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Informing Management Strategy for the Relict Leopard Frog (Rana onca): Insights Into Breeding Biology and an Attempt to Improve Augmentation Success Through Pre-Exposure and Clearance of an Emerging Amphibian Pathogen

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INFORMING MANAGEMENT STRATEGY FOR THE RELICT LEOPARD FROG (*RANA ONCA*): INSIGHTS INTO BREEDING BIOLOGY AND AN ATTEMPT TO IMPROVE AUGMENTATION SUCCESS THROUGH PRE-EXPOSURE AND CLEARANCE OF AN EMERGING AMPHIBIAN PATHOGEN

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ABSTRACT

The Relict Leopard Frog, *Rana onca*, is a species of conservation concern that suffered a decline sometime during the 20th century. Even after two decades of intensive management, the species currently only occupies 20 spring sites in southern Nevada and northwestern Arizona. The causes for the historical decline are mostly speculative, but relate to habitat loss, introduced predators, and emergent disease. Since 2001, *R. onca* has been under an intensive conservation program managed by a multiagency conservation team. There are several objectives specified in the program including the need to investigate the biology of the species and incorporate findings into management strategies. Presented in this thesis are two research projects intended to inform management of *R. onca*, including a life history assessment of breeding biology, and a proof-of-concept assessment aimed at improving survival of headstarted frogs in a landscape where an emergent amphibian pathogen is present.

The first project, on the breeding biology of *R. onca*, was initiated because there was limited information on the topic, with only a few summaries published in the literature and other information buried in relatively inaccessible government reports. Data on breeding biology were accumulated from 19 years of headstarting, translocation, and population monitoring efforts. To add new insights and clarify previous perspectives, this information was synthesized and assessed to determine breeding seasonality, egg mass size, time to hatching, time to metamorphosis, and time to reproductive maturity. In an iterative process, the knowledge gained on breeding biology was incorporated over time to improve the conservation program for the species.

The historical decline of *R. onca* may have been facilitated by the emergence of the amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*

(Bd). The second project was a proof-of-concept to determine if pre-exposure to Bd followed by clearance of infections (pre-exposure and clearance) improved survival in headstarted *R. onca* used to augment a population where the pathogen was present. The study incorporated 229 headstarted frogs separated into two groups, a group that underwent pre-exposure and clearance, and a control group treated identically but exposed to a sham inoculum. The groups were released to a study site where Bd was present and then monitored for subsequent infection and survival over 18 months. Mark-recapture and generalize additive modeling were used to analyze field data. Infection prevalence and intensity across treatment groups were predicted by survey date and air temperature, with Bd infections in frogs showing strong seasonal trends. The pre-exposure group had improved resistance at important points in time and showed a trend of moderately higher survival then the control group, although the difference in survival was not statistically supported. The findings from this research inform managers on the practicality of using pre-exposure and clearance on headstarted *R. onca* intended for release at sites with Bd, as well as provides insight into the potential dynamics of this amphibian pathogen in the southern Nevada region.

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I want to acknowledge all those that have participated on the Relict Leopard Frog Conservation Team (RLFCT). I was a greenhorn when I became a member of the team and I appreciated the warm welcome I received. I am grateful that my career began with this dedicated team and for the connections I made along the way. I present two projects in this thesis, so I will separate my acknowledgements for each chapter.

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DEDICATION

To my family:

To my mom, Rebeca, thank you for always being positive and for supporting me, especially with my schooling.

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Thank you all for giving me so much joy and for keeping me grounded.

A Relict Leopard Frog Poem

Grapevine Canyon

Searching for Leopard Frogs

By Rebecca Lee Peck

We came upon him
deep in the desert canyon
of cliffs and undercut limestone banks
near ferns and translucent pools
lined with dots of red monkey flower.

He was glistening like a jewel
motionless,
save for the water that
washed over him.

We moved slowly
following the shadows
downstream
the falls making the only sound
as he took in the warmth from the sky.

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CHAPTER 1

INTRODUCTION

The relict leopard frog, *Rana onca*, suffered a decline sometime during the 1900s. By the early 2000s the species was restricted to a few sites in southern Nevada, with an estimated overall population of about 1100 adult frogs (Bradford et al. 2004). Information on the historical distribution of the species, derived from a handful of museum collections and literature references, indicate that it occupied a narrow distribution in southwestern Utah into northwestern Arizona and southern Nevada (Bradford et al. 2004). The frog inhabited various aquatic habitats within the Virgin River drainage and its tributaries downriver to Black Canyon along the Colorado River. Speculation on the causes for the decline focus on several synergistic factors including: habitat degradation from agriculture and water development, loss of disturbance that allows the encroachment of vegetation into more open habitats, the introduction of amphibian and aquatic predators, along with disease, particularly the amphibian chytrid fungus disease (Bradford et al. 2004; Jaeger et al. 2017). The remaining populations of *R. onca* were studied by researchers in the 1990s and early 2000s to gather information on population status, systematics, and taxonomy of the species (e.g., Jaeger et al. 2001; Bradford et al. 2004). This research was the catalyst for the development of a management and conservation program for the species.

The program was initiated in 2001 by the formation of a multiagency conservation team called the Relict Leopard Frog Conservation Team (RLFCT). Members include participants from various federal and state resource and land management agencies, and partner entities, including biologists from the University of Nevada, Las Vegas (UNLV). The conservation team collaborated in the development of a 10-year conservation agreement, assessment, and strategy (CAS) signed in 2005, which was updated and renewed in 2016 (RLFCT 2016). The CAS

stipulates specific objectives, strategies, and actions aimed at ensuring the persistence of the species, as well as providing for the integration of new information on species biology following an adaptive management framework. The goal for the conservation program was eloquently summarized by Jon Sjoberg (formerly with Nevada Department of Wildlife) when, during an early meeting of the conservation team, he stated, “More frogs, more places, 10-years!”

In the following chapters, I describe two research projects that have management and conservation applications for *R onca*. I conclude this thesis in Chapter 4, where I summarize the applicability of my research to the management and conservation for the species. In Chapter 2, I summarize and assess over 19 years of data collected from the systematic monitoring of populations and from a headstarting and translocation program used to establish or augment populations of *R. onca*. The majority of these data were collected by UNLV personnel and student researchers led by Dr. Jef Jaeger, and I have been involved in this effort since 2010. These efforts have been guided by the conservation team and funded by various participating agencies and partner entities. My intent was to fill-in knowledge gaps about the life history of *R. onca* to clarify previous perspectives and expand understanding on aspects of breeding biology. Specifically, I investigated: breeding seasonality, egg mass size, time to hatching, time to metamorphosis, and time to reproductive maturity.

I derived data on breeding biology by assessing 1,396 monitoring survey records, 107 egg mass records, translocation records, and husbandry logs from headstarting. The number of field sites varied over time, as new sites were established or as translocation attempts failed, but from 2003–2021 a total of 26 sites were represented, including both remnant historical and translocation sites. I was able to clarify the breeding period of the species which peaks from January–May, but that *R. onca* can breed at any time; over the many years of sampling egg

masses were eventually detected during every month. I am unsure if an egg mass represents a clutch (an ovulation event) or whether *R. onca* has multiple clutches per year, but the average egg mass can contain 418 eggs, with individual egg masses having from 96–1,106 eggs.

Temperature affects all stages of anuran development (Zweifel 1968; Bradford 1990; Morrison and Hero 2003). I show that at water temperatures in the low-to-mid 20° C hatching of *R. onca* occurs in approximately 5–8 days, but field observations indicate that hatching may take much longer if eggs are oviposited in cold water that gradually increases in temperature as the season progresses. The time from hatching to metamorphosis was approximately 62 days in the laboratory under relatively favorable temperatures (i.e., 22–27° C). In the wild, metamorphosis appears to mostly occur within the same year as oviposition, but observations of very large tadpoles through winter months and into the spring indicate that overwintering by tadpoles is common. Using observations following initial translocation to unoccupied sites, I show that *R. onca* can reach sexual maturation in a little over a year (shortest observed time = 12.2 months). Support for this interpretation is provided by observations of both male and female frogs reaching adult sizes in a little over four months under favorable conditions. I conclude Chapter 2 by describing how these natural history observations have application to the management and conservation of *R. onca*.

In Chapter 3, I investigated the efficacy of an approach to increase resistance to an emerging amphibian disease, chytridiomycosis, in *R. onca*. The approach is to pre-expose animals to the disease and then clear them of infections, prior to subsequent exposure in the wild. Specifically, the aim was to improve survival of headstarted frogs used to augment a population of *R. onca* in which the disease was already present. The causal agent of chytridiomycosis is a chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (Bd; Longcore et al. 1999; Berger et

al. 1999). The pathogen is globally widespread and has been linked to amphibian species declines (Scheele et al. 2019). In southern Nevada, Bd has been detected in several anuran species, including *R. onca* (Forrest et al. 2015; Jaeger et al. 2017). Local isolates of Bd have been genetically identified as part of the highly pathogenic global panzootic lineage (Byrne et al. 2019), but the impact of Bd in wild anuran populations in southern Nevada is only speculative. What is known comes from laboratory experiments. Juveniles of *R. onca* are susceptible to chytridiomycosis when exposed to Bd isolated from *R. onca* and *Pseudacris regilla* (the Pacific tree frog) in southern Nevada (Waddle et al. 2019). Furthermore, laboratory findings have shown that *R. onca* has some resistance to Bd (Jaeger et al. 2017; Waddle et al. 2019), and that pre-exposure and infection clearance (using an anti-fungal agent) can significantly lower infection intensities and increase survivorship when subsequently exposed to the pathogen (Waddle et al. 2021).

The *R. onca* population I targeted for this study was known to be infected with Bd (Jaeger et al. 2017), and has been previously shown to have some form of Bd resistance (Jaeger et al. 2017; Waddle et al. 2019). The population is not thriving, and as part of conservation efforts has been receiving almost annual augmentations with headstarted animals. Given that pre-exposure to Bd followed by infection clearance was shown to improve survivorship of headstarted *R. onca* in the face of subsequent exposure, the U.S. Fish and Wildlife Service provided funding to UNLV for a proof-of-concept field study. The aim was to demonstrate the efficacy of such an approach to improve survival of frogs during an augmentation. The expectation was that animals undergoing pre-exposure and infection clearance would have lower overall subsequent Bd infection prevalence and intensities, and higher survival following release than unexposed animals.

In association with a headstarting program for the species, I acquired eggs from seven egg masses in the study area and surrounding springs. The animals were initially reared at a Bd-free facility until transferred as juvenile frogs to the animal facility at UNLV. The frogs were then separated into two treatment groups. One group (pre-exposed group) was exposed to live Bd zoospores, allowed to gain infections, and then treated with an anti-fungal agent to clear infections. The other group (control group) was treated exactly the same but exposed to a sham inoculum. A total 112 pre-exposed group and 117 control group frogs were eventually released to the study site. I implemented a mark-recapture method to individually identify frogs during field monitoring using photographs of spotting patterns (Zylstra et al. 2019). Following the release, I conducted 42 systematic recapture surveys over more than a year, sampling each frog captured for Bd. The mark-recapture method allowed me to track individuals and monitor infection prevalence and intensity, as well as estimate survival between treatment groups. Since a population of *R. onca* already existed at the study site, I also monitored resident frogs for Bd to provide a baseline on pathogen-host dynamics.

The findings of my study provide evidence that frogs gained some benefits from Bd pre-exposure and infection clearance. I analyzed infection prevalence and intensity between treatment groups in relation to time and air temperature, using generalized additive models. The models showed seasonal patterns in infection prevalence and intensity across all groups, with prevalence approaching zero during the summer months when air temperatures were highest, and reaching 100% during winter months when temperatures were the coldest. Infection intensity coincided with the seasonal pattern. The pre-exposed group showed significantly lower infection prevalence and intensity than the control group during the first month following release. During this period pre-exposed frogs were re-exposed to Bd in the wild, while control group frogs were

first exposed to the pathogen. The pre-exposed group also had significantly lower infection intensities than the control group during winter months. This is a period of time when *R. onca* is likely more susceptible to chytridiomycosis because temperatures favor Bd and food for *R. onca* is likely limited at the study site. The survival estimates from mark-recapture models showed slightly higher values for the pre-exposed group than the control group (4.5 or 5.6%), consistent with my expectation, but these differences were not statistically supported.

In my study, the overall benefit of using pre-exposure and clearance as an approach to improve survival of headstarted *R. onca* in the face of Bd was modest at best, especially given the lack of statistical support for differences in survival between pre-expose and unexposed groups. The sample size, however, was small for a field focused assessment, and I speculate that the difference in survival observed could have been statistically significant with a larger sample size. Pre-exposure did appear to increase host resistance to Bd infections at critical times, particularly immediately following release and again during winter months, periods when high infection intensities could presumably lead to fatal chytridiomycosis. The modest benefits, however, came with a high cost in terms of time invested, and an issue with clearing infections (see Chapter 3) that increased efforts. Efforts to further evaluate Bd pre-exposure and clearance in *R. onca* should use larger samples sizes, with the time invested mitigated by group housing and alternative group treatment to clear infections, such as heat treatment. As demonstrated by the research I present in Chapter 3, the approach used for this treatment was feasible at the scale of augmentations commonly used with *R. onca* and there was evidence of greater resistance to Bd with a potential increase in survival.

My intent when I started my thesis research was to publish Chapters 2 and 3 in peer-reviewed journals and I present these chapters as manuscript drafts. I am the lead author on these

manuscripts, and I plan to publish each with several coauthors (see below). To have these chapters appear as they may in publication, I use the plural nominative, “we”, throughout to reference myself and my coauthors. I have already submitted an earlier version of Chapter 2 to the journal, Herpetological Conservation and Biology, and the manuscript has been accepted, pending minor revisions. There are two potential coauthors on the publication. Dana L. Drake took an early interest in summarizing breeding biology of *R. onca* and collected a portion of the early data used in the assessment. I and Dr. Jef Jaeger conceptualized the research, collected and managed data, analyzed data, and co-wrote and edited the manuscript. I expect publication sometime in 2023, under the potential title “Insights into Relict Leopard Frog Breeding Biology”. Chapter 3 was initially written as a final performance report to the USFWS (the funding agency). I reworked this report for the thesis under the title “An Attempt to Improve Augmentation Success of the Relict Leopard Frog Through Pre-exposure and Clearance of an Emerging Amphibian Pathogen”. There are several potential coauthors on a future manuscript. Drs. Jaeger, Anthony Waddle, and Frank van Breukelen conceptualized the project. Dr. Jaeger, Dr. van Breukelen, and I wrote the proposal for funding. Kian Habashi, Una Karanovic, Sabrina Perkins, Robert Pelletier, along with I and Dr. Jaeger conducted the laboratory and field efforts. Sabrina Perkins conducted most of the photographic identification of animals, which I then confirmed. I managed datasets, conducted the survival modeling, and coordinated with Yorick Lambreghts and Dr. Waddle for GAM modeling. I took the lead in writing the initial report submitted to the funding agency, along with Drs. Jaeger and van Breukelen. I and Dr. Jaeger will be responsible for further editing of the manuscript.

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CHAPTER 2

INSIGHTS INTO RELICT LEOPARD FROG BREEDING BIOLOGY

Abstract

Information on the life history characteristics of organisms is often collected during management actions or through research facilitated by management programs. An example is the Relict Leopard Frog (*Rana onca*) for which aspects of breeding biology have been accumulated through long-term headstarting, translocation, and monitoring efforts. We reviewed survey data and headstarting and translocation records from 2003–2021 to determine breeding seasonality, number of eggs per egg mass, time to hatching, time to metamorphosis, and time to reproductive maturity. We determined that *R. onca* can breed year-round, but with a peak breeding period from January–May, when 97.5% of egg masses were observed. We estimated that egg masses contained around 418 eggs (± 57.7 SE), with individual egg masses consisting of 96–1,106 eggs. From field and laboratory data we inferred the general time from oviposition to hatching as 5–8 days in water temperatures in the low-to-mid 20° C. Time from hatching to metamorphosis in the laboratory was approximately 62 days ± 1.1 SE at 22–27° C. Our field observations indicated that the time from hatching to metamorphosis in the wild mostly occurred within the same year, but overwintering by tadpoles appeared common. Our monitoring at newly established translocation sites showed that *R. onca* can reach reproductive maturation in a little over a year (shortest observed time = 12.2 months) from oviposition of source animals to when evidence of breeding was first observed. Adult sizes for both males and females can potentially be reached in a little over four months. These insights on the breeding biology of *R. onca* have been used to better inform management actions.

Introduction

The natural history of organisms, encompassing life history characteristics, underlies and inspires various fields of science (Bartholomew 1986; Futuyma 1997; Arnold 2003). This area of research has found particular contemporary importance in resource management and conservation (Dayton 2003; Fleischner 2005; Bury 2006). Understandably, many species lack comprehensive life history descriptions (Bury 2006; Moore et al. 2013) because of the diversity of species and the practicality of gathering such information (Michaels et al. 2014). Moreover, available information can be difficult to access, as it may be in the gray literature (e.g., agency reports), embedded in other types of research (McCallum and McCallum 2006), or published piecemeal across multiple sources (Oliveira et al. 2017; Loughman 2020). In more recent times, life history investigations are predominately driven by conservation concerns (Wilson et al. 2009; Michaels et al. 2014). Gaps in knowledge for particular species are often filled by information gathered as part of management actions or research facilitated by management programs. One such example is the Relict Leopard Frog (*Rana onca*; Jaeger et al. 2001), a species of conservation concern for which information on its life history has been predominately accumulated during management efforts.

Rana onca is part of the *Rana pipiens* group and a sister taxon to the Lowland Leopard Frog, *Rana yavapaiensis* (Jaeger et al. 2001; Hillis and Wilcox 2005; Yuan et al. 2016). Populations of *R. onca* were known to occur historically in a narrow geographic range along the eastern fringe of the Mojave Desert, occupying springs and wetlands along the Virgin River drainage and adjacent portion of the Colorado River drainage (Jaeger et al. 2001; Bradford et al. 2004). The species declined during the 20th century, and by 2001 the range had contracted to a few geothermally influenced (hot) springs (with source temperatures generally above 30° C) in

two small areas of southern Nevada (Bradford et al. 2004). Potential causes for the decline are multiple and probably synergistic, with implicated factors including loss of habitat due to agriculture and water development, introduced predators, and disease (Bradford et al. 2004; Jaeger et al. 2017).

Management efforts for *R. onca* have been guided since 2001 by a multiagency Relict Leopard Frog Conservation Team (RLFCT), consisting of members from various land and resource management agencies and partners. The team developed a conservation agreement, assessment and strategy document (CAS) in 2005, which was updated and renewed in 2016 (RLFCT 2005, 2016, unpublished reports). Two main components of the strategy have been the systematic monitoring of populations and an intensive headstarting and translocation program. The latter has expanded the current distribution of *R. onca* to 19 sites in southern Nevada and northwestern Arizona within or near perceived historical range. Most of the translocation sites are springs with ambient water temperature (cold-water sites). Our focus herein is to synthesize life history information gained during these management actions to fill-in knowledge gaps related to aspects of breeding biology, expanding on our understanding of (1) breeding seasonality; (2) egg mass size; (3) relative incubation time; (4) time to metamorphosis; and (5) time to reproductive maturity. We summarize and assess information collected during management actions from 2003 through 2021. We present our findings within the context of what has already been published to clarify previous perspectives and offer new insights on components of *R. onca* breeding biology. Our intent is to better inform conservation strategy for *R. onca* and expand published knowledge on the life history of the species.

Materials and Methods

***Information sources*.**—We derived data to assess breeding seasonality and age to reproductive maturity from 1,396 population monitoring surveys conducted from February 2003 through December 2021. Some surveys over the last several years were targeted during summer months to specifically fill-in data gaps. Most of the data reflected visual encounter surveys (Crump and Scott 1994) conducted by crews usually consisting of two or more people, at least one with substantial experience surveying for the species. The number of occupied sites surveyed each year generally increased from eight to 22 as translocation sites were added; however, the number fluctuated when translocations failed to establish populations. Over time we surveyed a total of 26 sites, but we excluded data from two translocation sites where breeding by *R. onca* was never documented. Generally, we surveyed occupied sites three times annually, twice as seasonal temperatures warmed (predominately January–May) and once in fall as seasonal temperatures cooled (predominantly mid-September–mid-November). At any given site, however, the number of surveys we conducted potentially varied because of logistical issues or management objectives. Surveys occurred throughout the year, but were scant during June–mid-September when ambient temperatures were hot and mid-November–mid-January when temperatures were cold. Generally, we conducted surveys at hot springs earlier in spring and later in fall than at cold-water sites.

We assessed number of eggs per egg mass, time to hatching, and time to metamorphosis from information associated predominately with the headstarting and translocation program. An aggregate of eggs oviposited by *R. onca* has been described as a globular cluster (Bradford et al. 2005), and elsewhere as a spherical cluster or colloquially as an egg mass (Fig. 2.1); herein we use egg mass. We are unsure whether an egg mass represents an entire clutch or an ovipositional

bout (Altig and McDiarmid 2007). Each year we collected several egg masses (n = 4–10) for rearing, either whole or as partial masses. We collected partial masses to decrease the impact on source populations and increase genetic diversity of translocations. We derived data from field monitoring surveys associated with the collections, and integrated information from laboratory notes on the daily husbandry of headstarted animals, along with records from subsequent releases. We took measurements of water temperatures with different instruments over the years, including bulb thermometers (e.g, Rolf C. Hagen, Corp., Mansfield, MA) and digital pen thermometers (e.g., Thermopen MK4, ThermoWorks, American Fork, UT) with accuracies of 0.4–0.7° C. We reviewed records associated with 107 egg masses, although sample sizes varied across assessments because of missing data or inadequate descriptions.

Breeding seasonality.—We determined seasonality of *R. onca* breeding by the accumulated counts of egg masses during monitoring surveys from each month. We also separated data from hot and cold-water sites by month to explore potential differences in timing using a two-proportion z-test with an $\alpha = 0.05$ (Statology. 2020. Two Proportion Z-Test Calculator. Available from https://www.statology.org/two-proportion-z-test-calculator/ [Accessed 13 June 2022]). We did not determine the developmental stage (Gosner stage; Gosner 1960) of most egg masses in the field, so we assigned egg masses to the month of their observation. We also included spent egg masses that still had hatchlings (Gosner stages 20–25). In addition, we reviewed field observations on male calling.

FIGURE 2.1. Relict Leopard Frog (*Rana onca*) egg mass (clump) under water. Egg masses are spheroid and consistent with description by Altig and McDiarmid (2007) of aquatic "clumps". The diameter of *R. onca* egg masses have been reported as 4–6 cm (RLFCT 2005, unpublished report).

Egg mass.—We quantified the number of eggs in egg masses collected opportunistically for headstarting and translocation. For each mass, we counted hatchlings (Gosner stages 23–25) and unviable eggs following hatching. We determined numbers directly from 16 egg masses collected whole. We also estimated the number of eggs in 65 partial egg masses when the proportion collected was visually approximated at time of collection. In these cases, we counted as above and then multiplied by the estimated proportion of the mass collected.

Time to hatching.—To evaluate hatching time, we first determined the hatching period in the laboratory for whole and partial egg masses collected for headstarting. We determined hatching period as the time from egg mass collection in the field until hatching was first documented in the laboratory; this period did not include developmental time in the wild prior to collection. We observed hatching in *R. onca* to commence at Gosner stage 20, although hatching was not fully synchronized and many embryos at that point appeared to still be at Gosner stage 19. Variation of Gosner stage within an egg mass at hatching has been documented in other ranid species (Shumway 1940; Zweifel 1968; Beattie 1987). We recorded water temperature in the laboratory for each egg mass daily and then averaged these values for assessment. We reared egg masses at temperatures ranging from 18.0–25.1° C, with temperatures early in the conservation program being in the cooler range. Details on Gosner stages of embryos and date of hatching relative to laboratory rearing water temperatures were complete for 61 egg masses.

We collected egg masses at various stages of development, with these egg masses found in water temperatures ranging from 10.9–28.8° C (average = 20.4° C ± 0.5 SE, N = 56). Based on observations at time of collection, we grouped egg masses into four developmental categories: Gosner stages ≤ 10 (notes indicating the presence of vegetal and animal poles; n = 15), Gosner stages 11–12 (notes indicating eggs as black spheres; n = 21), Gosner stages ≥ 13 (notes indicating eggs breaking spherical shape; n = 20), or Gosner stage undetermined (notes lacking; n = 5). To evaluate complete hatching time in the wild, we reviewed field data for surveys and site visits that occurred at particular sites over short periods (2–10 days) where egg masses were detected and from which hatching times in the wild could be inferred. We identified only five such events at three hot spring sites that contained meaningful observations.

Time to metamorphosis.—We estimated the time (days) to metamorphosis in the laboratory from the date of hatching to the emergence of front limbs or when laboratory notes first indicated transfers of individuals from ‘tadpole’ to ‘frog’ tanks for a given egg mass. These events indicate that the animals had reached Gosner stages 42–46. For this assessment, we discarded most records of egg mass collections prior to 2012 because laboratory notes on metamorphosis were confusing or undescriptive; only one record from 2011 was kept. We also discarded records from two egg masses in 2019 and two egg masses in 2020 because details on the timing of metamorphosis could not be determined. In the end, we based our assessment on observations related to 55 egg masses collected whole or in part.

Time to reproductive maturity.—Initial translocations to unoccupied sites allowed an opportunity to evaluate time to reproductive maturity in *R. onca*. These sites were generally isolated with little to no chance of dispersal from neighboring populations. We reviewed monitoring data from 14 translocations where populations were established to the point of documented breeding. Initial translocations generally occurred in spring, using late-stage tadpoles, juvenile frogs, or both. We evaluated observations from subsequent monitoring surveys for evidence of reproductive maturity (i.e., egg masses or tadpoles hatched at the site). We focused on the shortest periods to reproductive maturity detected in our data, and conservatively calculated reproductive maturity as the time from egg mass collection for the oldest headstarted animals initially released at a site to the date when evidence of reproduction was detected.

Results

Breeding seasonality.—When assessed across years, we observed egg masses during surveys throughout the year; however, we encountered the majority of egg masses during January–May. Approximately 92.1% (338/367) of surveys with egg mass encounters occurred during this period, along with 97.6% (1,840/1,886) of all egg masses observed (Fig. 2.2A). The highest production appeared to be during February and March. There appeared to be little egg mass production during the hottest months (June–August), followed by a slight increase during fall months, prior to another short lull in winter when ambient temperatures were the coldest. When data were parsed between hot springs (Fig. 2.2B) and cold-water sites (Fig. 2.2C), we encountered more egg masses during surveys at hot springs earlier in winter, with the difference most pronounced in January (Z = -2.022, P = 0.022). During that month, we encountered egg masses during 50.0% (60/120) of surveys at hot springs, but only 18.2% (2/11) of surveys at cold-water sites (Figs. 2.2B & 2.2C). Similarly, the few egg masses (n = 8) we observed during the hottest months (June–August) were all at cold-water sites, while the few egg masses (n = 17) we observed in November and December were all at hot springs. We also heard calling by males throughout the year, with detections during surveys peaking in March (Fig. 2.3).

FIGURE 2.2. Seasonality of Relict Leopard Frog (*Rana onca*) breeding represented by egg masses counted during surveys per month from February 2003–December 2021 (solid line) across (A) sites, and separated between (B) hot springs and (C) cold-water sites. Bars represent the number of surveys in which egg masses were encountered (gray) or not encountered (white). Proportions associated with each bar reference the accumulated number of sites across years where egg masses were encountered (numerator) and number of sites monitored (denominator). For example, in panel A during the month of February, 229 surveys were conducted from 2003–2021, with 563 egg masses detected during 119 of those surveys. Of the 20 sites that were surveyed in February over the years, egg masses were observed at 18 of these sites.

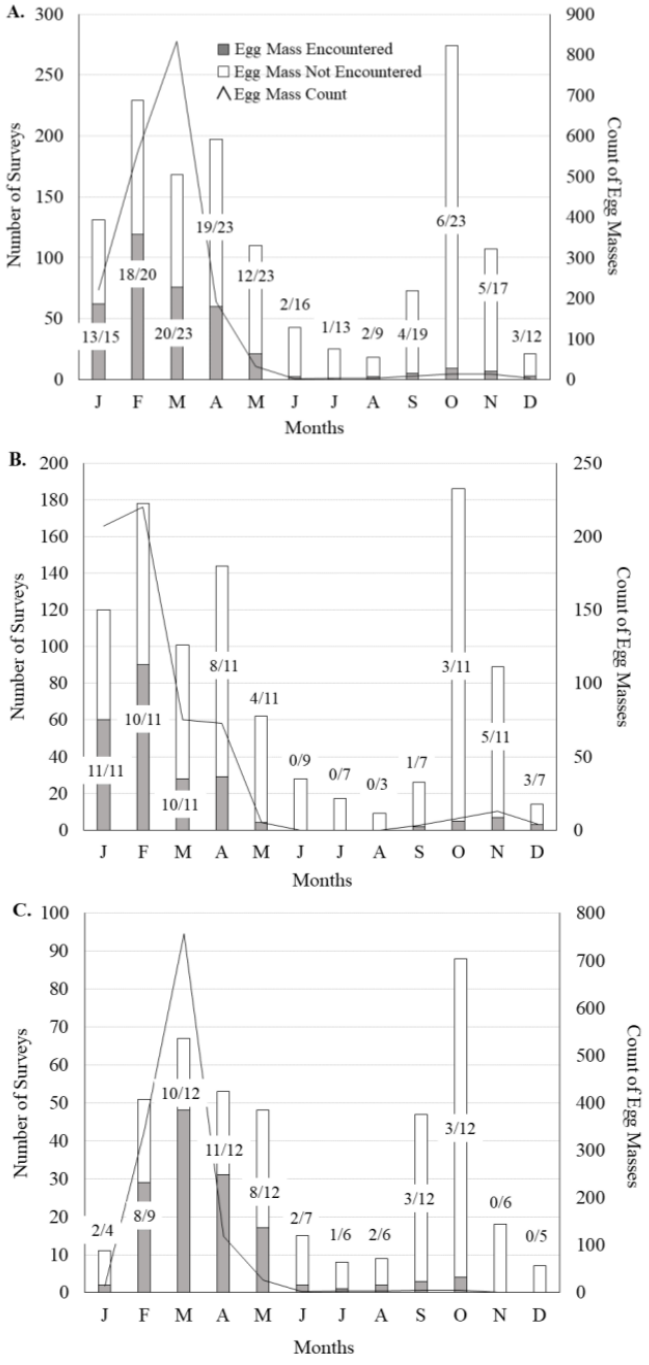
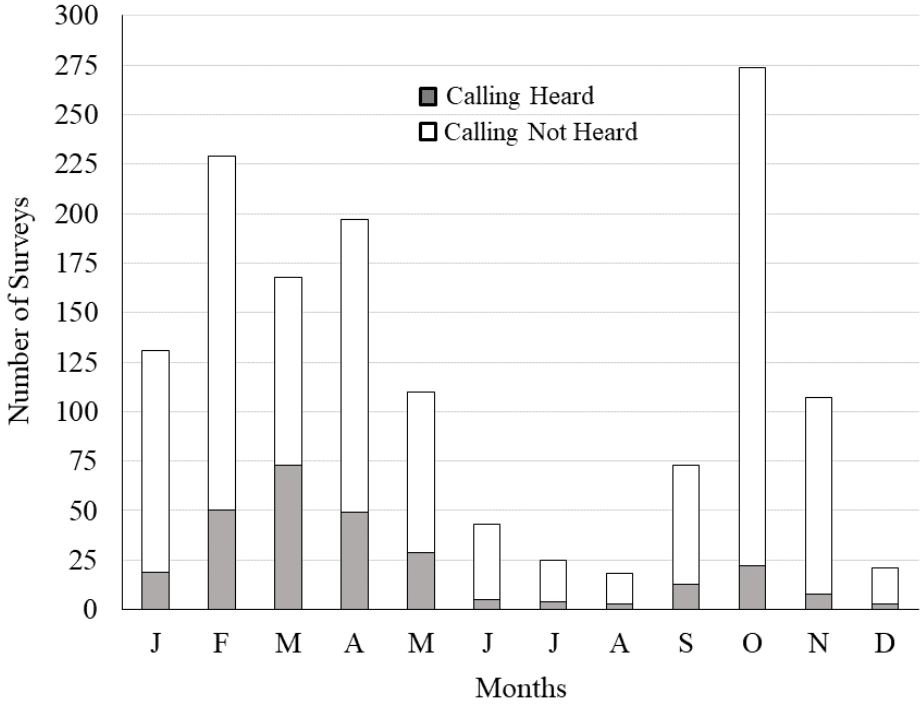


FIGURE 2.3. Breeding seasonality of the Relict Leopard Frog (*Rana onca*) represented by detection of calling males during surveys per month from February 2003–December 2021 at sites.



Egg mass.—Counts of eggs (viable and unviable) from 16 *R. onca* egg masses collected whole ranged from 96–1,106 (Table 2.1), with an average of 418 ± 57.7 SE. When we extrapolated from counts of 65 partial egg masses, where the approximate proportion collected had been recorded, the average number of eggs was estimated at 528 ± 28.9 SE. The proportion of viable eggs in masses collected whole averaged 0.855 (range, 0.311–1.00; Table 2.1), and in partial egg masses averaged 0.813 (range, 0.022–1.00).

Time to hatching.—Hatching period in the laboratory (not including developmental time in the wild prior to collection) was positively influenced by rearing water temperature and Gosner stage at time of collection, although marginally. The longest time to hatching in the laboratory

was eight days at 18.0° C for two egg masses that both lacked Gosner stage documentation. When we determined Gosner stages at collection, the youngest egg masses at Gosner stages ≤ 10 hatched after approximately five days (range, 4–6) at an average water temperature of 23.6° C (± 0.17 SE, n = 15). When collected at Gosner stages 11–12, hatching took approximately four days (range, 3–6) at an average water temperature of 23.1° C (± 0.2 SE, n = 21). Older embryos collected at Gosner stages ≥ 13 hatched after only three days (range, 2–4) at an average water temperature of 23.4° C (± 0.26 SE, n = 19); however, we excluded one sample that required six days to hatch at 21.2° C.

We inferred the time from oviposition to hatching in the wild from repeated observations of five egg masses. The shortest time to hatching was approximately 5.5 days at a water temperature of 21.9° C (recorded at Blue Point Spring, lower). We inferred the hatching of two other egg masses at the site to have occurred in 6.5–7.5 days at 19.9° C and eight days at 21.5° C. At a separate site (Rogers Spring), the time to hatching for an egg mass was approximately 6.5 days at water temperatures recorded at 19.1–22.0° C. We collected part of this egg mass for headstarting at Gosner stage ≤ 10 (probably 1.5 days old) and raised the eggs at 23.6° C; these eggs hatched in four days. For another egg mass found at a translocation site (Pupfish Refuge Spring), hatching occurred in approximately seven days at a water temperature around 18–21° C (based on previous temperatures taken around the oviposition area).

TABLE 2.1. Number of eggs per egg mass and proportion viable at hatching for 16 whole egg masses of the Relict Leopard Frog (*Rana onca*). Collection date and sample location within Clark County, Nevada provided; see map in Jaeger et al. (2017) for general location references.

General Area & Site	No. of Eggs	Proportion Viable	Date
Black Canyon			
Bighorn Sheep Spring	1106	0.931	01/22/2004
	496	0.990	01/17/2006
	476	1.00	01/17/2006
	551	0.976	02/06/2007
	184	0.978	02/20/2007
	457	0.941	02/20/2007
	348	0.957	03/09/2007
	409	0.311	03/09/2007
	383	0.945	03/09/2007
	269	0.877	03/09/2007
Black Canyon Spring (side spring)	347	0.559	01/24/2018
Salt Cedar Canyon Spring	121	0.802	01/30/2010
	503	0.881	01/24/2018
Northshore			
Blue Point Spring (upper)	517	0.952	02/05/2013
Blue Point Spring (lower)	427	0.761	01/23/2018
	96	0.823	01/23/2018

Time to metamorphosis.—In the laboratory, we observed hatchlings transition into metamorphs (Gosner stages ≥ 42) in an average of 62 days ± 1.1 SE (range, 47–82, N = 55) at water temperatures ranging from 22.0–27.0° C (average = 24.1° C ± 0.1 SE). We did not generally monitor the time required for tadpoles to transition from early metamorphosis (when front legs have emerged, Gosner stage 42) to completion of metamorphosis (when the tail is fully absorbed, Gosner stage ≥ 46), but in four cases where data was available the process took about 10–15 days at laboratory temperatures. From our field observations, the time from hatching to metamorphosis in the wild generally occurred within the same year, but overwintering of tadpoles appeared common. We detected large tadpoles across various surveys and years during

winter months (January–February) when overwintering provides the only explanation for their presence. Using this temporally restrictive set of observations, overwintering tadpoles have been observed at 7 hot springs and 4 cold-water sites.

Time to reproductive maturity.—Following translocation to new, unoccupied and isolated sites, we detected breeding by *R. onca* at seven sites to occur as early as January–April following initial releases during the previous spring (12.2–15.3 months from time of initial egg mass collections of the source animals). These translocations occurred at both hot and cold-water sites and were initiated with late-stage tadpoles, juvenile frogs, or both (Appendix Table 2.1). The shortest developmental time to reproductive maturity was at Goldstrike Canyon, a hot spring, where late-stage tadpoles were initially released on 9 April, 5 May, and 29 June 2004 from egg masses collected on 22 January and 15 March 2004. A viable egg mass was subsequently observed at the site during a survey on 27 January 2005, just over one year (372 days) from the earliest collection date of the source egg masses. Goldstrike Canyon is about 1 km downriver from nearest occupied site, Pupfish Refuge Spring, where *R. onca* had been established earlier by translocation; both sites are along the Colorado River below Hoover Dam. The river is not sustainable habitat for *R. onca* and dispersal from Pupfish Refuge to Goldstrike during the time of population establishment was unlikely. The second shortest time to reproductive maturity was at Grapevine Springs at just over 13 months (402 days) and there was no chance of dispersal to this site. Grapevine Springs is predominately a cold-water site, but a spring source emerges from an adit where water temperature in winter is just below 20 °C.

Discussion

Existing published information on the breeding biology of *R. onca* is limited. The majority of available information can be directly or indirectly tied to its conservation program, and to a handful of researchers and managers that have been associated with the program (including the authors herein). The information on breeding biology provided in the 2005 CAS were derived concomitantly with publications on the population status of the species and a short species account by Bradford et al. (2004; 2005). The data assessed in our current study included this early data, as well as subsequent data used to provide descriptions and summaries incorporated into the 2016 renewal of the CAS. Those earlier descriptions, however, were made predominately without presentation of the underlying data. Prior to the conservation program, there were only a handful of researchers working on the species, with much of the focus on distribution, demography, and systematics (e.g., Jaeger et al. 2001; Bradford et al 2004). There are a couple more recently published articles (Goldstein et al. 2017; Saumure et al. 2022) that we derive information from in our discussion below, but these too are tied closely to the conservation program.

Breeding seasonality.—Initial descriptions of breeding phenology in *R. onca* indicated that the species had an extended breeding period, with favored breeding times reported as being in spring and fall (Bradford et al. 2005). Most egg masses were reported to occur during the early seasonal period, defined as January or February through March or April (Bradford et al. 2005; RLFCT 2016, unpublished report), or possibly from March through May (Wright and Wright 1949). The later period was described as occurring in November (Bradford et al. 2005), although eggs have been reported in September (RLFCT 2005, unpublished report). Our assessment of

observations over 19 years indicates that *R. onca* is a prolonged breeder and oviposition can occur during any month, along with calling by males. There is, however, a clear breeding period from January through May. More specifically, overall breeding appears to increase towards the latter half of January and remains high through April, extending into early May. Breeding activity was broadly associated with temperature, increasing as ambient temperatures warmed in January and declining as temperatures increased towards summer extremes in May. The connection to temperature was most apparent in the temporal shift towards breeding earlier in the season at hot springs and later in the season at cold-water sites. This interpretation, however, may be somewhat biased by the pattern of early-season surveys which generally started at hot springs around mid-January and then shifted towards cold-water sites as the season progressed.

Breeding seasonality in the sister taxon *R. yavapaiensis* in Arizona has been described as "bipartite" with a major breeding period in spring and a lesser period in fall (Sartorius and Rosen 2000). A review of the reported timing for breeding in *R. yavapaiensis* generally supports this perspective, although at geothermally influenced springs or low elevation sites there has been speculation that the species may be reproductively active year-round (Sredl 2005). The seasonal pattern is similar to that in *R. onca* with a major breeding period in spring followed by a slight uptick in egg mass production in fall (predominately late-September–early November), but we are hard pressed to describe this as bipartite given the limited number of egg masses observed during the latter period. Similar to the temporal shift in breeding observed earlier in the year, the small amount of late season breeding by *R. onca* appears to occur earlier at cold-water sites and later at hot springs. We have no information on the number of clutches that females may produce over a year, but given that breeding is possible throughout the year, there appears to be the potential for more than one ovulation event.

The lack of breeding during summer months in *R. yavapaiensis* has been postulated as a mechanism to avoid seasonal declines of surface water or loss of egg masses during floods caused by summer rains (Sartorius and Rosen 2000). Summer monsoons extend into the eastern Mojave Desert, although with less predictability than in the Sonoran Desert. The monsoon usually starts in late-June and often extends through summer in a "halting and episodic" manner (Redmond 2009). Rain events have been implicated as an environmental cue for stimulating oviposition in some anurans, including a ranid species with prolonged breeding (Saenz et al. 2006). Anecdotal observations suggest similar behavior in *R. onca*, and we have detected egg masses at cold-water sites targeted for surveys in summer within days following rains. If this is a general phenomenon in *R. onca*, the potential mechanism is not clear and may relate to changes in relative humidity, air temperature, water temperature, or habitat disturbance.

Egg mass.—General descriptions of egg mass size in *R. onca* have reported "up to 250 eggs" (RLFCT 2005, unpublished report) or "many hundred eggs" (Bradford et al. 2005). We estimated an average egg mass size of 418 eggs ± 57.7 SE from a collection of 16 egg masses, but believe this estimate may be biased low. The egg masses were gathered opportunistically to facilitate the programmatic needs of headstarting and translocation, and the collection appears to have an overrepresentation of small egg masses. Our estimated average of 528 eggs ± 28.9 SE from a collection of 65 partial egg masses may better reflect egg mass size, even if the methodology was less accurate. The range of 96–1,106 eggs per egg mass from the collection of whole egg masses seems representative, but bigger egg masses are likely given the large size that females can reach under exceptional conditions (Saumure et al. 2022). We found no comparable estimate of egg mass size in *R. yavapaiensis*, but the average for *R. onca* was much lower than

the 1,600 eggs reported for the "clutch size" (referencing egg mass size) for *Rana magnaocularis* (Frost and Bagnara 1977), another closely related species from Mexico (Yuan et al. 2016).

Time to hatching.—Temperature dependence of anuran embryo development is well documented (Moore 1939; Zweifel 1968; Bradford 1990), and water temperatures experienced by populations of *R. onca* are certainly affected by seasonal weather patterns in the Mojave Desert, along with specific site conditions (e.g., geothermally influenced or not). Our data predominately focused on developmental time under laboratory conditions, not including developmental time of the egg masses in the wild prior to collection. Egg masses collected at the earliest Gosner stages (≤ 10) and reared in the laboratory at water temperatures from 22.2–24.4° C, hatched in 4–6 days. Our assessment of egg masses collected at later Gosner stages and reared at similar temperatures were consistent with this developmental timing. Based on a very early subset of the data included herein, hatching period in the laboratory was reported as 5–7 days for egg masses collected in the field at Gosner stage < 14 and reared at "room temperature" (RLFCT 2005, unpublished report). Original notes on rearing temperatures were lacking from that time but can be assumed to be 21–22° C from peripheral information (Drake 2010; Goldstein et al. 2017). The variation in the estimated development times between the two assessments could be easily explained by the temperature differences. At colder water temperatures embryonic development takes longer, as was evidenced by two egg masses collected in 2007 that took eight days to hatch in water temperatures of 18° C.

To estimate the hatching time from oviposition using the laboratory data, we need to include the developmental time in the wild prior to collection. The earliest egg masses collected (Gosner stage ≤ 10) were thought to range from about 0.5–2 days old when encountered. In *R.*

pipiens, development to the equivalent of Gosner stage 10 was experimentally reached in 26 h following fertilization at a water temperature of 18° C (Shumway 1940). Adding likely developmental times prior to collection to our laboratory data indicates that *R. onca* takes approximately 5–8 days to go from oviposition to hatching at water temperatures in the low-to-mid 20s° C. This estimate is consistent with the previous account that indicated this transition takes approximately 1 week (RLFCT 2016, unpublished report). From observations made on five egg masses in the field, time to hatching in the wild was inferred to occur in approximately 5.5–8 days in water temperatures of 19.1–22.0° C. Recent observations at a cold-water site indicate much longer hatching times are possible. Young egg masses were observed in water as cold as 10.4° C, and 22 days later many of the masses were observed with hatchlings or small tadpoles at water temperatures of around 16.5–17.8° C, although many eggs appeared unviable (Jef Jaeger, unpublished data). For comparison, the incubation period of four egg masses of *R. yavapaiensis* observed in the field reportedly took 15–18 days at a water temperature of 14.2° C (Sartorius and Rosen 2000).

Time to metamorphosis.—As with embryonic development, temperature has an effect on tadpole development, as well as growth of frogs towards sexual maturity (Morrison and Hero 2003). Development and growth rates vary across populations, influenced by site-specific conditions and temporal shifts in resource availability, among other factors (Jørgensen 1992; Gotthard 2001). In *R. onca,* the time required for tadpoles to reach metamorphosis after hatching was previously suggested to take "several months" (Bradford et al. 2005). Under laboratory conditions, when fed *ad libitum,* tadpoles of *R. onca* reportedly metamorphosed (ostensibly at Gosner stage 46) in 2–3 months at water temperatures of 24–25° C (RLFCT 2016, unpublished

report) or much longer (approximately 6.5 months; RLFCT 2005, unpublished report) at presumably colder temperatures (probably not higher than 21–22° C). We estimated that hatching to metamorphosis in *R. onca* took on average 62 days ± 1.1 SE (range, 47–82) in water temperatures around 24° C.

Our estimate of the time to metamorphosis was faster than the times observed during a laboratory study on tadpole development in *R. onca* (Goldstein et al. 2017). In that study, young tadpoles were assessed at temperatures from 15–35° C at 5° C increments. At 15 and 35° C tadpoles appeared to be outside their optimal temperature range and exhibited very limited growth and development. The tadpoles maintained at 35° C failed to survive, but the tadpoles that started at 15° C were eventually warmed to 25° C and many reached metamorphosis (an attempt to mimic a potential natural pattern). At temperatures closer to those experienced in the headstarting laboratory, tadpoles reached metamorphosis most quickly at 25° C. The timing of metamorphosis reported in that study, however, did not include the age of tadpoles when first entered into the experiment, which we determined to be 10 days based on the listed hatching date and the date tadpoles were received from the headstarting program. We were unable to determine if the time required to reach acclimation temperature was incorporated, but based on the reported acclimation rate this would have been two days at 20° C, three days at 25° C, and eight days at 30° C. Thus, time from hatching to forelimb emergence in that study averaged 274–276 days at 20° C, 77–80 days at 25° C, and 108–116 days at 30° C.

The time to metamorphosis in *R. yavapaiensis* has been reported to be as short as 3–4 months or as long as nine months (Sredl 2005). Overwintering has been documented in that species (Collins and Lewis 1979) and has also been reported in *R. onca* (O'Toole et al. In press). Overwintering of tadpoles is a characteristic of many temperate anurans (Collins and Lewis

1979; Walsh et al. 2015). Our review of monitoring survey data indicated that overwintering by tadpoles is common in *R. onca*, but the timing of the process and the mechanism that drives it are unstudied in the species. We speculate, however, that tadpoles associated with late season breeding of *R. onca* in fall may often overwinter.

Time to reproductive maturity.—Reproductive maturation in anurans depends on juvenile growth rates and body size rather than age specifically (Jørgensen 1992; Ryser 1996). In some ranid species, males tend to reach sexual maturity at smaller sizes and more quickly than females (Berven 1990; Ryser 1996; Hughes and Meshaka 2018). In field collections of *R. onca*, the smallest identified males were reported at 44 mm, presumably snout-vent length (SVL), and the smallest females at 48.5 and 51 mm (Wright and Wright 1949). Males have been reported to reach reproductive maturity at approximately 42 mm SVL when swollen, pigmented thumb (nuptial) pads appear (Bradford et al. 2004, 2005) and can reach this size during their first year (Bradford et al. 2005; Saumure et al. 2022). Females can also reach adult sizes within a single year, at least under exceptional conditions. At a newly established, artificial pond system, several juvenile frogs released at around 32 mm SVL as part of an initial translocation were recaptured as adults just over four months later, specifically a male at 68 mm and three females ranging from 75–84 mm SVL (Saumure et al. 2022).

Time to reproductive maturity in *R. onca* has been previously described from observations of breeding behavior at newly established translocation sites. Egg masses or young tadpoles have been observed about a year after initial translocations at several newly establish populations, indicating that both males and females were capable of breeding in a little less than 1.5 years (Saumure et al. 2022) from when they were oviposited. In our assessment, however, the

shortest time required from oviposition of the source individuals to reproduction was just over one year (12.2 months) at a newly established hot spring site. We should emphasize that all these observations were associated with source animals reared through metamorphosis under favorable conditions before release (e.g., around 24° C water temperature, abundant food).

Importance to management.—The conservation program for *R. onca* started as an urgent endeavor to improve the status of the species and inform on population responses to management actions. The collection of data on breeding biology was subsidiary to those aims, but over time provided the major basis for our understanding. The information gained has directly informed management actions for the species. Specifically: (1) the determination of breeding seasonality has improved the efficiency of egg mass collections for headstarting and translocation and has informed the scheduling of habitat maintenance to avoid conflicts, (2) estimates of egg mass size and viability have been used in the planning of collection quotas, (3) determination of developmental times has informed the scheduling of rearing activities and coordination of releases, (4) the previous determination of optimal temperature ranges for tadpole growth has improved transit times for headstarting, and (5) the understanding of time to reproductive maturation has been used to organize efficacy monitoring, along with governing expectations for the timing of success at translocation sites. These examples demonstrate the contemporary application of life history information to adaptive management in species conservation.

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CHAPTER 3

AN ATTEMPT TO IMPROVE AUGMENTATION SUCCESS OF THE RELICT LEOPARD FROG THROUGH PRE-EXPOSURE AND CLEARANCE OF AN EMERGING AMPHIBIAN PATHOGEN

Abstract

The disease chytridiomycosis is linked to amphibian declines. One approach to mitigating susceptibility to this disease is to improve resistance in individuals through prior exposure to the causal agent, the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). The Relict Leopard Frog (*Rana onca*) is a species of conservation concern for which headstarting and translocation are used to augment or establish populations in a landscape where Bd is present. Our aim was to determine the efficacy of using prior exposure to Bd followed by drug-mediated clearance (pre-exposure and clearance) to improve survival of headstarted *R. onca* used to augment a population. We acquired juvenile frogs that were headstarted from eggs collected at a study site where Bd was present. We divided these frogs into two treatment groups, infecting one group with Bd (pre-exposed) and then clearing the infections with itraconazole (an anti-fungal agent). We handled the other group identically except that these frogs were given a sham inoculum prior to clearance treatment (control group). In total, we released 112 pre-exposed and 117 control group frogs to augment the study site. We monitored these groups across 42 sampling events over 18 months. We used photographic references of spotting patterns to identify individual frogs, and estimated survival of treatment groups using mark-recapture modeling. We analyzed the relationships of Bd prevalence and intensity across sampling events (date) and air temperatures using generalized additive models. We found that both of these variables significantly predicted Bd prevalence and intensity across treatment groups. In July and August, when ambient daytime temperature at the study site persisted above the thermal tolerance of Bd

(> 30° C), infection probabilities and intensities were close to zero values. During December–February when ambient air temperature was the coldest, but within Bd thermal tolerance, infection probabilities reached 100% and intensities reached highest values. We also observed statistically supported differences between treatment groups at times that may be important for survival. Soon after release in June, the pre-exposed group had lower infection probability and intensity than the control group. The pre-exposed group also had lower infection intensity in early December–early February when overall infections were high. We estimated higher survival in the pre-exposed group than in the control group, but the difference of 4.5 or 5.6% was not statistically supported. Even if the difference is real, we are unsure whether such a modest increase would be biologically meaningful to the population over time. We determined that pre-exposure and clearance provided some increased resistance to Bd, but our findings provide only limited validity for improved survival of headstarted frogs.

Introduction

The relict leopard frog, *Rana onca* is currently managed under a conservation agreement, and associated assessment and strategy implemented by a multiagency conservation team (Relict Leopard Frog Conservation Team; RLFCT 2016). Headstarting and translocation to establish or augment populations has been a successful component of management strategy for *R. onca*; however, finding suitable translocation sites has become increasingly challenging. The pathogenic amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) has been detected in anuran species across much of the historical range of *R. onca* and in one historical population of the species (Jaeger et al. 2017). The fungus causes the amphibian disease, chytridiomycosis, which has been broadly implicated in amphibian species declines (Scheele et al. 2019). Disease

was not initially considered a factor in the historical decline of *R. onca* (Bradford et al. 2004), but researchers later questioned that perspective (Forest and Schlaepfer 2011), with speculation focusing on the possibility of Bd. Two attempts have been made to establish *R. onca* at translocation sites where other anuran species were infected with Bd. In both cases populations of *R. onca* failed to establish, although there were other confounding issues and threats at those sites (Jef Jaeger, unpublished data). The current management strategy for *R. onca* is to avoid translocation to sites where Bd is present, which greatly limits options for the species.

Laboratory experiments to assess the susceptibility of *R. onca* to Bd have shown mixed results. Juvenile frogs challenged with Bd strains isolated during epizootics in other ranid species from California showed strong resistance to chytridiomycosis (Jaeger et al. 2017). In contrast, when juvenile frogs were challenged with Bd isolated from local anurans in southern Nevada, including a wild *R. onca*, survivorship was negatively affected (Waddle et al. 2019). Isolates of Bd collected from anurans in southern Nevada were genetically identified as part of the highly pathogenic Global Panzootic Lineage (Byrne et al. 2019). Interestingly, there were observed differences in resistance to Bd and chytridiomycosis between source populations of *R. onca* used in the laboratory experiments (Jaeger et al. 2017; Waddle et al. 2019). Frogs sourced from an area in southern Nevada where Bd was present (herein Northshore) were 5.5 times more likely to survive Bd infections and 10 times more likely to clear infections than frogs sourced from an area of southern Nevada where Bd had not been detected (Waddle et al. 2019). These results indicate potential adaptation of the local host population to the presence of Bd.

Variation in host-susceptibility to Bd has been well documented, and there are populations and species that display some level of resistance (e.g. Searle et al. 2011; Bradley 2015; Voyles et al. 2018). There is also evidence that some species have immunological

responses to Bd, but that these responses can be overwhelmed during initial infections, leading to chytridiomycosis (Ellison et. al. 2014; Grogan et al. 2018). A promising approach to mitigating susceptibility to chytridiomycosis has been to stimulate resistance in frogs by eliciting infection through pre-exposure to Bd and then clearing the individuals of the infection. Laboratory experiments with *R. onca* using a medical antifungal agent to clear infections greatly decreased intensity of subsequent infections and increased survivorship following re-exposure to the Bd strain (Waddle et al. 2021). These laboratory results provide baseline evidence that *R. onca* has some level of resistance to Bd and is capable of developing an immune response to the pathogen.

Herein, we describe our efforts to evaluate the efficacy of pre-exposure to Bd and clearance of infection using itraconazole, an antifungal medical agent, to improve augmentation success in a large number of *R. onca* released to a site where Bd is present. The population at this site was identified by the U.S. Fish and Wildlife Service as being of critical conservation concern (RLFCT 2016), and the population is being augmented using headstarted animals. We collected eggs from the Northshore system, raised them to juvenile frogs, and divided these animals into two groups. In one group, we elicited Bd infections through exposure to isolates of Bd collected from *R. onca* at the study site, and then subsequently cleared these frogs of infections using itraconazole treatments. The other group was treated identically, except that we exposed them to a sham inoculum. We subsequently released both treatment groups, and monitored Bd prevalence and infection intensity (load) through skin swabs and a common molecular assay (Jaeger et al. 2017). We used reference photographs of spotting patterns to monitor individuals and groups. This allowed us to differentiate among treatment groups and wild frogs captured in the study site, filter-out duplicate Bd samples during sampling periods, and implement mark-recapture methods. We assessed survival, subsequent infection prevalence, and infection

intensity from June 2020–November 2021. We predicted that pre-exposure would increase survival of frogs when compared to unexposed (control) frogs following release. We also predicted that pre-exposed frogs would have lower infection prevalence and intensity over time when compared to controls.

Materials and Methods

Study site.—We targeted our research at a lower stream stretch of Blue Point Spring within Lake Mead National Recreation Area. This spring is part of Northshore, located along the base of the Muddy Mountains near the Overton Arm of Lake Mead. A general description of the aquatic conditions was provided in Bradford et al. (2004), and a map showing the regional location was presented in Jaeger et al. (2017). We chose this site because previous field monitoring had detected presence of Bd in the population of *R. onca* on numerous occasions (Jaeger et al. 2017) and augmentation of animals headstarted from the system was a management strategy. The Northshore system generally lacks other anurans.

The stream stretch we targeted was densely covered in emergent vegetation, particularly cattail (*Typha*), reed (*Phragmites*), sedges (*Scirpus*), and sawgrass (*Cladium californicum*). The water was thermally influenced, with temperatures measured at capture sites ranging from 26.6° C (± 0.1 SE) during the hottest months (July–September) to 20.7° C (± 0.1 SD) during the coldest months (January–March). Prior to the beginning of the project, we reduced emergent vegetation along approximately 70 linear meters of stream using a mechanical weed-whacker and hand tools to improve habitat condition. Sampling efforts quickly expanded along the stream to include heavily vegetated areas above and below the original stretch, forming a total area of

approximately 350 linear meters of stream. Habitat conditions improved in the extended areas as we opened-up dense vegetation during sampling efforts.

Source animals and laboratory housing.—We derived juvenile frogs from seven separate egg masses collected from sites in Northshore in January and February 2019. We maintained animals through metamorphosis at a Bd-free facility in the Lake Mead State Fish Hatchery until we transferred them to the Animal Facility at the University of Nevada, Las Vegas during May–August 2019. We housed juvenile frogs in an environmental chamber maintained at 19.6–20.3° C, with 12 h per day of UVB lighting. During the early period of the study while procedures were being conducted, we housed frogs individually following the protocol described by Jaeger et al. (2017). Frogs were first acclimated to the environmental chamber for at least 13 d during which time they were confirmed to be free of Bd (see below).

Bd inoculations.—We assigned juvenile frogs to either a treatment group (N = 124) that received the Bd inoculum (herein pre-exposed group) or to a control group (N = 120) that was treated identically but with a sham inoculum (thus unexposed). The total number of animals for this study was limited by the available space in the environmental chamber. At the time of inoculations, all frogs were over three weeks post-metamorphosis. We assigned frogs to groups semi-randomly, with individuals from particular egg masses split roughly equally between groups; size was also considered. To infect frogs, we used four isolates of Bd collected from *R. onca* at Northshore; the stock cultures were on their 6–11th transfer prior to use in this experiment.

We prepared inoculum of active Bd zoospores and exposed frogs following a protocol similar to that described by Jaeger et al. (2017). We dribbled a total of approximately 1 million live Bd zoospores on to each frog for 3 consecutive days (approximately 3 million zoospores total exposure). During this process, we did not change water in the housing containers. For the frogs in the control group, we followed the exact same protocol, but used a sham inoculum made by flooding sterile agar plates with autoclaved water (Waddle et al. 2019). None of the control frogs tested positive for Bd through the laboratory period. The exposures occurred in late July–early August 2019. We allowed infections to develop for approximately 2.5 weeks after exposures, towards the end of which we observed pre-exposed frogs developing clinical symptoms of chytridiomycosis (e.g., excessive skin shedding, erythema; Berger et al. 1998). All frogs in this group tested positive for Bd. Four frogs died from chytridiomycosis during the infection phase, so we added four new frogs to the study, exposing these frogs to Bd in early September.

Bd monitoring.—To monitor Bd infection and intensity during laboratory treatments and later following field release, we sampled skin cells from each frog using a swabbing protocol similar to that described by Jaeger et al. (2017). We systematically swabbed frogs a total of 32 times: 10 times on each ventral side, five times on each foot, and one time on each hand. Swab samples were frozen at - 20° C until assayed. During laboratory treatment, we monitored infections approximately weekly following exposure and through clearance. We analyzed samples using an assay based on quantitative real-time polymerase chain reaction (qPCR; Boyle et al. 2004), and created specific Bd standards of an isolate (LBP114) collected at Northshore (following Longo et al. 2013; Rebollar et al. 2017). We multiplied resulting genomic equivalent values by 80 to

estimate Bd zoospore equivalents per sample (ZE: Vrendenburg et al. 2010). We considered any detection of Bd ≥ 0.1 ZE as an infection (e.g., Lambertini et al. 2016). We re-assayed all field samples that were negative for Bd (0 ZE) to confirm results, with 2.5% (13/514) being positive on retesting at very low values (ZE ≤ 2.5).

Bd clearance.—To clear frogs of Bd infections prior to release in the wild, we used itraconazole as a treatment. We started by treating both the pre-exposed and control groups in mid-August 2019, using an itraconazole concentration of 0.0025% in a 0.6% NaCl solution, with frogs individually soaked in this solution for 20 minutes over nine consecutive days; a slight increase in the number of days from the protocol described by Waddle et al. (2021). This initial treatment, however, failed to clear all infections in the pre-exposed group. We conducted a second treatment of both pre-exposed and control groups in mid-September, but extended the number of treated days from nine to 11. This treatment also failed to clear all infections, and we conducted a third treatment of both groups starting at the end of October 2019. In this last successful treatment, we extended the number of treated days from 11 to 14 to encompass two life cycles of the fungal pathogen as observed in our laboratory and increased the itraconazole concentration to 0.01%. We did not observe any apparent toxic effects from the itraconazole treatments. After the third treatment, we confirmed that all frogs were negative for Bd across four weekly tests. The four replacement frogs in the pre-exposed group only went through the second and third treatments. Over the course of the earlier treatments a total of 11 frogs from the pre-exposed group died, all with clinical signs of chytridiomycosis, and one additional frog sustained an injury and was considered unfit for release.

Delay in release.—Our issue with clearing Bd from infected frogs delayed the initial plan to release frogs to the wild in early fall 2019, and we instead held animals through the winter for release the following spring. We felt that releasing in winter would lower overall survival of the translocated frogs and reduce monitoring success at a critical early stage of the research. Furthermore, a spring release aligns with the general timing of augmentations conducted for management at this site. We held frogs under similar conditions to that during treatments, but co-housed them in groups of 10–11 frogs in 34-quart size, clear plastic containers (60.9 cm W x 42.69 cm x D x 16.76 cm H) with modified lids to allow direct UVB light. During this period, three frogs in the control group died. We could not determine the causes of these deaths, but we ruled out chytridiomycosis.

Frog identification and field sampling.—Prior to release, we photographed and batch marked frogs for individual identification. We took three reference photographs of spotting patterns on the dorsal and lateral sides of each frog (Zylstra et al. 2019), creating a photographic database. In the field, we took similar photographs of captured frogs and then later identified individuals by visually comparing field to reference photographs. Initial identifications were confirmed by a second dedicated reviewer for quality assurance. We also photographed wild frogs captured at the study site over time adding these animals to the database (herein resident group). We batch marked frogs prior to release by clipping a single toe-tip on the left or right side depending on treatment group, rotating among digits but excluding thumb and longest rear toes. The batch marking improved efficiency of photographic identification.

We released a total of 112 pre-exposed and 117 control frogs to the study site on 23 May 2020. We conducted field sampling about every 10 days (range, 6–18) from 2 June 2020–12

June 2021 for a total of 37 sampling events. We conducted an additional five events about every five days (range, 3–7) from 15 October–4 November 2021. We sampled at night when frogs were more active and easier to catch. We practiced sterile handling and sampling techniques, and disinfected equipment and clothing between visits. Field crews consisted of three to five persons, with swabbing, measuring, and data collection managed by a single delegated person across sampling events. We measured weather conditions at the start of sampling events (using a Kestral 5500 Weather Meter, Kestrel Instruments, Shawnee On Delaware, PA), and measured water temperature at capture sites for each frog (or at the closest water in the few cases when frogs were caught on bank) using an electronic thermometer (accuracy 0.4° C; Thermopen MK4, ThermoWorks, American Fork, UT). We then measured frogs for snout-vent-length (SVL) and mass, determined sex, and visually evaluated relative health (e.g., symptoms of chytridiomycosis). The frogs were then swabbed for Bd testing, photographed for individual identification, and then released.

Infection prevalence and intensity.—We used generalized additive models ('GAMs'; Zurr et al. 2009) to analyze the non-linear relationships of infection prevalence and intensity (response variables) across the first year of sampling events. Predictor variables were date of sampling and air temperature at the start of sampling event. We maintained treatment group as a fixed-effect (i.e., intercept estimated for each group) and date or temperature as a smoothing function (splines) for each group (i.e., interaction between group and date or temperature). Models for the resident group only included date or temperature splines. We included individual identification of frogs as a random effect to control for non-independence of multiple measurements. For infection prevalence, we used binomial error distribution with logit link. For infection intensity,

we $\log_{10}$-transformed the ZE results and used a Gaussian error distribution with identity link function (Whitfield et al. 2012). We developed GAMs using the mgcv package in R (Wood 2006; R Core Team 2021). To provide comparative insight into Bd dynamics among resident frogs, we used the same models, but in separate assessments from the treatment groups. We used Wald tests to determine significance of model terms. For descriptive statistics, we calculated average maximum ZE for periods of interest based on the highest ZE for frogs captured during the period.

Survival.—We estimated survival probabilities from mark-recapture data using a Cormack-Jolly-Seber approach (Lebreton et al. 1992) implemented in program MARK (White and Burnham 1999). We pooled capture data from sampling events to create individual capture histories across six periods representing the 18 months of monitoring starting with the initial release. Pooling was required because samples sizes were small during particular sampling events and months (Appendix Table 3.1). We pooled unevenly to better equilibrate sample sizes across periods and incorporated sampling intervals into our models. To select appropriate models that best explained the variation in the data, we performed a goodness-of-fit procedure on a global candidate model (maximum parameterization) to determine the median variance inflation factor ($\hat{c}$). We used $\hat{c}$ to quantify, and correct for, overdispersion to minimize the possibility of overparameterization (Cooch and White 2014; Sandercock 2020). We subsequently used an adjusted delta Akaike's Information Criterion (QAICc) to compare relative support for candidate models (Burnham and Anderson 2004) and used a step-down approach to estimate survival (Doherty et al. 2012). We determined statistical and biological significance of the treatment effect (i.e., pre-exposure vs control groups) using associated Beta estimates of survival from a

model where we set survival to be fixed through time between treatment groups (Cooch and White 2014).

Results

Sampling.—Of the frogs released we recaptured 55.4% (n = 62) of the pre-exposed group and 63.2% (n = 74) of the control group at least once. Capture probabilities were determined to be relatively equal (see modeling of survival below). The number of unique resident frogs captured was 149 and of these 69.8% (n = 104 frogs) were subsequently recaptured at least once. By the latest sampling events in October and November 2021, we recaptured only a total of nine pre-exposed frogs and four control group frogs. Of these frogs, the majority had been recaptured previously with the exception of one control group frog.

Survival.—Overdispersion of the data (high variance) was a factor in our modeling of survival. The median variance inflation factor ($\hat{c}$) for the global candidate model was 1.8368 (indicating overdispersion; Sandercock 2020), and we adjusted the likelihood term using this value to correct for the overdispersion. There were 2 models with a delta QAICc ≤ 2, which indicated that the models had substantial support explaining the variation observed in the data (Burnham and Anderson 2002). In these 2 models the survival parameter (probability) was fixed through time for both groups, but the models differed as to whether the capture parameter was equal (Model 1) or differed for each group (Model 2).

In both models, estimated survival was marginally but consistently higher for the pre-exposed group (Model 1 = 0.8552; Model 2 = 0.8519) than for the control group (Model 1 = 0.8087; Model 2 = 0.8143). The difference between the group estimates for Model 1 was 0.0559

and for Model 2 was 0.0452. The odds-ratios for both models indicated slight increases in survival for the pre-exposed group (Model 1 = 4.8%; Model 2 = 3.8%), but the differences were not significant because confidence intervals of the Beta estimates for these models confined zero (Model 1 = - 0.0209–0.1139; Model 2 = - 0.0411–0.1164). Survival estimates, however, may be biologically meaningful for both models because neither of the confidence intervals for the Beta estimates confined the estimated difference (Cooch and White 2014).

Infection prevalence and intensity.—We detected a statistically significant temporal trend for infection prevalence in both treatment groups (pre-exposed: $\chi^2 = 40.71$, ref. df = 7.518, $p < 0.0001$; control: $\chi^2 = 46.38$, ref. df = 10.969, $P < 0.0001$), although we found no significant difference between the groups over time ($Z = - 1.515$, $P = 0.1298$). There did, however, appear to be some biologically meaningful differences in infection prevalence. Shortly following releases early in the study, infection prevalence differed between the pre-exposed and control groups (Fig. 3.1A; no overlap in 95% CI). Many of the frogs in the control group appeared to have gained Bd infections, with 76.5% (39/51 unique frogs) of the sample in June having infections (Appendix Table 3.1). In contrast, only 20.0% (6/30 unique frogs) of the pre-exposed sample at that time were infected (Appendix Table 3.1). Infection prevalence for both groups dropped into the summer (hot) months, reaching infection probabilities close to zero (Fig. 3.1A), with 8.6% (3/35 unique frogs) of the pre-exposed group and 22.2% (6/27 unique frogs) of the control group in July–September having infections (Appendix Table 3.1). Infection prevalence began to rise for both groups into the following fall and winter months (Fig. 3.1A), reaching 100% infection probabilities during the coldest months starting in December 2020 through February 2021. Infection probabilities dropped for both groups into spring 2021. At that time more than half of

each group were estimated to remain infected, but the trend showed a likely lower probability of infection for the pre-exposed group (Fig. 3.1A).

We detected a significant difference in infection intensity between treatment groups over time ($t = -5.511$, $P < 0.0001$), as well as statistical significance of temporal trends in both groups (pre-exposed: $F = 23.455$, ref. df = 7.680, $P < 0.0001$; control: $F = 30.655$, ref. df = 11.180, $P < 0.0001$). Infection intensity in the control group showed a spike in the GAM following release in June, peaking just before July 2020 (Fig. 3.1C). Average maximum intensity for the Bd positive frogs in the control group during June was 476.3 ZE (± 2274.1 SD) compared to 3.0 ZE (± 2.3 SD) in the pre-exposed group (Appendix Table 3.1). As with infection prevalence, infection intensity was relatively low for both groups during the summer months and then rose substantially into fall and winter (Fig. 3.1C). Infection intensity during fall and winter reached significantly higher levels in the control group than the pre-exposed group, before infection intensities dropped in both groups in the following spring. Around the height of infections during January–March, average maximum infection intensity in the control group was 11,063.2 ZE (± 15,127.2 SD) and 716.2 ZE (± 1159.8 SD) for the pre-exposed group (Appendix Table 3.1).

For resident frogs, we detected a statistically significant temporal trend in both infection prevalence ($\chi^2 = 99.59$, ref. df = 6.534, $P < 0.0001$) and intensity ($F = 26.723$, ref. df = 7.314, $P < 0.0001$). In general, prevalence probabilities were relatively high in June 2020, dropping into summer months, and then increasing substantially into the winter months before declining again in spring 2021 (Figs. 3.2A). Infection intensity showed a similar pattern (Fig. 3.2C). Of the resident frogs sampled in June 2020, 45.2% (19/42 unique frogs) showed infections, with an average maximum intensity of 2825.2 ZE (± 10,788.6 SD) (Appendix Table 3.1). During the summer months (July–September) average maximum infection intensity was generally low

(1202.3 ± 1795.7 SD; Appendix Table 3.1), but unlike the treatment groups the probability of infection remained above zero (Fig. 3.2A). During the winter, the infection probability was near 100% (Fig. 3.2A), with an average maximum intensity of 1027.5 (± 3451.0 SD) in January–March (Appendix Table 3.1).

For both treatment groups, we detected significant relationships between infection prevalence and air temperature (pre-exposed: $\chi^2 = 28.07$, ref. df = 5.326, $P < 0.0001$; control: $\chi^2 = 28.44$, ref. df = 1.009, $P < 0.0001$), but between the groups, the overall relationship was not significant (Z = -0.229, $P = 0.81862$). The probability of infection was at or near 100% at the lowest temperatures about < 12.5° C, and was approaching zero at the highest temperatures > 35° C (Fig. 3.1B). At more intermediate temperatures from about 17–32° C the groups mostly differed, with the pre-exposed group showing significantly lower infection probabilities than the control group (Fig. 3.1B).

We also detected significant relationships between infection intensity and air temperature for each group (pre-exposed: F = 17.222, ref. df = 6.264, $P < 0.0001$; control: F = 45.699, ref. df = 2.231, $P < 0.0001$), as well as between the groups (t = -5.26, $P < 0.0001$). Both groups showed declines in infection intensity with increasing air temperature, but the pre-exposed group had lower infection intensity overall, except at the highest temperatures where infection intensities for both groups were at single digits (Fig. 3.1D). Infection intensity in the pre-exposed group were at single digits as temperatures increased above 17° C, while the control group maintained substantially higher intensities (Fig. 3.1D). At the lowest temperature < 10° C average maximum infection intensity in the control group was 2606.0 ZE (± 2674.1 SD), while that in the pre-exposed group was 129.1 ZE (± 115.1 SD).

Figure 3.1. Relationships between probability of infection (A) across 37 sampling events from 2 June 2020–12 June 2021 (date) and (B) over starting air temperature (° C) for treatment groups. Also shown are the relationships between infection intensity (C) across date and (D) over temperature. Gray ribbons represent 95% confidence interval for the estimated fit. Data for infection intensity is $\log_{10}$-transformed. Predictions are derived from the generalized additive model.

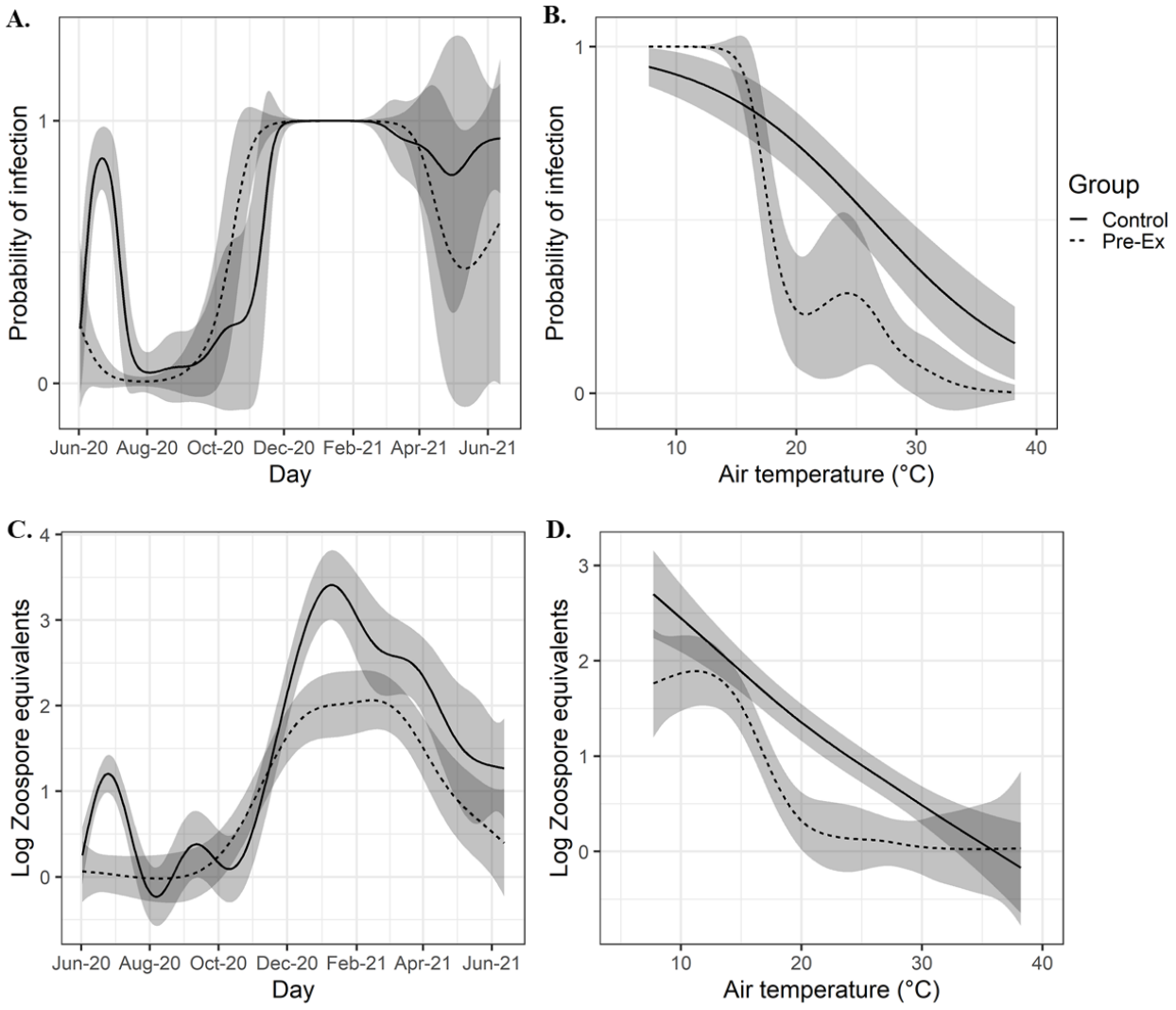
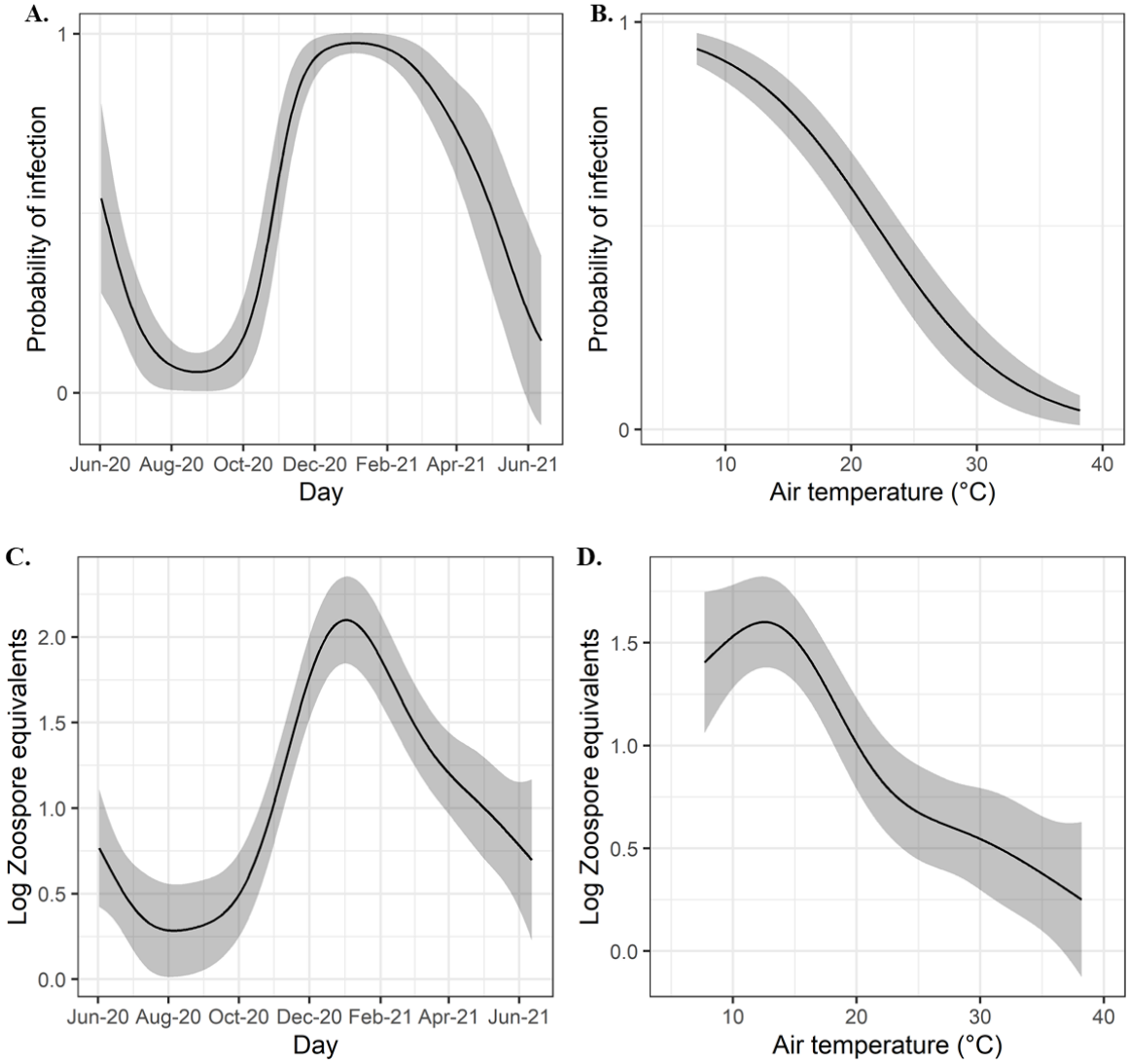


Figure 3.2. Relationships between probability of infection (A) across 37 sampling events from 2 June 2020–12 June 2021 (date) and (B) over starting air temperature (° C) for resident frogs from the site. Also shown are the relationships between infection intensity (C) across date and (D) over temperature. Gray ribbons represent 95% confidence interval for the estimated fit. Data for infection intensity is $\log_{10}$-transformed. Predictions are derived from the generalized additive model.



For the resident group, we also detected significant relationships between infection prevalence and air temperature ($\chi^2 = 78.34$, ref. df = 1.001, $P < 0.0001$), as well as infection intensity and temperature (F = 23.25, ref. df = 4.489, $P < 0.0001$). Infection prevalence and

intensity both showed relatively smooth declines from higher infection values at colder temperature to very low infection values at hotter temperatures (Figs. 3.2B & 3.2D). Unlike the patterns in treatment groups, however, probabilities of infection prevalence for the resident frogs remained < 100% during the coldest temperatures and > 0% at the hottest temperatures (Fig. 3.2B). Estimates of infection intensity were highest at about 12.5° C and were in the single digits as temperatures increased above 21° C (Fig. 3.2D).

Discussion

We investigated the efficacy of pre-exposure to Bd followed by subsequent clearance of the infection to improve augmentation success in *R. onca* released to a site where the pathogen is present. This approach had the potential to provide short-term priming of the immune system prior to subsequent exposure to Bd, but there is some evidence in *R. onca* that pre-exposure and clearance may initiate an adaptive immune response potentially providing longer term resistance (Waddle et al. 2019; Waddle et al. 2021). As a management strategy this approach seemed most appropriate to investigate in *R. onca* at Northshore where the population appears to have some level of inherent resistance to Bd (Jaeger et al. 2017; Waddle et al. 2019).

Seasonal trends in Bd infection prevalence and intensity driven by temperature were clearly present in our data, as has been shown in other systems (Berger et al 2004; Turner et al 2021). Our GAMs indicated that during the hottest months of summer, Bd infection prevalence and intensity were very low, with values close to zero across treatment groups and resident frogs. During the coldest months of winter, Bd infection probabilities reached, or were close to, 100% in all groups, with infection intensities also being highest during the winter months.

Our GAMs indicated that there was no statistical difference in the temporal trend in infection prevalence between treatment groups, but there was an apparent difference during June 2020 following releases when temperatures were moderate. At that time, infection prevalence was much higher in the control group than in the pre-exposed group, with non-overlapping 95% confidence intervals. Early in the study, the control group frogs were 13 times (odds ratio) more likely to have infections than the pre-exposed group, indicating that the pre-exposed frogs were more resistant to initial Bd infection. Infection intensities were statistically different between treatment groups, particularly in June just after release and again during winter when the control group had much higher infection intensity (more than an order of magnitude) than the pre-exposed group. The importance of these differences to survival, however, has only slight support from our mark-recapture models given only marginal and nonsignificant differences in overall estimated survival between treatment groups.

The survival estimates indicate that frogs in the pre-exposed group may have been 3.8% or 4.8% more likely (odds ratios) to survive than frogs in the control group. We detected overdispersion of data in our global mark-recapture model and the adjustment we made to the likelihood term subsequently supported models with few parameters. Prior to the adjustment, models incorporated time, group, or both group and time effects. Survival estimates from these more parametrized models also tended to be higher in the pre-exposed group than the control group (data not shown). Our sample size for estimating survival was small and we suspect that the necessity of holding animals in captivity for a year may have reduced initial survival following release. Both these issues likely affected our ability to detect significance between small differences in survival.

The seasonal pattern of Bd infection observed at this site supports an interesting hypothesis proposed by Waddle et al. (2019) regarding the development of life-stage-dependent mechanisms of resistance to chytridiomycosis observed in *R. onca*. The scenario those authors present also provides a functional model for how the population at Northshore may persist in the presence of Bd. Those authors conducted laboratory experiments challenging *R. onca* with several isolates of Bd, including one derived from a *R. onca* at Northshore. In experiments on various life-stages, Waddle et al. (2019) showed very high survivorship of late-stage tadpoles, very low survivorship of newly metamorphosed frogs, but relatively high survivorship of somewhat older juvenile frogs. Those authors suggested that low survivorship in metamorphs may be explained by reduced immunocompetence within the first 8-week post-metamorphosis when the immune system is in transition between life-stages. Those authors speculated that seasonally high temperatures at Northshore may temporarily lower the selective pressure of Bd. Our data support this perspective in that Bd infection prevalence and intensity were both at, or close to, zero values during the hottest months of summer. Metamorphs emerging during summer months experience an environment with a very low presence of Bd and temperatures that disfavor the pathogen's growth, which may increase their survival. We speculate that juvenile frogs that experience and clear mild Bd infections during summer may gain resistance similar to that induced by pre-exposure and clearance. Such natural immunization may improve survival in winter when Bd prevalence and intensity are greatest. This scenario has implications for management which should avoid releasing headstarted tadpoles, metamorphs, and juvenile frogs to sites with Bd during the spring in hot desert environments, as is commonly practiced at Northshore, and instead hold animals for release until temperatures rise in early summer.

We observed a surprising disappearance of Bd at the site in October and November 2021, following relatively continuous detections through June 2021. We conducted a series of rigorous quality assurance steps to confirm that the non-detection was not an artifact of sampling or laboratory error. We also confirmed non-detection of Bd in an additional sample of 20 frogs in November 2022. Previously the pathogen had been consistently detected at this site during intermittent sampling since 2010 (Jaeger 2017; Jef Jaeger unpublished data). We speculate that Bd disappeared from the site during the summer of 2021 when high temperatures may have pushed Bd prevalence below a threshold of persistence. Given our data on Bd infections in resident frogs, we do not think "herd immunity" (Barnett and Civitello 2020) was a factor in the decline. We note, however, regarding one obvious change in habitat condition, the loss of a small fish-free pond. The pond had been artificially maintained since 2007, but dried in between 15 February–29 April 2020 when water inflow was lost. The pond was favored by *R. onca* and frog densities appeared high over the years (Jef Jaeger unpublished data); however, if the pond was a factor, this leaves the question of why Bd did not disappear in summer 2020, following the pond's desiccation.

Conclusion.—Our results indicate that there are some benefits from pre-exposure and clearance of Bd as a strategy to improve augmentation or translocation success at sites where Bd is present. Pre-exposed frogs showed slightly higher survival, although not statistically significant there was support for the potential difference from our measures of disease process. Infection prevalence and intensity were significantly lower in the pre-exposed group early in the study at a potentially important time following release. Infection intensity was also lower in the pre-exposed group in winter when temperatures favor Bd persistence in the region and *R. onca*

appeared to be more susceptible to infection. For management purposes, pre-exposure and clearance should lead to overall higher survival of released animals. The observed potential slight increase in survival of the pre-exposed frogs, however, was not statistically supported under our current mark-recapture models. Furthermore, we are unsure if an increase of 3.8% or 4.8% (odds ratios) in the likelihood of survival is biologically meaningful in improving the long-term status of this population.

Protocol optimization.—We developed our protocol for exposure and clearance as part of the research project; however, modifications could be made to simplify the protocol for management application. For example, we housed frogs individually which allowed us to monitor infections of each individual, this substantially increased efforts required for husbandry and pathogen monitoring. For management application, such efforts could be greatly reduced by housing animals in groups, treating animals in groups, and randomly sampling subsets of individuals from each group to monitor infections. Regardless of potential modifications, the process requires considerable effort and time. We struggled with clearing infections across a large number of frogs using our initial itraconazole treatment protocol. We ended up modifying dosage and duration twice during retreatments before finally succeeding in clearing all exposed frogs of Bd, which greatly increased treatment time. An alternative approach may be to clear infection using heat treatments at temperatures above the thermal tolerance for Bd, which is generally lower than the thermal tolerance of anurans. Finally, we used isolates of Bd that we acquired from frogs at the site where we conducted our project. Virulence is known to vary across Bd strains (Berger et al. 2005) and to avoid spreading various strains of Bd (if clearance fails), any

attempt to use pre-exposure and clearance as an inoculum should require an isolate from the population being targeted.

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CHAPTER 4

CONCLUSION

The implementation of an effective management and conservation program for the Relict Leopard Frog, *Rana onca*, requires a strategy that promotes our understanding of the species' natural history. Ideally, assimilation of new information generated from management actions or targeted research should be incorporated in an iterative process to evaluate current strategies and actions. Such a scientifically rigorous framework should allow improvement of the program over time (Holling 1978). The conservation agreement, assessment, and strategy (CAS) for *R. onca* stipulates specific objectives and actions aimed at ensuring the persistence of the species (RLFCT 2016). The objectives can be summarized as: (1) address threats to populations; (2) maintain or enhance habitat; (3) establish new or augment existing populations; (4) assess status of populations through long-term monitoring; (5) investigate conservation biology and incorporate findings into management strategy; and (6) provide information to the public and decision makers. Our efforts in Chapter 2 focused on deriving and synthesizing information on the breeding biology of *R. onca*, and in Chapter 3, we assessed the efficacy of an inoculation approach to improve translocation outcomes for the species. These projects were designed to inform several of the objectives of the CAS, including providing recommendations to the Conservation Team managing the species (Objective 5). Furthermore, we intend to publish both Chapters 2 and 3 in peer-reviewed journals to promote information dissemination about the species (Objective 6). As completed, my thesis work will eventually touch upon all of the objectives in the CAS, expanding knowledge on *R. onca*, informing management, and the public.

The information we synthesized in Chapter 2 was derived from almost two decades of systematic population monitoring and annual headstarting-translocation actions. As such, the

synthesis was clearly intended to have implications for improving these management strategies. The information on breeding seasonality improves the efficiency of egg mass collections for headstarting (Objective 3), as well as informs on which months to avoid habitat work to mitigate impacts on early life stages and recruitment (Objective 2). Knowledge of egg mass size and viability promotes better planning on annual collections to meet headstarting quotas determined by the Conservation Team (Objective 3). Our early understanding of developmental times of eggs and tadpoles, as well as specific knowledge of temperature on growth, improves the logistics of rearing activities in the laboratory and translocations (Objectives 3 & 4). Lastly, our insights into when reproductive maturation occurs in newly established translocated populations improves interpretation of efficacy monitoring and expectations of translocation success (Objective 4).

The research in Chapter 3 provides insights into an immunization approach to improve resistance in headstarted *R. onca* to a fungal disease, chytridiomycosis, through pre-exposure to the causal pathogen, *Batrachochytrium dendrobatidis* (Bd), followed by clearance of infection. The project was envisioned as a proof-of-concept at a scale relevant to current augmentation efforts and was focused at a site where the pathogen was present (Objectives 1 & 5). We observed significant seasonal trends in Bd prevalence and intensity among groups, with probabilities very close to zero, during the summer months of July and August when ambient air temperatures at the study site often persisted above the upper thermal tolerance of Bd (Johnson et al. 2003; Piotrowski et al. 2004). The prevalence of Bd reached 100% and intensity reached its peak values for all groups during winter months (December–March) when ambient air temperatures were within Bd thermal tolerance. We detected that frogs pre-exposed to the pathogen had low infection prevalence and intensities at times that may be important for

survival, especially when infections appeared prominent in the environment. Soon after the release in June, the pre-exposed group had significantly lower infection prevalence and intensity. Later during winter months the probability of infection was 100% for both groups, but Bd intensity was significantly lower in the pre-exposed group. Although there was no statistical support for the differences in survival estimates from the mark-recapture models, our two models indicated a potential modest increase in survival of the pre-exposed animals (3.8% or 4.8%, odds ratios). Although we included 229 frogs in the project, roughly split between those that were pre-exposed and the control, the sample size may have been too small to detect the observed difference in survival resulting from the treatment. Furthermore, initial survival of the released frogs in both groups may have been reduced by their captivity for a year prior to release.

What is also not clear is whether the observed modest increase in survival, if real, had longer-term implications for the population. We aimed to assess the efficacy of this approach for management, and we conclude that our findings provide limited validity for improved survival of headstarted frogs. The project also came at a high cost in terms of time, particularly during the treatment process in the laboratory. If further evaluation of the pre-exposure and clearance approach in *R. onca* is desired, then larger samples sizes should be used, with the time invested mitigated by modifications of laboratory protocols, including group housing and an alternative group treatment to clear infections, such as a heat treatment.

Another recommendation that derives support from the research in Chapter 3 is that managers should avoid releasing headstarted animals at the study site (Northshore) in spring, as commonly practiced. Probabilities of Bd infections in *R. onca* are relatively high in the spring when temperatures favor Bd growth, and late-stage tadpoles may be particularly at risk if they metamorphose in spring. Metamorphs may lack fully functioning immune systems and are quite

susceptible to chytridiomycosis (Waddle et al. 2019). Instead, this scenario supports an approach at the study site to release animals during the beginning of summer, when ambient temperatures often persist above the thermal tolerance of Bd. In addition, young frogs that experience and clear mild infections in summer may gain resistance similar to that induced by pre-exposure and clearance, which may improve survival in the following seasons when Bd prevalence and intensity are relatively high.

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Appendix Table 2.1. Days to detection of breeding by the Relict Leopard Frog (*Rana onca*) at translocation sites, determined as the time from initial (earliest) egg mass collections of source animals to first detection of reproduction by these animals. Also provided are the date of first release at a site, life stages of the animals released (T = late-stage tadpoles or early stage metamorphs, J = juvenile frogs), and the life stages of animals at first detection of reproduction (E = egg mass, H = hatchlings, Tb = tadpoles born at site). The number of surveys conducted prior to detection of breeding is also specified.

Site	Earliest Date of Source Egg Collection	Date of First Release	Date of Detection	Days to Detection	Release Stage	Stage Detected	No. of Surveys Prior to Detection
Goldstrike Canyon, NV	01/22/2004	04/09/2004	01/27/2005	372	T	E	2
Grapevine Springs, NV	02/11/2020	04/11/2020	03/18/2021	402	T, J	Tb	1
Quail Spring, NV	01/23/2008	04/24/2008	04/02/2009	436	J	Tb	1
Horse Spring, NV	02/07/2012	05/17/2012	04/26/2013	445	T	H	1
Las Vegas Springs Preserve, NV	01/24/2018	05/29/2018	04/25/2019*	457	J	Tb	3
Grapevine Spring (lower), NV	01/17/2006	03/30/2006	04/22/2007	461	T	Tb	3
Kaolin Spring, NV	01/18/2016	05/26/2016	04/27/2017	466	T, J	E	2
Grapevine Spring, AZ	01/22/2004	04/05/2004	08/25/2005	582	T	E	4
Union Pass Spring, AZ	01/21/2011	04/15/2011	09/20/2012	609	T, J	E	4
Tassi Spring, AZ	01/17/2006	08/24/2006	10/02/2007	624	J	Tb	2
Pupfish Refuge Spring, NV	02/27/2003	10/22/2003	01/19/2005	693	J	E	4
Bearpaw Poppy Spring, NV	02/07/2012	05/01/2012	02/11/2015	1101	J	E	7
Red Rock Spring, NV	01/22/2004	04/22/2005	03/28/2007	1162	J	E, H	6
Lime Spring, NV	01/30/2012	06/07/2012	05/20/2015	1207	T, J	H	7

* Not from monitoring survey data, date of detection reported by Saumure et al. (2022).

Appendix Table 3.1. Infection prevalence and intensity (load) of *Batrachochytrium dendrobatidis* (Bd) in the Relict Leopard Frog (*Rana onca*) samples at Northshore from 2 June 2020–12 June 2021 and 15 October–4 November 2021 for control, pre-exposed, and resident frogs. Frogs released on 23 May 2020. The number of unique individuals per sampling period is indicated, along with total number of samples. Maximum mean Bd intensity (Zoospore Equivalents; ZE) with standard deviation (SD) are based on samples testing positive for the pathogen.

Control Group

Periods	Month	No. of Events	No. Unique	No. Samples	Bd Positive	Bd Prevalence (%)	Max. Mean Bd Load ZE (SD)
May–June 2020	June	5	51	75	39	76.5	476.3 (2274.1)
July–September 2020		9	27	47	6	22.2	47.6 (68.0)
	July	3	15	22	3	20.0	34.5 (57.5)
	August	3	12	13	1	8.3	16.7 (0)
	September	3	10	12	2	20.0	82.5 (112.3)
October–December 2020		8	19	34	17	89.5	1985.8 (2458.1)
	October	3	8	11	3	37.5	3.7 (3.5)
	November	3	9	11	7	77.8	314.3 (503.5)
	December	2	10	12	10	100	3167.5 (2607.1)
January–March 2021		8	10	28	9	90.0	11,063.2 (15,127.2)
	January	3	6	12	6	100	11,044.1 (15,275.0)
	February	2	3	3	3	100	797.7 (895.9)
	March	3	8	13	7	87.5	5656.1 (11,750.8)
April–June 2021		7	8	13	7	87.5	6997.0 (12,095.9)
	April	3	4	5	4	100	11,812.1 (14,828.3)
	May	2	1	1	1	100	0.6 (0)
	June	2	5	7	4	80.0	462.1 (815.2)
October–November 2021		5	4	16	0	0	0 (0)
	October	4	4	13	0	0	0 (0)
	November	1	3	3	0	0	0 (0)

Appendix Table 3.1 (continued).

Pre-exposed Group							
Periods	**Month**	**No. of Events**	**No. Unique**	**No. Samples**	**Bd Positive**	**Bd Prevalence (%)**	**Max. Mean Bd Load ZE (SD)**
May–June 2020	June	5	30	47	6	20.0	3.0 (2.3)
July–September 2020		9	35	51	3	8.6	5.3 (7.6)
	July	3	21	22	0	0	0 (0)
	August	3	13	15	0	0	0 (0)
	September	3	14	14	3	21.4	5.3 (7.6)
October–December 2020		8	23	39	21	91.3	247.7 (468.2)
	October	3	14	17	10	71.4	9.2 (8.9)
	November	3	12	13	12	100	188.8 (410.7)
	December	2	8	9	8	100	374.5 (569.6)
January–March 2021		8	13	32	13	100	716.2 (1159.8)
	January	3	5	6	5	100	79.0 (106.1)
	February	2	7	9	7	100	115.6 (105.5)
	March	3	12	17	12	100	724.8 (1218.0)
April–June 2021		7	9	13	5	55.6	40.0 (61.7)
	April	3	5	5	2	40.0	6.5 (8.1)
	May	2	4	4	2	50.0	91.7 (79.1)
	June	2	4	4	3	75.0	5.1 (2.2)
October–November 2021		5	9	27	0	0	0 (0)
	October	4	9	22	0	0	0 (0)
	November	1	5	5	0	0	0 (0)

Appendix Table 3.1 (continued).

Resident Group Periods	Month	No. of Events	No. Unique	No. Samples	Bd Positive	Bd Prevalence (%)	Max. Mean Bd Load ZE (SD)
June 2020		5	42	60	19	45.2	2825.2 (10,788.6)
July–September 2020		9	60	93	9	15.0	1202.3 (1795.7)
	July	3	23	28	6	26.1	1246.5 (2105.8)
	August	3	27	27	4	14.8	653.0 (704.3)
	September	3	29	38	3	10.3	795.6 (1261)
October–December 2020		8	67	111	51	76.1	6358.7 (19,401.2)
	October	3	35	45	14	40.0	3294.2 (5923.9)
	November	3	32	44	28	87.5	7067.1 (24,678.9)
	December	2	19	22	19	100	4313.2 (8949.3)
January–March 2021		8	63	122	58	92.1	1027.5 (3451.0)
	January	3	31	38	28	90.3	1177.1 (2763.4)
	February	2	17	19	14	82.4	1687.8 (5083.4)
	March	3	46	65	36	78.3	651.9 (3008.5)
April–June 2021		7	39	54	20	51.3	718.1 (1844.5)
	April	3	29	36	16	55.2	339.7 (752.1)
	May	2	7	7	3	42.9	2976.0 (4361.9)
	June	2	9	11	2	22.2	2.3 (1.9)
October–November 2021		5	32	72	0	0	0 (0)
	October	4	30	56	0	0	0 (0)
	November	1	16	16	0	0	0 (0)

CURRICULUM VITAE

Rebeca Rivera
Rivera488@gmail.com

EDUCATION

BS	**Biological Sciences**, University of Nevada, Las Vegas Concentration in Ecology and Evolutionary Biology GPA: 3.64/4.00 Dean's Honor List – multiple semesters	**December 2010**
MS	**Biological Sciences**, University of Nevada, Las Vegas Concentration in Ecology and Evolutionary Biology GPA: 3.92/4.00	**Expected graduation Summer 2023**

PROFESSIONAL EXPERIENCE

Graduate Student Research Assistant **2023**
University of Nevada, Las Vegas (UNLV). Dr. Jef R. Jaeger (Supervisor)
10-20 hours of work per week (hours reduced to complete master's degree)

Consultation and Writing Efforts

- Offer feedback and assistance with population monitoring surveys, and headstarting and translocation activities for the Relict Leopard Frog.
- Provide updates to protocols used for headstarting and translocation activities.
- Assistance with writing a final report for a conservation genetic assessment of the Relict Leopard Frog.
- Co-writer of technical and final reports for information dissemination to the Relict Leopard Frog Conservation Team.
- Co-writer of meeting minutes for the semiannual Relict Leopard Frog Conservation Team meetings, as well as co-editor of team reports.
- Co-writer of a species account for the Relict Leopard Frog for the Arizona Game and Fish Department (AGFD).

Data Collection and Management

- Collect data and maintain long-term datasets from population monitoring surveys and headstarting and translocation activities through Microsoft Excel and Access.
- Implement QAQC on datasets.

Educating and Training

- Educate, train, and oversee UNLV students and seasonal employees on field surveys, and in headstarting/translocation activities.
- Co-lead weekly research meetings for undergraduate and graduate students assisting with Relict Leopard Frog activities.

Research Assistant, School of Life Sciences **2011 to 2022**
UNLV. Dr. Jef R. Jaeger (Supervisor)
40–56 hours of work per week (depending on seasonal activities)

Field Activities

- Organize and conduct diurnal and nocturnal monitoring surveys for the Relict Leopard Frog semiannually in southern Nevada and northwestern Arizona (i.e., Mojave Desert).
- Coordinate and conduct mark-recapture population estimation for Relict Leopard Frog.
- Conduct field sampling of regional anuran species to detect amphibian chytrid fungus (i.e., Clark, Lincoln and Nye counties in NV).
- Collect information and measurements on site conditions for projects (e.g., GPS coordinates, weather variables, vegetation and environmental measurements).
- Conduct small-scale habitat management efforts for the Relict Leopard Frog, along with invasive species control (e.g., American Bullfrog).
- Coordinate and conduct habitat site assessments with various land and resource managers for the Relict Leopard Frog.
- Implement disease and invasive aquatic species prevention protocols (e.g., HACCP), with emphasis on amphibian chytrid fungus, quagga mussel, hydroids, and snails.
- Operate a rigid-hull inflatable boat with a 15 HP outboard engine.
- Operate a 4x4 truck, including off-road driving and towing of boat.

Headstarting/Translocation, Research, and Laboratory Activities

- Collect eggs of the Relict Leopard Frog for annual headstarting/translocation activities.
- Organize, delegate and conduct headstarting/translocation activities for the Relict Leopard Frog (e.g., husbandry maintenance on hundreds of animals, implementation of pre-release treatments, and transportation of animals).
- Assisted with laboratory experiments into the effects of an amphibian chytrid fungus on the Relict Leopard Frog and Northern Leopard Frog (e.g., disease sampling, DNA extractions and dilutions, microbial transfers and cell counting using a hemocytometer).
- Spearheaded laboratory and field efforts related to assessing the efficacy of pre-exposing the Relict Leopard Frog to the amphibian chytrid fungus to increase survivorship of headstarted frogs.
- Data mined information collected from long-term monitoring surveys and management activities for the Relict Leopard Frog to inform on its breeding biology.
- Organized and led field efforts for micro-tissue sampling of the Relict Leopard Frog as part of a conservation genetic assessment.
- Aided with various molecular techniques for a population genetics project on Desert Bighorn Sheep (e.g., DNA extractions, PCR, gel electrophoresis).

Training and Coordination of Personnel

- Educate, train, and supervise UNLV students and seasonal employees on field surveys, and in headstarting/translocation activities.
- Educate, train, and supervise UNLV students and seasonal employees with database input and quality assurance.
- Coordinate with Federal, State, and Local agency personnel with implementation of conservation and management actions for the Relict Leopard Frog (e.g., Las Vegas

Springs Preserve, Southern Nevada Water Authority, Clark County Desert Conservation Program, AGFD, Nevada Department of Wildlife [NDOW], Utah Division of Wildlife Resources, U.S. Fish and Wildlife Service [USFWS], Bureau of Reclamation [BOR], Bureau of Land Management [BLM], and National Park Service [NPS],).

Data Collection, Management, and Analysis

- Implement QAQC for data collection in the field and laboratory and entry into Access and Excel.
- Manage and maintain databases in Microsoft Access and Excel related to population and disease monitoring, and headstarting/translocation activities.
- Analyze and interpret information and data collected from field and laboratory activities
- Analyze mark-recapture data and estimate population sizes using Program MARK.
- Use Google Earth to confirm the reliability of GPS coordinates.
- Use ESRI ArcGIS for map production.

Technical Writing and Information Dissemination

- Minute Taker at semiannual meetings of the Relict Leopard Frog Conservation Team, and co-editor of annual team reports and work plans.
- Coauthor of annual and semiannual reports on monitoring and management activities for the Relict Leopard Frog, which include recommendations on habitat management and whether a site is suitable for species translocation.
- Coauthor of the renewal of the conservation agreement, assessment, and strategy plan for the Relict Leopard Frog (see Publications & Reports below).
- Assist with compiling information and writing, including biological species assessments, various project reports, grant proposals and project permits (see Publications & Reports below).
- Coauthor of several scientific publications (see Publications & Reports below).
- Present information on project activities and findings at regional and local meetings, in university courses, and to the public (see Formal Presentations below).
- Update project protocol and technique documents associated with field and headstarting/translocation activities for the Relict Leopard Frog.

Environmental Legislation and Policies

- Participation on the Relict Leopard Frog Conservation Team involves discussions of legislation and policy issues relating to the conservation and management of the Relict Leopard Frog and federal rights-of-way (e.g., ESA, NEPA, EA, EIS, Clean Water Act, National Historic Preservation Act, Candidate Conservation Agreement with Assurances).
- Co-edited policy and law sections of the Conservation Agreement and Conservation Assessment and Strategy for the Relict Leopard Frog.

Undergraduate Research Assistant, Public Lands Institute **2010 to 2011**
UNLV. Dr. Jef R. Jaeger (Supervisor)
20 hours of work per week

Field Activities

- Assist with nocturnal and diurnal monitoring surveys, and mark-recapture efforts for the Relict Leopard Frog.
- Assist with nocturnal collection efforts on scorpion species for phylogeographical research.

Research and Laboratory

- Assist with implementing molecular techniques (e.g., DNA extractions, gel electrophoresis, PCR, sequencing) on an effort to assess nuclear DNA differences between the Relict Leopard Frog and its sister taxa, the Lowland Leopard Frog.
- Assist with headstarting/translocation activities for the Relict Leopard Frog.

Veterinary Technician Assistant, Sunrise Vet Clinic **2009 to 2011**
Las Vegas, NV. Dr. David Henderson (Supervisor)
20–25 hours of work per week

Clinic Experience

- Conduct front desk responsibilities and provide customer service.
- Translate for Spanish-speaking pet owners.
- Monitor recovering cats and dogs following surgery.
- Conduct sanitation and sterilization techniques of kennels, exam rooms, surgical suites and tools.
- Data recording and tracking of animals for feral cat clinic events.

Teacher Assistant, Lynn Bennett Early Childhood Education Center **2007 to 2009**
UNLV. Eileen Quinn, M. Ed (Supervisor)
15–20 hours of work per week

Child Care Experience

- Monitor and care for children < 5 years old.
- Stimulate growth and development of children using age-appropriate curriculum.

Cashier and PetCare Assistant, PetsMart **2005 to 2007**
Henderson, NV. Patricia Rivera (Supervisor)
20–32 hours of work per week

Retail Experience

- Ring-up customers, handling customer's money, and balancing a register.
- Provide customer service, answer phone calls, and face store.

Animal Care Experience

- Husbandry responsibilities on a variety of fish, small mammals, birds, and reptiles.
- Provide customer service, advice on pet care, and answer phone calls.
- Restock store items and face store.

PUBLICATIONS & REPORTS

Peer-reviewed Journal
My name in bold

O'Toole, T., R.A. Saumure, A.R. Bennett, A. Ambos, **R. Rivera**, K. Guadalupe, J.R. Jaeger. *In press*. *Rana onca* (Relict Leopard Frog). Metamorphosis and Overwintering Tadpoles. Herpetological Review.

Saumure, R.A., A. Ambos, A.R Bennett, T. O'Toole, **R. Rivera**, K. Guadalupe & J.R. Jaeger. 2022. *Rana onca* (Relict Leopard Frog). Growth, Sexual Maturity, and Size. Herpetological Review 53: 108–110.

Waddle, A.W., **R. Rivera**, H. Rice, E.C Keenan, G. Rezaei, J.E. Levy, Y.S. Vasquez, M. Sai, J. Hill, A. Zmuda, Y. Lambreghts & J.R. Jaeger. 2021. Amphibian resistance to chytridiomycosis increases following low-virulence chytrid fungal infection or drug-mediated clearance. Journal of Applied Ecology 0: 1365 –2664. https://doi.org/10.1111/1365-2664.13974.

Saumure, R.A., **R. Rivera**, J.R. Jaeger, T. O'Toole, A. Ambos, K. Guadalupe, A.R. Bennett & Z. Marshall. 2021. Leaping from extinction: rewilding the Relict Leopard Frog in Las Vegas, Nevada, USA. IUCN: 76–81.

Bennett, A.R., **R. Rivera**, R.A. Saumure, T. O'Toole, J.R. Jaeger & P.R. Bean (2020) *Rana onca* (Relict Leopard Frog). Diet and Mortality Note. Herpetological Review 51: 302-303.

Waddle, A.W., J.E. Levy, **R. Rivera**, F. van Breukelen, M. Nash, and J.R. Jaeger. 2019. Population-level resistance to chytridiomycosis is life-stage dependent in an imperiled anuran. EcoHealth 16: 701–711.

Jaeger, J.R., A.W. Waddle, **R. Rivera**, D.T. Harrison, S. Ellison, M.J. Forrest, V.T. Vredenburg, and F. van Breukelen. 2017. *Batrachochytrium dendrobatidis* and the decline and survival of the Relict Leopard Frog. EcoHealth 14:285–295.

Forrest, M.J., M.S. Edwards, **R. Rivera**, J.C. Sjöberg, and J.R. Jaeger. 2015. High prevalence and seasonal persistence of amphibian chytrid fungus infections in the desert-dwelling Amargosa Toad, *Anaxyrus nelsoni.* Herpetological Conservation and Biology 10:917–925.

Submitted for Peer-reviewed Journal

Rivera, R., D.L. Drake, J.R. Jaeger. *In peer-review 2023*. Aspects of Relict Leopard Frog breeding biology. Submitted to Herpetological Conservation and Biology Journal.

Selected Reports

Jaeger, J.R. and **R. Rivera**. 2022. Conservation Implementation for the Relict Leopard Frog (*Lithobates onca*) in Nevada, Arizona, and Utah. Unpublished final report submitted by UNLV to the Nevada Department of Wildlife, as a sub-grantee under Competitive State Wildlife Grant agreement with Nevada Department of Wildlife. Las Vegas, NV.

Jaeger, J.R. and **R. Rivera**. 2022. BLM NV CESU Relict Leopard Frog Research and Site Work. Unpublished final report submitted by UNLV to the Bureau of Land Management. Las Vegas, NV.
Relict Leopard Frog Conservation Team. 2016. Conservation agreement and conservation assessment and strategy for the Relict Leopard Frog (*Rana onca* [= *Lithobates onca*]). Available online. Note authorship contribution in the Acknowledgements.

Jaeger, J.R. and **R. Rivera**. 2015. Relict Leopard Frog conservation. Unpublished final report submitted by the UNLV to the National Park Service, as a sub-grantee under agreements with National Park Service and Nevada Department of Wildlife. Las Vegas, NV.

Jaeger J.R. and **R. Rivera**. 2013. Expanding efforts to quantify the status of the Relict Leopard Frog. Unpublished final report submitted by the UNLV to U.S. Fish and Wildlife Service. Las Vegas, NV.

MEDIA ACTIVITY

Creatures of the Night, 10/2014. Featured in an 8 minute segment on the natural history of amphibians. Directed and produced by Dr. Michael Webber, UNLV with funding from National Science Foundation. Available online at: https://vimeo.com/112526223.

FORMAL PRESENTATIONS

My name in bold indicates Lead Presenter.

Status of Relict Leopard Frog Management. Jaeger, J.R. and **R. Rivera**. Mohave Desert Network Science Symposium, November 2–3, 2022. Boulder City, NV.

Aspects of Relict Leopard Frog Development and Breeding Biology. **Rivera, R.** UNLV Research Colloquium, October 12, 2022. Las Vegas, NV.

Improving amphibian resistance to an emerging fungal disease through pre-exposure and clearance: an effort to inform management strategy. **Rivera, R.** UNLV Research Colloquium, UNLV, September 15, 2021. Las Vegas, NV.

Aspects of Relict Leopard Frog Development and Breeding Biology. **Rivera, R.** UNLV Research Colloquium, September 23, 2020. Las Vegas, NV.

Relict Leopard Frog Conservation Planning and Implementation. **Rivera, R.**, and J.R. Jaeger. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 28, 2019. Las Vegas, NV.

Leopard Frogs of Southern Nevada, from Extinction to Understanding**. Rivera, R.** and J.R. Jaeger. Clark County World Wetlands Day, January 30, 2019. Las Vegas, NV.

Relict Leopard Frog Conservation Planning and Implementation. **Rivera, R.**, and J.R. Jaeger. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 15, 2018. Las Vegas, NV.

Relict Leopard Frog Susceptibility to Chytridiomycosis Varies by Collection Site. Waddle, A.W., G. Rezaei, N. Pattni, J. Levy, Y. Vasquez, **R. Rivera**, F. van Breukelen, and J.R. Jaeger. California-Nevada Amphibian Populations Task Force Meeting, January 11–12, 2018. Auburn, CA.

Relict Leopard Frog Conservation Planning and Implementation. **Rivera, R.** and J.R. Jaeger. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 28, 2017. Las Vegas, NV.

Conserving a Rare Mojave Desert Endemic, the Relict Leopard Frog. **Rivera, R.** and J.R. Jaeger. UNLV Guest Lecture, Principles of Human Ecology, November 7, 2016. Las Vegas, NV.

Relict Leopard Frog Recovery Conservation and Planning. Jaeger, J.R. and **R. Rivera**. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 23, 2016. Las Vegas, NV.

A Questionable Role for Amphibian Chytrid Fungus in the Decline of the Relict Leopard Frog. Waddle A., J.R. Jaeger, and **R. Rivera**. Colorado River Terrestrial and Riparian Meeting, January 26–28, 2016. Laughlin, NV.

Assessing the Relict Leopard Frog to Inform the Upcoming ESA Listing Decision. Jaeger, J.R., **R. Rivera**, M. Burroughs, J.C. Sjöberg, R. Haley, and M.J. Sredl. California-Nevada Amphibian Populations Task Force Meeting, January 7–8, 2016. University of California, Davis, CA.

A Questionable Role for Amphibian Chytrid Fungus in the Decline of the Relict Leopard Frog. Jaeger, J.R., **R. Rivera**, A. Waddle, D.T. Harrison, S. Ellison, M.J. Forrest, V.T. Vredenburg, and F. van Breukelen. California-Nevada Amphibian Populations Task Force Meeting, January 7–8, 2016. University of California, Davis, CA.

Relict Leopard Frog Conservation, Finishing Almost Five Years of Implementation. **Rivera, R.**, J.R. Jaeger, R. Haley, and J. Sjöberg. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 13, 2015. Las Vegas, NV.

Relict Leopard Frog Recovery Conservation. Jaeger, J.R., **R. Rivera,** R. Haley, and J. Sjöberg. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 21, 2014. Las Vegas, NV.

Developing the Relationship Between Relative and Actual Abundance to Estimate Population Size of the Relict Leopard Frog. **Rivera, R.** and J.R. Jaeger. Invited Presentation, Colorado River Terrestrial and Riparian Meeting, January 28-30, 2014. Laughlin, NV.

Calibrating Indices of Relative Abundance to Estimate Population Size of the Relict Leopard Frog. **Rivera, R.** and J.R. Jaeger. California-Nevada Amphibian Populations Task Force Meeting, January 9-10, 2014. Beatty, NV.

Relict Leopard Frog Recovery Monitoring. Jaeger, J.R., **R. Rivera**, R. Haley, and J. Sjöberg. Invited Presentation, Lake Mead Ecosystem Monitoring Workgroup, August 15, 2013. Las Vegas, NV.

Relict Leopard Frog Recovery Conservation. **Rivera, R.**, J.R. Jaeger, R. Haley, and J. Sjöberg. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 22, 2013. Las Vegas, NV.

Update on Monitoring and Management of the Relict Leopard Frog. **Rivera, R**, J.R. Jaeger, R. Haley, and J. Sjöberg. Colorado River Terrestrial and Riparian Meeting, January 29–31, 2013. Laughlin, NV.

Relict Leopard Frog Conservation. **Rivera, R**. UNLV Guest Lecture, Introduction to Human Ecology, April 2013. Las Vegas, NV.

Relict Leopard Frog Conservation. Jaeger, J.R., **R. Rivera**, R. Haley, and J. Sjöberg. Clark County Multiple Species Habitat Conservation Plan Annual Project Progress Report Symposium, August 16, 2012.

Conservation of the Relict Leopard Frog. **Rivera, R**. UNLV Guest Lecture, Introduction to Human Ecology, November 2011. Las Vegas, NV.

A Further Investigation into the Relationship of *Rana onca* and *Rana yavapaiensis* Based on Nuclear Markers. **Rivera, R**. UNLV Lab Presentation, November 2010. Las Vegas, NV.

PROFESSIONAL DEVELOPMENT

Wildlife First Aid Training
Sierra Rescue International, Reno, NV, 4/7–4/8/2022

Biometry, learned to use Program R (3 credits, BIOL 628)
UNLV, NV, Fall 2019

Geographic Information Science and Systems: Theory and Application, learned to use ESRI ArcGIS (4 credits, GEOL 430)
UNLV, NV, Spring 2016

Introductory-Level Short Course on Design and Analysis of Mark-Recapture/Resight Studies with a Focus on Using Program MARK
Utah State University, Logan, UT, May 7–10, 2013

Boating Safety Education Certificate
Nevada Department of Wildlife, August 2012

PROFESSIONAL AFFILIATIONS

Relict Leopard Frog Conservation Team, 2011–Present
Team member, Minute Taker, Co-editor of team reports and annual work plans

CA/NV Amphibian Populations Task Force, 2012–2020
Meeting registration organizer – annual volunteer

COMMUNITY SERVICE

Latino Youth Leadership Conference, Invited Guest
UNLV, June 21, 2018

LANGUAGES

English: native language

Spanish: Intermediate-fluent listener and speaker, intermediate-fluent reading and writing

COMPUTER SKILLS

Proficient in Microsoft Word, Excel, PowerPoint and Access

ADDITIONAL PROFESSIONAL EXPERIENCE

- Assisted BLM with road cruising for snakes (1-2 nights from 2019–2021).
- Participated with workshop and field survey on the Kingman Springsnail with AGFD (11/15/2017).
- Aided AGFD with genetic sampling on the Northern Leopard Frog (06/2017, 08/2017).
- Supported USFWS with Columbian Spotted Frog mark-recapture surveys (07/2017).
- Assisted BLM with catching hummingbirds for banding (1–2 days each spring/summer 2015–2017).

- Supported NDOW with Amargosa Toad mark-recapture surveys (1–2 days each spring 2011–2014, 2016).
- Assisted BOR with mist netting bats (09/2014).
- Assisted UNLV graduate student with butterfly surveys and collections (spring/summer 2012).
- Assisted UNLV graduate student with scorpion collecting and road cruising for snakes on various occasions (2011–2014).
- Conducted Northern Goshawk surveys under a task agreement between UNLV and NPS (summer 2011).
- Assisted UNLV, with collecting Desert Bighorn Sheep fecal samples for a populations genetic project (summer 2011).
- Volunteered for NPS on Bald Eagle and Peregrine Falcon surveys (spring 2011).
- Aided U.S. Geological Survey (USGS) with spring site assessments and vegetation surveys (05/2011).
- Assisted USGS with mountain lion trapping (02/2011).
- Assisted UNLV with bird taxidermy (2010).

PROFESSIONAL REFERENCES

1. Dr. Jef R. Jaeger, Assistant Professor in Residence
   School of Life Sciences, UNLV
   jef.jaeger@unlv.edu
   702.895.2463

2. Joe Barnes, Wildlife Staff Biologist
   Nevada Department of Wildlife
   jbarnes@ndow.org
   775.688.1404

3. Mark Slaughter, Supervisory Natural Resources Specialist
   Bureau of Land Management, Nevada
   mslaught@blm.gov
   702.515.5195

4. Michael Burroughs, Wildlife Biologist – Retired
   U.S. Fish and Wildlife Service, Nevada
   burroughsm@aol.com
   702.656.5906