2.7g (EB), with EB showing a significantly greater mass than ER (Tukey HSD p=0.02), LA (Tukey HSD p=0.03), and NF (Tukey HSD p=0.007); no other groups significantly differed.

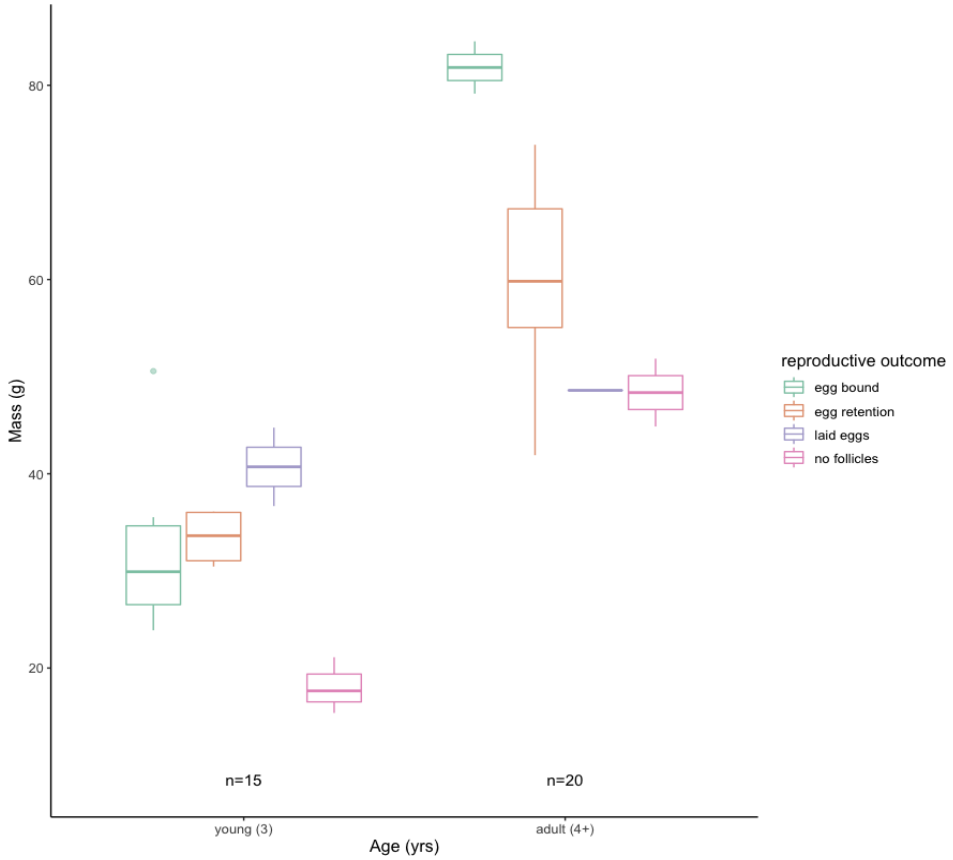


Figure 2.10. Post-brumation mass of female OSF held at Vancouver Aquarium (n=35). Frogs were weighed in March 2022, immediately prior to their breeding season. Age, from the time of weighing, is grouped by 3 years-old (typically first-time breeders) and 4+ years-old to aid analysis.

Despite the significant differences in post-brumation mass between age and reproductive outcome (Fig. 2.10), neither of these variables proved to be significant predictors of EB. In fact, the best-fit GLM included only initial follicular grade (from day 00) as an explanatory variable. As follicular grade on day 00 increased (from 0 to 3) the chance of egg binding significantly increased (p=0.02; full model output in Appendix II.III). The other potential explanatory

variables that did not show any effect were: i) age, ii) spring mass, iii) previous breeding history, iv) number of eggs laid, and v) provenance.

Amplexus Behaviours by Follicular Grade

None of the measured amplexus variables explained a significant amount of the variation in egg binding incidence in the 30 OSF with recorded behaviour at VA in 2022. The null GLM, *egg binding ~ 1* proved a better fit (lowest AIC) than any model with amplexus variables included. While not predictive of egg binding, analysis of the amplexus variables against the previously described life history characteristics revealed some trends in mating behaviours even if significance was lacking. Individuals with an initial follicular grade of 0 took the longest time to be amplexed (mean latency = 12.7 hrs), while females with a grade 2 took the least amount of time (mean latency = 3.5 hrs), although these differences were not significant (ANOVA p=0.186). These same OSF with follicular grade 0 spent an average of 0.21 hours in amplexus over the first 59 hours which was significantly less than the grade 2 females, who spent an average of 27.8 hours in amplexus (Kruskal-Wallis with Dunn test; p = 0.028). Females with an initial follicular grade of 3 spent an average of 29.2 hours in amplexus, but this was not significantly different than any other group due to variance in the data (Appendix II.II, Fig. A2.2). There were no significant differences in the number of amplectants for each follicular grade (0-3) on each ultrasound day (for day 00-54, ANOVAs: p=0.286, p=0.637, p=0.408, p=0.0868, respectively). The number of each contact phase event (*i.e.* touch, attempt, extended) increased with increasing follicular grade (on day 00) up to grade 2, whereupon it decreased for follicular grade 3s (Fig. 2.11). Overall, grade 3 females experienced fewer contact events than grade 1 or 2 females. No significant differences were observed in the total time in amplexus, time to first amplexus contact or total number of amplectants between EB and any other reproductive outcome (*i.e.* ER, LA, NF; ANOVA p > 0.05; Fig. 2.12). For 25 of the 30 OSF, the number of touch contacts (duration < 0.14 hrs) outnumbered any longer contact (attempt and extended), regardless of reproductive outcome.

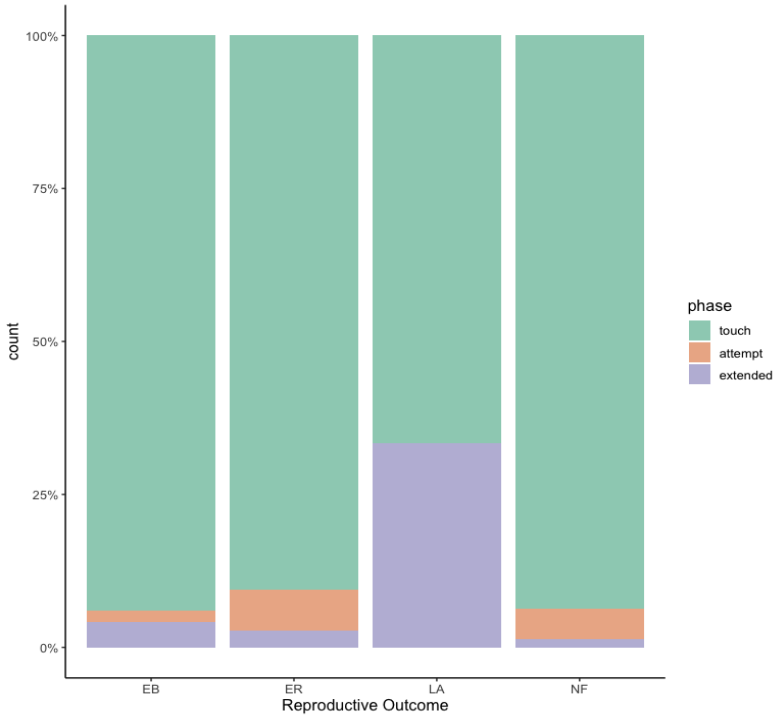


Figure 2.11. Distribution of amplexus contact events by follicular grade. Event durations are touch < 0.14 hrs, 0.14 < attempt > 4 hrs, extended > 4 hrs. Sample sizes differ between follicular grade: grade 0 (n=5), 1 (n=11), 2 (n=12), 3 (n=7). Count scales (y-axis) differ, with the majority of contact events being touch.

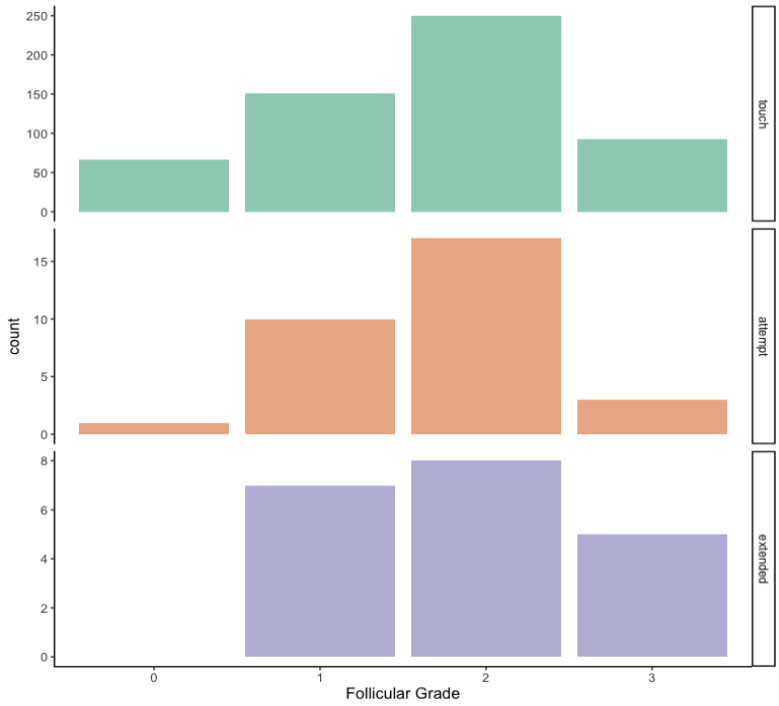


Figure 2.12. The percentage of contact events according to OSF reproductive outcomes. Each count represents contact made on a female OSF by a male. The duration of this contact is classified as either touch (< 0.14 hrs), attempt (0.14 < x > 4 hrs) or extended (> 4 hrs). The same male may contact a female more than once in a row; each is counted as unique contact. Sample sizes differed between reproductive outcomes: EB (n=8), ER (n=12), LA (n=3), NF (n=5).

Discussion

Egg retention may well be a natural phenomenon in amphibians, but escalation to the reproductive disorder known as egg binding has been uniquely observed in zoological populations, which affords an opportunity to monitor and study this anomaly. While no primary causes for egg binding have been elucidated herein, many potential avenues were explored in OSF. Here, we highlight the differing impact of increased mass on reproductive outcome in young and old frogs, with only the latter increasing the risks of egg binding. Further, the grade of follicular development at the onset of the breeding season was significantly associated with subsequent egg binding incidence, with potential correlations to reabsorption capacities. With the possibility for practical application in ongoing OSF recovery actions, this study sheds light on a reproductive health concern impacting many conservation breeding programs.

Scaled Mass Index

The post-brumation SMI estimates from VA and TZ were significantly greater than the mean wild SMI, aligning with the increased body condition values often found in animals under human care compared to wild conspecifics due to a consistent and high-calorie diet, the elimination of predation, and/or controlled climate (Blanco and Sherman, 2005; Kummrow *et al.*, 2010). These increased SMI estimates may reflect greater reproductive output (Necas, 1999), a larger body mass, or both. On the other hand, post-brumation SMI was lower at GVZ than the wild estimate, contrasting with expectations. SMI estimates are meant to be comparable across age groups by accounting for relative allometric growth (Peig and Green, 2009), but reproductive output often increases with increasing female age (Liao *et al.*, 2014) and a smaller reproductive output in the relatively young cohort at GVZ in 2020-2021 would reduce its overall SMI in comparison to VA and TZ. While gravidity was accounted for by comparing SMI within pre-brumation and post-brumation seasons (Prado and Haddad, 2005), it is possible the level of follicular development impacted measurements within the post-brumation season as well. Follicular development was not monitored or graded in spring of 2021 and while SMI performs better than other body condition indices at measuring the presence of eggs, it does not distinguish stages of egg development which intrinsically denotes an increasing density and size of follicles (Calatayud *et al.*, 2018). Thus, if zoo and wild frogs were measured at different follicular grades, this might translate to significant differences in SMI. Zoo frogs were measured prior to being put in breeding groups, meaning oviposition may not have occurred for 2-4 weeks, while wild frogs are assumed to oviposit within hours to days of capture. This latter assumption is based on field observations where females are rarely seen unless actively breeding and prior research suggests females only enter the breeding area when they are ready to breed (Licht, 1969). Further, ultrasound images from wild OSF collected in 2022 indicated all had grade 3 follicular development, suggesting imminent oviposition. Taking the potential difference in follicular grades into account, each zoo SMI represents a conservative estimate for the post-brumation season which might have continued to increase over the next couple weeks, while wild OSF were at their highest potential SMI for the season. The wild SMI estimates, therefore, likely represent more of the reproductive output while those from the zoos represent more of the frog's fat reserves, assuming there would be no significant

differences in reproductive output at grade 3 between wild and zoo populations. VA and TZ, then, have significantly greater fat reserves than wild conspecifics but not necessarily the increased reproductive output hypothesized to cause egg binding due to surpassed physical capacities (Kummrow *et al.*, 2010). At GVZ, if follicular development was delayed relative to wild individuals this would help explain their lower SMI. Further, increased enrichment and activity from the more naturalized, larger holding tanks (Chum *et al.*, 2013) and communal breeding setup at GVZ might account for lower fat reserves (as represented in SMI estimates) relative to VA and TZ but would not explain the difference from wild estimates.

Despite the lower SMI at GVZ, egg binding occurred there at a similar rate as in the other zoos and no significant differences were found between EB and non-EB frogs at VA or GVZ. While overweight OSF at VA and TZ might be of concern as a phenotypic divergence from wild conspecifics, these results suggest increased body condition is not a primary contributor to egg binding in OSF. However, this conclusion may be inflated by small sample size as there were only 3/42 and 13/45 EB frogs at GVZ and VA respectively, from 2020-2022. A further phenotypic inconsistency is the mean SMI at GVZ markedly decreasing from pre- to post-brumation (52.4 to 40.9 g) while at VA the SMI increased from pre-brumation. Temperate anurans do not feed during brumation (Pinder *et al.*, 1991) and OSF do not resume foraging until after the breeding period so the decreased SMI might reflect the loss of body weight over the winter. For female anurans, however, most of the energy reserves stored up prior to brumation are channeled into egg production (Pinder *et al.*, 1991), which should have increased their post-brumation SMI since this index accounts for reproductive output (Calatayud *et al.*, 2018). While potential differences in follicular grade may help account for the lower post-brumation SMI at GVZ, it does not explain the decrease from fall to spring and it would be important to determine whether these differing patterns of SMI change are consistent year-to-year at each zoo. The OSF at all three zoos should be weighed and measured according to a common standard (*i.e.* SVL, not SUL) at least twice a year, pre-brumation and post-brumation. This will allow more accurate comparison of body condition trends within and across zoos; an important first step in determining the cause of any differences confirmed therein.

Body Mass in Young OSF

Larger frogs typically have a larger reproductive output consisting of fewer but larger individual eggs than smaller frogs of the same age (Duellman, 1989). As frogs age they direct more energy toward reproduction instead of growth, increasing their overall reproductive output relative to younger frogs, reflecting positive trends between reproductive output, age, and body size a (Jørgensen, 1981; Gibbons and McCarthy, 1986; Liao *et al.*, 2014). While egg size and number were not estimated in this study, theory implies that the 3-year-old OSF at VA in 2022 would have a lower reproductive output than older OSF, reducing their risk of egg binding as a result of exceeded reabsorption capacities (Kummrow *et al.*, 2010). Yet, most egg binding occurred in 3-year-old OSF with a lower body mass (though not significantly) than ER or LA frogs of the same age. The greater mass of ER and LA 3-year-old OSF could be a result of increased growth - indicating reduced reproductive investment due to energy trade-offs (Berven, 1988; Liao *et al.*, 2016) - or a result of increased reproductive output in the form of larger but fewer eggs (Duellman, 1989). If the former is true, OSF with reduced fecundity (*i.e.* lower reproductive

output) in their first breeding season devote more energy to continued growth over a longer lifespan (Williams *et al.*, 2006), with this reduced early fecundity consequently reducing their early risk of egg binding. However, if the latter assumption is true, then smaller OSF producing a greater number of albeit smaller eggs are at higher risk of egg binding than OSF producing fewer but larger eggs, perhaps due to a greater space-occupying effect - with the vast numbers of eggs interfering with other internal organ functions (Hedley, 2016). Both of these potential explanations should be investigated through counting and measurement of both laid and unlaid eggs in first-time breeders particularly, as these hypotheses hold very different implications for OSF husbandry. If speeding up growth limits early fecundity and reduces egg binding risks, the impacts of omitting the brumation period in favour of growth (Calatayud *et al.*, 2015, 2020) should be carefully investigated. These conclusions on the impact of body mass on egg binding risks should be observed cautiously, however, as EB OSF had lower body mass than ER and LA frogs, but these differences were not significant, nor were there significant pre-brumation mass differences for 2-3-year-old EB frogs at VA and TZ over 2010-2021. Therefore, OSF in their first breeding season should be carefully monitored for signs of egg binding regardless of body mass until the impact on young frogs is more clearly delineated.

Body Mass in Old OSF

In the long-term dataset from VA and TZ (2010-2021), mass differences became visible for OSF over the age of 5, and in 2022 at VA, older EB frogs (4+ years-old) had significantly higher post-brumation body mass than frogs with other reproductive outcomes (ER, LA, NF). Although weakly, the modelling of long-term data from VA (2010-2021) echoes a dichotomous relationship between mass and age. An increasing mass elevated the risk of egg binding only as a frog aged, but as standalone predictors it was lower age that predicted EB with mass on its own having negligible impact. The modeling of long-term TZ data, while again not significant, showed the same pattern in the former two predictors (*i.e.* age and its interaction with mass) but mass had a stronger positive relationship with EB, indicating that an increased body mass at any age increased the risk of egg binding, albeit slightly. This difference could be explained by differences in population demographics, since TZ generally has an older cohort of frogs, thus limiting the sample size of 2-3-year-old frogs and thus the full distribution of ages. It should be noted that the long-term datasets for both VA and TZ use pre-brumation body mass and while a frog's nutritional status outside of the breeding season can be related to egg size and/or number (Jørgensen, 1981), this is not always the case (Lüddecke, 2002). In fact, there is generally a trade-off between somatic maintenance (*i.e.* maintenance of the structural body) and reproduction (Kirkwood, 2001). Decreased early fecundity (ex. by the delay of maturation) favours longevity and increased body size (Williams *et al.*, 2006; Liao *et al.*, 2016). While there is evidence of OSF living up to 10 years in the wild (unpublished data), they are generally believed to live much shorter lives than those in captivity; Stark and Meiri (2018) reported animals held *ex situ* live an average of 17% longer than wild conspecifics. However, with the longevity of zoo animals comes a decline in performance and function (*i.e.* senescence) which is not generally seen in the wild due to predation and other extrinsic pressures (Saino *et al.*, 2002). Aging frogs have also been seen to retain more eggs for a longer time than younger frogs, perhaps no longer able to clear apoptotic eggs from their genital tract (Iguchi *et al.*, 2013). The slower metabolism of larger animals combined with the impacts of senescence (Saino *et al.*, 2002) and

greater numbers of retained eggs may overwhelm the physical capacity of older OSF to reabsorb unlaid eggs (Kummrow *et al.*, 2010), leading to egg binding later in life. This reiterates the need for *ex situ* OSF morphometrics to be measured on a more consistent basis using common methodology to allow further investigation of these trends at all zoos. Diet and activity levels of *ex situ* OSF should also be adjusted to slow the over-conditioning (*i.e.* high body mass) of OSF as they age, since matching the phenotype of wild conspecifics should be a goal of conservation breeding programs (Schulte-Hostedde and Mastromonaco, 2015), let alone the concomitant benefit of reducing egg binding risks.

Follicular Development and Breeding Behaviours

The follicular grade of OSF prior to breeding proved to be the only significant predictor of egg binding among all tested variables for VA in 2022, which included life history characteristics (ex. age, breeding history, mass) and amplexus behaviours (ex. latency, number of amplectants, time in amplexus). Increasing follicular grade (from 0 to 3) increased the chance of egg binding such that females who started the breeding season with late-gravid ovaries and mature eggs (grade 3) were the most likely to become egg bound. However, this result is likely an artefact of females not laying the mature eggs they started the breeding season with rather than an initial grade 3 follicular development being a direct cause of egg binding. First, a greater proportion of OSF at GVZ (in comparison to VA) started their breeding season with grade 3 follicular development and nearly all these females laid their eggs without incident before their next ultrasound. Second, the two OSF at VA with an initial grade of 3 who did not become EB (1 ER and 1 LA) both laid > 50% of their eggs over the course of the breeding season. While these females started with mature eggs, any of the eggs remaining after oviposition were reabsorbed or retained without incident. Ovulated eggs are generally retained in the anuran ovisac for only a day or two before being expelled during amplexus (Rugh, 1951). Cell death occurs in oviposited anuran eggs that are not fertilized within a few days (Tokmakov *et al.*, 2011) and this same cell death is thought to be the method of reabsorption used for retained eggs (Iguchi *et al.*, 2013). Most reabsorption of eggs has been noted in the genital tract (ex. uterus and oviduct), although degradation of a few eggs free-floating in the body cavity has also been noted (Iguchi *et al.*, 2013). Post-mortem reports and photos of EB OSF (see Fig. 2.3B) often revealed half the OSF's retained eggs forming a viscous mass in the ovisac while the other half remain free-floating in the body cavity. The high number of eggs filling the body cavity of EB frogs could be responsible for subsequent lethargy and dysfunction (Hedley, 2016) and may interfere with the typical reabsorption process. OSF with delayed follicular development, never surpassing grade 2, were less likely to become egg bound, suggesting follicles at this stage were easier to reabsorb. Unfortunately, we were unable to distinguish the location of follicles and eggs (ex. un-ovulated, ovisac, body cavity) via ultrasound but this should be investigated further as the duration eggs are retained, and the number and location of their retention may reveal much about the reabsorption capacity of OSF. Furthermore, if the number of retained eggs is critical to reabsorption capacity, this could explain why smaller 3 year-old OSF, who likely produced a greater number of smaller eggs than larger 3 year-olds, became egg bound.

So, what prevented OSF at VA in 2022 from laying their mature eggs? A frequent cause for delayed oviposition in reptiles is unsuitable environmental conditions (Kummrow *et al.*, 2010;

Pimm *et al.*, 2015). In most anurans, the only form of parental care is the selection of oviposition sites, the suitability of which can directly impact larval survival and thus reproductive success (Resetarits and Wilbur, 1989). OSF breeding is explosive, with females laying egg masses in communal piles (Phillipsen *et al.*, 2010), often in the same location year after year. The more a zoo environment differs from its wild context, the more reproductive performance is likely to decline (Schulte-Hostedde and Mastromonaco, 2015) as important stimuli and behaviours are altered (O'Regan and Kitchener, 2005; Asa *et al.*, 2011). The changes made to the holding conditions at VA in 2022 (ex. removal of vegetation) for the concurrent overwintering study may have created unsuitable conditions for oviposition, causing female OSF to retain their eggs rather than lay them. Interestingly, the few OSF who did lay eggs, whether they were in amplexus or not, produced no viable eggs, suggesting mates may have been inadequate as well. Sexual selection, mate choice, and mating cues are often removed, altered, or obscured in *ex situ* environments, which can also lead to reproductive failure (Asa *et al.*, 2011; Schulte-Hostedde and Mastromonaco, 2015). While this study did not find any amplexus behaviours to be predictive of egg binding, the most common amplexus lasted less than 8 minutes (touch phase). This short duration contrasts with observations of seemingly successful amplexus (leading to viable oviposition) of OSF in both the wild and other zoos where a pair remains in amplexus until eggs are deposited and fertilized, which might take a few hours to many days, depending on the female's stage of follicular development (Calatayud *et al.*, 2018). While none of the amplexus behaviours recorded and analysed here were a primary cause of egg binding, they themselves may have been impacted by the altered environmental conditions or might have acted synergistically with them, as egg retention is likely multifaceted (Stacy *et al.*, 2008). Calatayud et al. (2018) found egg retention was most common for anurans who experienced inconsistent or negligent amplexus so continued research on mating behaviours is warranted. Husbandry staff at zoos should continue to monitor OSF amplexus behaviours where they can do so unobtrusively, and a standard of natural breeding behaviour should be determined from wild populations for comparison.

Implications for Conservation

While the incidence of egg binding in current OSF conservation breeding populations likely does not pose an imminent threat to their viability, this should not lessen the conservation concern. Genetic management is often prioritized in conservation breeding and reintroduction programs, but reproductive success and individual wellbeing must also be considered (Asa *et al.*, 2011) lest the genetic material being managed be lost as a result of reproductive dysfunction. More research should be conducted into the optimal environmental conditions for OSF, including ways to maximize natural mate selection and minimize the mismatch between zoo and wild conditions, thereby limiting unintended changes brought about by *ex situ* management (O'Regan and Kitchener, 2005; Schulte-Hostedde and Mastromonaco, 2015). Amphibians in their first breeding season should be carefully monitored and perhaps given more time to reach full sexual maturity (and the ensuing increased body size) before being placed in the active breeding program. The body condition of older frogs should also be monitored and body mass stabilized or decreased after age 5 to avoid late-onset egg binding. Where possible, ultrasound imaging should continue to be used to track follicular development and females retaining mature eggs should be carefully monitored to ensure all or most are laid. Finally, an underlying

emphasis throughout this study is the need for standardized data collection. Tracking and comparing breeding strategies in zoos is only possible when data are collected regularly, according to consistent standards across all zoos. Collaboration and communication are necessary for all effective recovery programs but particularly where multiple *ex situ* and *in situ* partners are involved. Thus, the continuation of such collaboration in ongoing conservation breeding and reintroduction programs will surely prove critical to the persistence of OSF in Canada.

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Tables, Figures, and Appendices

Tables

Figures

Figure 2.10. Post-brumation mass of female OSF held at Vancouver Aquarium (n=35). Frogs were weighed in March 2022, immediately prior to their breeding season. Age, from the time of weighing, is grouped by 3 years-old (typically first-time breeders) and 4+ years-old to aid analysis.

Figure 2.11. Distribution of amplexus contact events by follicular grade. Event durations are touch < 0.14 hrs, 0.14 < attempt > 4 hrs, extended > 4 hrs. Sample sizes differ between follicular grade: grade 0 (n=5), 1 (n=11), 2 (n=12), 3 (n=7). Count scales (y-axis) differ, with the majority of contact events being touch.

Figure 2.12. The percentage of contact events according to OSF reproductive outcomes. Each count represents contact made on a female OSF by a male. The duration of this contact is classified as either touch (< 0.14 hrs), attempt (0.14 < x > 4 hrs) or extended (> 4 hrs). The same male may contact a female more than once in a row; each is counted as unique contact. Sample sizes differed between reproductive outcomes: EB (n=8), ER (n=12), LA (n=3), NF (n=5).

Appendix II.I: summary of available morphometrics

Table A2.1. OSF morphometrics

			pre-brumation		post-brumation	
population	**established**	**documented egg binding**	**mass**	**SVL**	**mass**	**SVL**
VA	2010	2011-2022	2012-2022	2010-2022*	2021-2022	2021
GVZ	2017	2018-2022	2021	2020-2021	2021-2022	2020-2021
TZ	2010	2010-2022	2010-2022		2010-2022	2021
Wild					2010-2022	2010-2022

**length measurements at VA were taken as snout-urostyle length (SUL) but later converted into SVL*

Appendix II.II: supplementary figures

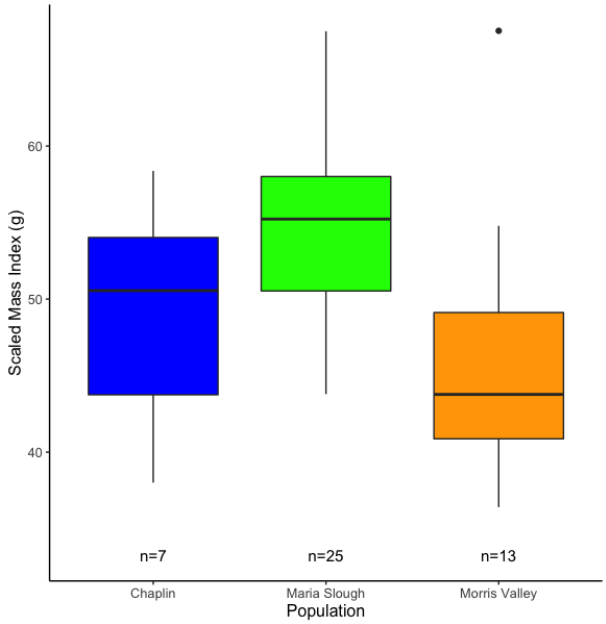

Figure A2.1. Scaled mass index for the three wild populations (CH, MS, MV) of OSF measured in the spring of 2021. Measurements were collected during the breeding season (Mar-Apr) and only gravid females were included. A post-hoc Dunn test indicated no significant differences in mean SMI between these populations.

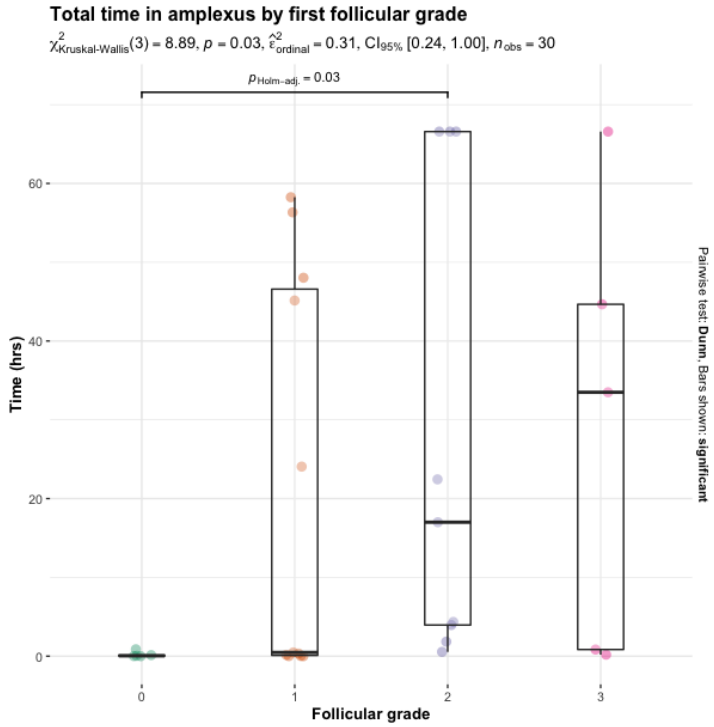

Figure A2.2. The total time in amplexus over the first 59 hours of the OSF breeding season at VA in 2022 according to initial follicular grade. Time in amplexus indicates the cumulative time a female was being amplexed by a male. Follicular grades vary from not-gravid (0) to late-gravid (3). These grades were assigned to ultrasound images taken the day before females were exposed to males (day 00). A test of significance (Kruskal-Wallis) is indicated at the top of the figure, with the significant p = 0.03 (from post-hoc Dunn test) indicated between grades 0 and 2.

Appendix II.III: final GLMs and GLMM outputs

Table A2.2. GLMM - full model
EB ~ scaled mass * scaled age + population + scaled mass : population + (1 | ID)

Fixed Effects	**Estimate**	**Std. Error**	**Z**	**p**
Intercept (Control)	-13.25230	3.35851	-3.946	7.95e-05 ***
scaled mass	1.12042	3.79887	0.295	0.768
scaled age	-0.49365	1.37997	-0.358	0.721
population (VA)	0.55773	3.52984	0.158	0.874
interaction: scaled mass - scaled age	0.01388	0.85754	0.016	0.987
interaction: scaled mass - pop VA	-0.97752	3.94274	-0.248	0.804
Random Effects	**Variance**	**Std. Dev.**	**N**	
frog ID (intercept)	3138	56.42	556	
AIC	**BIC**	**logLik**	**deviance**	**df.resid**
194.4	224.6	-90.2	180.4	549

Table A2.3. GLMM - Vancouver Aquarium subset
EB ~ scaled mass * scaled age (1 | ID)

Fixed Effects	**Estimate**	**Std. Error**	**Z**	**p**
Intercept (Control)	-12.66787	1.49992	-8.446	<2e-16 ***
scaled mass	-0.00855	1.19133	-0.007	0.994
scaled age	-0.19879	1.44686	-0.137	0.891
interaction: scaled mass - scaled age	0.04804	0.86608	0.055	0.956
Random Effects	**Variance**	**Std. Dev.**	**N**	
frog ID (intercept)	3251	57.02	470	
AIC	**BIC**	**logLik**	**deviance**	**df.resid**
31.6	43.8	-10.8	21.6	81

Table A2.4. GLMM - Toronto Zoo subset
EB ~ scaled mass * scaled age (1 | ID)

Fixed Effects	**Estimate**	**Std. Error**	**Z**	**p**
Intercept (Control)	-13.068145	5.116111	-2.554	0.0106 *
scaled mass	3.331353	6.942322	0.480	0.6313

scaled age	-2.572018	5.573885	-0.461	0.6445
interaction: scaled mass - scaled age	0.005718	3.024546	0.002	0.9985
Random Effects	**Variance**	**Std. Dev.**	**N**	
frog ID (intercept)	2266	47.6	86	
AIC	**BIC**	**logLik**	**deviance**	**df.resid**
168.5	189.2	-79.2	158.5	465

Table A2.5. GLM - Vancouver Aquarium 2022 life history variables
EB ~ follicular grade (day 00), family = binomial(link = “logit”)

(Intercept)	-3.8934	1.3718	-2.838	0.00454
follicular grade (day 00)	1.4016	0.6015	2.330	0.01979
Null deviance	37.628 on 34 degrees of freedom			
Residual deviance	29.993 on 33 degrees of freedom			
AIC	33.993			

Table A2.6. GLM - Vancouver Aquarium 2022 amplexus variables (NULL model)
EB ~ 1, family = binomial(link = “logit”)

(Intercept)	-1.1896	0.4317	-2.756	0.00585 **
Null deviance	32.596 on 29 degrees of freedom			
Residual deviance	32.596 on 29 degrees of freedom			
AIC	34.596			

General Conclusions

Species at risk often require human intervention to mitigate the excessive threats they face due to human disturbance. When the mitigation of *in situ* threats is insufficient for species recovery, *ex situ* programs can be integrated with ongoing conservation efforts to provide assurance against immediate extinction and offspring for future reintroduction efforts (Johnson *et al.*, 2020). Amphibians face a particularly serious extinction crisis (Stuart *et al.*, 2004) and an increasing number of amphibian species are being brought into captivity in attempts to establish conservation breeding and reintroduction programs (Dawson *et al.*, 2016). There remains significant room for improvement, however, as the suitability of many of these amphibians for *ex situ* conservation has recently been called into question due to low success rates and a lack of assessment prior to program establishment (Griffiths and Pavajeau, 2008; Tapley *et al.*, 2015; Bradfield *et al.*, 2022).

The importance of maintaining demographically robust *ex situ* populations with adequate representation of wild population genetics to avoid loss of genetic diversity and inbreeding depression is well known (Soulé *et al.*, 1986; Frankham, 1995; Lees and Wilcken, 2009). The genetic management required to attain genetic sustainability, however, sometimes precipitates a decline in reproductive performance when natural mate selection is sacrificed for optimal genetic pairings (Asa *et al.*, 2011). Furthermore, unreliable, or insufficient breeding without additional founders can impact genetic sustainability (Coloma and Almeida-Reinoso, 2012), and with only 55% of amphibian conservation breeding programs producing viable offspring (Smith and Sutherland, 2014), the efficacy of these programs is questionable. Knowledge gaps can further compromise both reproductive and genetic success of conservation breeding programs and ideally should be addressed prior to program establishment (Bradfield *et al.*, 2022).

The Oregon Spotted Frog is a species at risk in Canada for which *ex situ* efforts have been deemed necessary to prevent local extinction. Despite differences in the established conservation breeding and reintroduction programs, all ex situ locations displayed poor reproductive performance and a high incidence of egg binding. While two of the three zoos (VA, TZ) closely managed the genetics of their OSF populations, they did so without prior assessment of the genetic diversity of either wild or zoo OSF populations. The third zoo (GVZ) allowed for more natural mate selection but still experienced reproductive dysfunction (*i.e.* egg binding) and the lack of information around this issue has proved detrimental to the reproductive sustainability of all three programs. My results showed there were no significant differences in the genetic diversity of the three zoos, but rather that all three had stable genetic diversity and inbreeding levels relative to the wild populations. Thus, careful genetic management (at VA and TZ) has not significantly improved population genetic health compared to the more communal, hands-off approach of GVZ, and the benefits of pre-selected breeding groups should be carefully assessed and reconsidered. Population structure results also indicated low to moderate differentiation among the wild OSF populations, suggesting that the risk of outbreeding depression from mixing lineages is lower than expected for small, isolated populations (Hitchings and Beebee, 1997; Lesbarrères *et al.*, 2006). However, the possibility of outbreeding and inbreeding depression should still be investigated by monitoring offspring

survivorship before final conclusions are made or programs are changed irreversibly. It would be important to closely monitor inbreeding levels as values were high in both zoo and wild populations and there is evidence of increased inbreeding and mutation loads in unmanaged animal populations relative to genetically managed ones (Humble *et al.*, 2023).

With regards to egg binding, the causes behind this reproductive dysfunction remain multi-faceted and elusive. Zoological institutions have the unique ability to monitor and study this reproductive disorder *ex situ* and should share this opportunity by reporting on reproductive challenges in platforms accessible outside the zoo community. For conservation breeding and reintroduction programs, such as those underway for the endangered OSF, every individual plays an important role in demographic stability and genetic sustainability such that all mortalities have far-reaching repercussions (Asa *et al.*, 2011). While no primary cause for egg binding in OSF has been fully elucidated, many potential avenues were explored. The lower body condition of GVZ frogs relative to other zoo and wild conspecifics could be a result of the larger tank sizes and more communal breeding approach. While such conditions have not eliminated egg binding, the benefits of allowing natural mate selection (Asa *et al.*, 2011) should be strongly considered in light of the similar genetic outcomes across zoos. The importance of adequate environmental conditions and stimuli to reduce egg binding should also be investigated in these populations and more research conducted on the interaction of mass and age. Where possible, ultrasound should be used to monitor the follicular development of female OSF, and oviposition induced in first-time breeders if eggs are retained beyond the breeding season.

Underlying all these results, this study highlights the importance of collaboration not only between *in situ* and *ex situ* partners (Byers *et al.*, 2013), but also among the various zoos involved in *ex situ* programs. While there are inevitable limitations due to differences in facilities, resources, and locations, effort should be given to ensuring a cohesive approach across facilities no matter when populations are established. For instance, discrepancies in data collection and measurement techniques across *ex situ* populations of OSF greatly impeded the analysis and clarity of results surrounding body condition in this study. In fact, some of these differences were not discovered until analysis was attempted and might have gone unnoticed for another decade. Communication and feedback should be increased to avoid such oversight in the future. A greater balance between genetic and reproductive health should also be sought for conservation breeding and reintroduction programs as the prioritization of one can lead to the decline or exclusion of the other (Asa *et al.*, 2011). With the predicted genetic diversity declines in wild OSF populations, the importance of continued *ex situ* efforts is clear, but the causes of egg binding should continue to be investigated lest these mortalities threaten the long-term sustainability of these zoo populations. Overall, this study contributes to improving the cohesiveness of recovery actions for OSF in Canada, providing critical assessments of both the genetic and reproductive health among ongoing OSF conservation breeding populations. These results will inform not only the strategies and protocols of the zoos involved, but also provide recommendations applicable to the global amphibian conservation breeding effort.

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