Biology and Captive Husbandry of *Andinobates geminisae*, a Critically Endangered Dart Frog

by

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B.Sc., University of British Columbia, 2016

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Summer 2019

Approval

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Abstract

One third of amphibians are threatened with extinction and the number of amphibian captive breeding programs (CBPs) is increasing as components of recovery strategies. However, the success rate of amphibian CBPs is only 50%, and may be limited by health problems resulting from inadequate husbandry knowledge. I investigated the role of husbandry in the occurrence of Spindly Leg Syndrome (SLS), a limb development disease, in a population of captive-bred *Andinoabes geminisae* tadpoles at the Panama Amphiban Rescue and Conservation Project (PARCP). I found that that vitamin supplementation and filtration method of tadpole rearing water may affect SLS prevalence and that decreasing tadpole husbandry intensity delays the development time of tadpoles. A fortuitous accident during one of my experiments provided compelling evidence that phosphate exposure may also be a key factor in the occurrence of SLS. Accompanying my SLS work, I described the tadpoles of *Andinobates geminisae* and *Oophaga vicentei* (Vicente's dart frog) using tadpoles that died of baseline mortality during my experiments. Combined, my work demonstrates that CBPs can serve beyond their immediate conservation purpose to facilitate important research on the biology of the species they hold.

Keywords: amphibian conservation, tadpole, captive breeding programs, ex situ conservation, poison dart frogs, spindly leg syndrome.

Dedication

I dedicate this thesis to Ducky. Thank you for reminding me to take time to stop and sniff around every once in awhile.

Acknowledgements

I would like to thank Dr. Arne Mooers for taking me on as his student and giving me the freedom to choose a project that suited my interests and goals, even though it was in an unfamiliar field. Thank you Arne, for helping me develop my interests, build my career path, and become a better scientist, through your guidance and genuine support and care for my well-being. This project would not have been possible if it were not for Dr. Roberto Ibáñez and Dr. Brian Gratwicke, who welcomed me to carry out my research at the Panama Amphibian Rescue and Conservation Project. Thank you Roberto, for taking me under your wing, answering my endless questions, and providing me with countless opportunities to learn about amphibians in the field as well as at the PARCP – thanks to you, I am the budding herpetologist I am today. Thank you Brian, for meeting me to discuss project ideas whenever you were in town, for serving as a vital lifeline when things didn't turn out as expected, and for your feedback on my manuscript. I also want to thank Dr. Wendy Palen, for her support and involvement on my committee and for all her help with experimental design and data analysis.

Quiero agradecer al personal del PARCP: Jorge Guerrel, Nancy Fairchild, Elliot Lassiter, Nair Cabezón, Lanki Cheucarama, Jennifer Warren, Guadalupe Ureña,Orlando Ariel Garcés, y Valeria Franco, por tan caluroso recibimiento y sus amistades; nuestros almuerzos de cumpleaños, paseos por la playa, noches de película, y cenas en la ciudad, están entre mis memorias más preciadas de Panamá. Nunca olvidaré el esfuerzo que hicieron para hacerme sentir bienvenida y en casa durante mi tiempo en Panamá. Gracias, Jorge, por todo el tiempo que te quedaste tarde en el PARCP para ayudarme con mis experimentos o para superar problemas inesperados. Thank Elliot Lassiter, for all your extra help and for teaching me to identify so many new species in the field in your free time.

Lastly, I want to thank all of the volunteers at the PARCP for the extra help they provided, especially my two amazing friends, Ryan Ikeda and Scott Walker, who flew down to visit me and help me collect water quality data that was critical for my thesis discussion when I was running out of time – you are life savers and true friends.

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Chapter 1. Introduction

Originating over 300 million years ago and comprising over 8,000 species, the Class Amphibia (or the amphibians) is one of the most ancient and diverse groups of vertebrates today (AmphibiaWeb 2019; Dodd 2010; Wells 2007). The number of amphibian species described has more than doubled since 1985 (AmphibiaWeb 2019) and continues to grow by 128-172 new species per year (Tapley et al. 2018). Ironically, amphibians are simultaneously undergoing global declines with projections of reducing the number of frog species by 7% over the next century, based on extinction data from 1971-2001 (Alroy 2015). This “profoundly disturbing paradox” (AmphibiaWeb 2019; Hanken 1999) challenges modern herpetologists, who are faced with the task of studying a class of animals that may be disappearing faster than it can be described (AmphibiaWeb 2019; Alroy 2015; Crawford et al. 2010).

Amphibian declines affect everyone; amphibians are an important source of protein in some cultures (Ribas & Poonlaphdecha 2017; Gonwou & Rödel 2008), and their skin secretions, which have been used in traditional medicine for millennia to treat inflammation, infection, parasites and cancer, have vast pharmaceutical potential (Rodríguez et al. 2017); amphibians may control insect agricultural pests and vectors of human disease (Hagman & Shine 2007; Kagemann & Han 2015.; Khatiwada et al. 2016; Raghavendra, Sharma, & Dash 2008) and their disappearance is documented to have cascading effects on ecosystem structure and function, (Connelly et al. 2008; Ranvestel et al. 2004; Whiles et al. 2006). Lastly, amphibians hold aesthetic and cultural value. Amphibians are represented in film and literature, inspire artwork, and are important symbols of fertility, good fortune, and rebirth in many cultures (Crump 2015; Tapley et al. 2011). Amphibians are also increasingly popular as pets (Tapley et al. 2011), and childhood encounters with amphibians can provide formative learning experiences about the natural world.

While extinction is a natural process, current amphibian declines are far from this - the extinction rate of amphibians is four times measured background rates, and declines are primarily caused by human activity (Tapley et al. 2015; Gascon et al 2007). Many threats to amphibians can be mitigated through policy and law enforcement (eg. habitat loss, pollution, invasive species, and overexploitation), but climate change and

the spread of fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) has triggered a seemingly irreversible series of species extinctions, even in areas of pristine and protected habitat (Scheele et al. 2019; Tapley et al. 2015; Stuart & Sutherland 2014; Gascon et al. 2007). In these cases, the only way to safeguard some species from extinction is to keep them in conservation breeding programs (CBPs) *in addition* to ongoing *in situ* conservation efforts until we find a way for them to persist in the wild.

Captive breeding can be thought of as the open-heart surgery of conservation biology: it is expensive, risky, and should only be used when preventative measures and less invasive treatments have failed. Unlike open-heart surgery, captive breeding is not a well-established emergency procedure; each new case brings novel complications that are overcome largely through trial and error, and subsequently, the success rate of amphibian CBPs is only 54% (Biega 2017). Another contrast between these two emergency practices is their level of research activity. The Journal of Cardiac Surgeons has produced 34 volumes since 1980 with articles on methodology, case reports, and cardiac health that continue to inform the treatment and prevention of cardiac disease, while little to no information gleaned from amphibian CBPs is published, and the knowledge pool on amphibian CBPs is stagnating in comparison. While captive breeding programs may be necessary to prevent the extinction of some species, it is currently hard to justify such expensive and drastic procedures when the success rate of these programs is only 54%. I argue that the success rate of CBPs is at least partially constrained by idiopathic (of unknown cause) amphibian diseases, but that CBPs have the potential to determine effective treatments for these diseases using a scientific approach. I also argue that CBPs could increase their value by increased publication of information on the rare species they hold, thus further justifying the existence of such programs. This thesis addresses the problem of low success in captive breeding programs due to captivity-specific health problems.

Of course, a high likelihood of captive breeding success alone cannot ensure the long-term persistence of a species. The success of captive breeding programs in reducing the extinction risk of wild populations depends on a variety of other factors, including the size of the source population, the number of release events, the number of captive bred individuals released, and the survival and reproductive success of captive-bred individuals post-release (McCleery et al. 2014; Heinrichs et al. 2019). Furthermore, if the

in situ conditions responsible for wild population declines are not addressed, then any positive effects of CBPs on wild populations will only be temporary (Heinrichs et al. 2019). Captive breeding and release programs also pose risks. CBPs can negatively impact wild populations through the removal of scarce remaining wild individuals for use in founder populations (McCleery et al. 2014; Heinrichs et al. 2019). There is always a risk due to the transmission of new diseases (Woodford & Rossiter 1994). A further concern is unwanted genetic change:(Willoughby and Christie 2018; Araki et al. 2009; Araki et al. 2007). For example, the relative reproductive success of steelhead trout (*Oncorhynchus mykiss*) is reduced by as much as 40% per generation bred in captivity due to genetic effects, and interbreeding between wild and first generation captive-bred individuals results in offspring with an overall reproductive fitness that is only 55% that of the an individual born of two wild born parents (Araki et al. 2007). Overall, the potential benefits of CBPs should therefore be carefully weighted against their risks (Araki et al. 2007; Araki et al. 2009; McCleery et al. 2014; Heinrichs et al. 2019) when deciding whether to include CBPs in conservation initiatives.

Hindlimb paralysis, Spontaneous Metamorph Death (SMD), edema, and various skeleto-muscular deformities are just a few examples of the idiopathic diseases that commonly affect amphibians held in captivity (Ferrie et al. 2014; Wright and Whitaker 2001; Jorge Guerrel pers. comm.). Superficially, many of these diseases appear to occur spontaneously, but their high frequency in captivity and their absence in the wild suggests that they are the result of failure to meet some specific husbandry need - water quality, nutrition, substrate choice, temperature, or something else (Ferrie et al. 2014; Wright & Whitaker 2001). These diseases threaten the success of captive breeding programs. For example, the Oregon Spotted Frog Recovery Team lost 30% of their head-starting population of tadpoles at Canadian facilities to body edema of unknown etiology in 2008 (Gielens et al. 2008), and the Panama Amphibian Rescue and Conservation Project (PARCP) lost over 50% of captive-born metamorphs of a critically endangered species of poison dart frog to Spindly Leg Syndrome, a deformity of the limbs of unknown etiology (PARCP unpublished data 2017). Scientific investigation of the potential husbandry factors influencing the incidence and severity of idiopathic captivity-related diseases could raise the success rate of captive breeding programs and improve our understanding of the general needs of captive amphibians.

Many of the species brought into amphibian CBPs are cryptic, with little to no published information available on them from the wild. For example, the vanishing robber frog (*Crugastor evanesco*) is appropriately named because it had nearly disappeared from its home range due to the *Bd*-caused fungal disease chytridiomycosis by the time it could be described and taken in for captive breeding at the Panama Amphibian Rescue and Conservation Project (PARCP), where all remaining individuals now reside. Likewise, the last remaining female of an unknown *Craugastor* species at the PARCP died before anything on her species could be described (Ryan et al. 2010; Roberto Ibanez pers. comm.). The possession of these cryptic species in captivity provides a unique opportunity to publish novel data on their behaviour, natural history, and biology, including descriptive information (calls, behaviour, adult or tadpole morphology) that would otherwise be unattainable and could be used in species-specific search and recovery efforts in the wild. Sufficient time and energy should be allocated into processing and publishing these data not only for conservation initiatives, but also for the field of herpetology in general.

My thesis exemplifies the process of developing evidence-based treatments of captivity-related amphibian disease and the production of publishable CBP-based information. Under the auspices of the NSERC CREATE RenewZoo internship program, I worked at the Panama Amphibian Rescue and Conservation Project (PARCP) for 15 months to carry out the two components of my thesis work. First, I experimentally investigated the possible causes of Spindly Leg Syndrome (SLS), a leg deformity that was severely limiting the survival of young poison dart frog metamorphs at the PARCP. I carried out two experiments between July and December 2017 and March and August 2018 to test if qualities of the tadpole rearing water or tadpole husbandry, respectively, were important factors in the prevalence of SLS. I spent my remaining time at the PARCP describing the eggs and tadpoles of the two species of dart frog held at the PARCP, with a view to improving the scarce taxonomic knowledge base for dendrobatid tadpoles. My work resulted in significant improvement in the reproductive output and survival of poison dart frogs at the PARCP and the production of two manuscripts in under two years, demonstrating the importance and feasibility of such studies.

Chapter 2. Two dendrobatid tadpole descriptions from Panama with notes on their eggs (Anura: Dendrobatidae; *Andinobates* and *Oophaga*[1]

Abstract

Dendrobatids (family Dendrobatidae) are a highly diverse group of neotropical frogs whose systematics are complex and unstable. Like most amphibians, dendrobatids are under severe pressure from habitat loss and disease and are in critical need of conservation action, including the generation of up-to-date ecological range data. Tadpole morphology can help resolve phylogenetic questions and so contribute to conservation, but few dendrobatid tadpoles have been described to date. Due to the small size, cryptic behaviour, and remote habitat locations of many species, finding the tadpoles of dendrobatids in the wild can be very difficult. Due to an absence of information, few biologists have attempted to identify free-living dendrobatid tadpoles in the field, limiting the sensitivity of search efforts for species of conservation concern. Likewise, few dendrobatid eggs have been officially described, and the detection of egg masses in the wild as a useful survey tool has not been explored. We describe the eggs and tadpoles of *Andinobates geminisae* and *Oophaga vicentei*. We found that the tadpoles of both species are morphologically indistinguishable from congeneric species and that their morphologies are consistent with existing hypotheses on their systematics and evolution.

Key words: Amphibia, Anura, Dendrobatidae, larvae, tadpoles, eggs, morphology, *A. geminisae, O. vicentei,* Poison Dart Frog, Panama

[1] This chapter is a draft of a manuscript of the same name, authored by K. Higgins and R. Ibáñez, written for the journal *Zootaxa*.

Introduction

The superfamily Dendrobatoidea contains 314 species of Poison-Dart Frog, divided between families Dendrobatidae (194 spp.) and Aromobatidae (120 spp.) on the basis of genetic similarity and the differential ability to produce skin toxins (Frost 2018; Grant *et al.* 2006). Known for their aposematic coloring and complex parental care behaviours, dendrobatids (family Dendrobatidae) have been studied extensively in behavioural ecology, evolution, and diversity, especially to answer questions on the evolution of reproductive behaviours (e.g., Brown *et al.* 2008; Sánchez 2013; Weygoldt 1987; Silverstone 1975; Savage 1968) and color polymorphism (e.g., Richards-Zawacki, Wang and Summers 2012; Rudh, Rogell and Höglund 2007; Wang and Summers 2010). The skin toxins produced by dendrobatids are also of great biomedical interest because of the precise physiological effects of the compounds in their skin (Daly et al. 2000; Myers and Daly 1976).

Family Dendrobatidae is a taxonomically complex and diverse group subject to a great deal of phylogenetic debate. Grant *et al.* (2006) placed Dendrobatidae as a family within the superfamily Dendrobatoidea, along with several major revisions including the division of the genus *Dendrobates* (Silverstone 1975) into 5 smaller genera (*Dendrobates*, *Adelphobates*, *Oophaga* and *Ranitomeya*), the division of genus *Epipedobates* (Myers 1987) into *Allobates, Ameerega*, and *Epipedobates*, and the division of the genus *Colostethus* (Cope 1866) into *Allobates, Anomaloglossus, Colostethus, Hyloxalus, Rheobates*, and *Silverstoneia* (Grant *et al.* 2006, Grant *et al.* 2017). Despite the initial critiques to Grant *et al.*'s 2006 phylogeny, it was eventually accepted by most. Further division of *Ranitomeya* and *Dendrobates* resulted in the creation of new genera *Excitobates* (Twomey and Brown 2008) and *Andinobates* (Brown *et al.* 2011) and, most recently, Grant *et al.* (2017) proposed the creation of two additional genera: *Ectopoglossus* and *Leucostethus*. It is clear there are still many unresolved questions in poison dart frog systematics (Grant *et al.* 2017).

Tadpole morphological traits can be used to hypothesize phylogenetic groupings in dendrobatids (Grant *et al.* 2017; Myers and Daly 1980; Sánchez 2013), but

researchers historically have shown little interest in tadpoles, and tadpole information is scarce. This lack of interest is probably at least in part due to personal preference for more charismatic adults, but also because locating tadpoles can be challenging, especially for species who deposit single tadpoles into distantly separated phytotelmata in logistically and physically challenging environments (Grant *et al.* 2017). We don't have enough knowledge of dendrobatid tadpoles to identify them to species based on morphology; therefore, we rely on witnessing a positively identified nurse frog transporting the tadpole(s) for its' identification. Consequently, tadpole descriptions are usually based on specimens from early developmental stages when tadpoles are being transported (Grant *et al.* 2017; Sánchez 2013). However, tadpoles may not display all taxonomically relevant morphological characters at such early stages (e.g., tail fin size, body proportions and tooth row number (Grant *et al.* 2017). This limits the utility of these specimens in phylogenetic, developmental or evolutionary studies. The ability to morphologically distinguish free-living dendrobatid tadpoles could also aid in the generation of ecological and range data for species of conservation concern by means of (a) determining species presence and mating based on tadpole presence, and (b) locating high density tadpole nursery sites and prioritizing them for protection. Therefore, more complete data on tadpole morphology, from a variety of developmental stages and localities, would facilitate ecological and behavioural studies, contribute to conservation, as well as to the development of new phylogenetic hypotheses for this interesting and highly diverse taxon (Grant *et al.* 2017; Sánchez 2013).

Dendrobatid egg size and morphology represent another knowledge gap. These data are seldom mentioned in the literature. Descriptive data on egg masses is also scarce – descriptions are typically of eggs removed from dissected females and rarely describe more than the color of the preserved eggs after their removal. As with tadpole data, basic information on the size and appearance of different dendrobatid eggs might allow to detect species present in the field without relying on sighting adults. Dendrobatid eggs from a variety of species are available for measurement and description in captive populations - it is just a matter of prioritizing the publication of this information.

The Smithsonian's Panama Amphibian Rescue and Conservation Project houses assurance populations for two species of dendrobatid frogs: *Andinobates geminisae* and *Oophaga vicentei*. As part of a study on tadpole health issues, tadpole deposition, development, and survival from both captive populations have been monitored at this

facility. The frequency of the monitoring allowed us to find and preserve several dead tadpoles from baseline mortality events before their bodies decomposed (usually no more than 24 hours dead before preservation). We took advantage of this opportunity to describe the tadpoles of these two species, using specimens from a variety of life stages. We also provide brief description of the eggs of these two species, along with the size of the eggs and embryos.

The tadpoles of both species were morphologically typical of their genera, and could not be distinguished from their closest congeneric relatives on the basis of the observed and recorded character states. These findings are consistent with the pattern that tadpole morphology is very similar, if not the same, for closely related species, suggesting that tadpole similarity is indicative of evolutionary proximity within this family. We photographed the eggs of both species and used digital imaging software to measure the embryos and eggs, when possible.

2.1. *Andinobates geminisae* Tadpole and Egg Description

Introduction

Originally considered part of the genus *Ranitomeya*, the dendrobatid genus *Andinobates* is fairly new (Brown *et al.* 2011). It currently contains fifteen species, with the most recent species described in 2017 (Brown *et al.* 2011; Frost 2018; Márquez *et al.* 2017). Known members of this genus occur in the forests of Colombia, Ecuador and Panama (Frost 2018). Andinobates geminisae is distinct from other congeneric species because of its uniform orange coloration and its' unique male advertisement call (Batista et al. 2014). The Four species of the genus *Andinobates* are present in Panama: *A. claudiae, A. fulguritus, A. geminisae* and *A. minutus* (Batista *et al.* 2014; Brown *et al.* 2011), *A. geminisae* being the most recently described species (Batista *et al.* 2014). Nothing has been published on the reproductive biology, behaviour or larval morphology of *A. geminisae* since its description in 2014. *A. geminisae* is endemic to a small area in the Caribbean lowlands of Panama (Roberto Ibáñez, pers. comm.; Batista *et al.* 2014), where copper mining operations are being developed. This potential threat creates a pressing demand for more biological information pertinent to the conservation of this species (Batista *et al.* 2014).

Tadpole descriptions are available for seven out of fifteen species of *Andinobates* (Table 2-1). Of these seven descriptions, only three are complete, i.e., they have a sample size greater than one and are not limited to back-riding individuals of stage 25 (Gosner 1960). Of the four *Andinobates* species from Panama, only the tadpole of *A. minutus* has been described, with a complete description in Silverstone (1975). So far, within *Andinobates*, sympatric species cannot be distinguished on the basis of free-living tadpole morphology, but this may be due to a lack of adequate data. For example, free-living tadpoles suspected to be those of *A. altobueyensis* and *A. fulguritus* were found in water-filled leaf axils in Alto Del Buey and Camino de Yupe, Colombia, respectively (Silverstone 1975). These individuals could not be morphologically distinguished from *A. minutus* because *A. altobueyensis* and *A. fulguritus* tadpoles had yet to be described (Silverstone 1975).

Here we describe the tadpoles of *Andinobates geminisae* using n = 40 tadpoles ranging between stages 25-43 (Gosner 1960). Based on this new description, the tadpoles of *A. geminisae* are distinguishable from all other sympatric phytotelm-dwelling dendrobatid larvae on the basis of their size, colour, and features of the oral apparatus, with the exception of the closely related *A. minutus*, which is morphologically identical to *A. geminisae* for the traits examined in this description, but of least concern for conservation, according the IUCN (2018). We also provide a brief description of the size and appearance of *A. geminisae* eggs. This is the first egg description from the freshly laid eggs of a captive female for this genus; the only previous description (Silverstone 1975) was for dissected eggs from wild-caught *A. minutus* females.

Materials and Methods

Tadpoles

As part of ongoing experiments to determine the cause of limb deformities in the Panama Amphibian Rescue and Conservation Project's (PARCP) assurance population of *A. geminisae* between the months of May 2017 and July 2018, we monitored tadpoles for mortality three times a week, and dead individuals were collected and preserved as they were found. All tadpoles were preserved in 10% formalin or ≥ 70% ethanol. We took measurements of body length (BL) and total length (TL) using a Leika dissecting microscope and 1 mm^2 graph paper under a petri dish containing each specimen (n =

40). We examined two additional specimens for comparison from the Círculo Herpetológico de Panamá (CH 3026 and CH 3042, collected from Altos de Campana, and Llano Cartí Rd. in the provinces of Panamá Oeste and Panamá, Panama, respectively). Figure 2-2 is based on Anganoy-Criollo's (2014) presentation of length data for tadpoles. This method of presenting size data effectively demonstrates length variation in each stage and illustrates a general length growth pattern over the course of development. Terminology follows that of McDiarmid and Altig (1999). For labial tooth row formulas (LTRF), we used the "fractional designation" LTRF described in McDiarmid and Altig (1999) and exemplified by Altig (1970). Developmental stages refer to Gosner (1960) and summary statistics of the mean and standard deviation for body and total lengths were calculated using R Statistical Software (R Core Team 2018).

Eggs

Breeding tanks were also monitored for the appearance of new clutches three times weekly. During the last two weeks of work we placed freshly laid eggs in Gosner stages ≤ 3 on laminated 1 mm^2 graphic paper and photographed them before returning them to their original deposition sites for their continued development. The measuring process had no apparent effect on the fertility of these clutches and they continued their development into tadpoles normally. The diameter measurements for the embryos and jelly capsules represent the mean diameter of two diameter measurements that cross at a 90-degree angle. Egg embryo diameter and total egg diameter were measured using Fiji digital imaging software (Schindelin *et al.* 2012).

Results

Tadpoles

The body is oval-shaped and depressed. Eyes and nares are positioned dorsally, oriented dorso-laterally. The spiracle is sinistral and vent tube is dextral. The oral disc is round with a very slight lateral indentation (Figure. 2-1). The posterior labium is lined with a single row of oral papillae that extend the bottom lateral corners of the anterior labium. Dental formula is 2(2)/3[1], although the apparent variation in the presence of a posterior labial tooth row gap may have been due to the loss of denticles in lower quality specimens. The beak is massive and serrate, and the mouth is oriented antero-ventrally.

In 10% formalin, the larvae are greyish brown dorsally and light grey to transparent ventrally. The tail fin is light grey or translucent and nearly reaches the body (Figure. 2-1). Live tadpoles were dark grey to black dorsally, with a lighter grey ventral side. The tail tip is rounded and translucent (Figure. 2-1). Table 2-1 presents this information alongside corresponding morphological information available for 14 other species of *Andinobates* for comparison.

A total of 10 different stages were measured for BL and a total of 8 stages were measured for TL (Table 2-2). Fewer samples were included in total body length measurements because of decomposition or damage to the tail tips. TL ranged between 9.6 and 24.4 mm from stages 25 through 42, respectively (Table 2-2). BL was less variable compared to total length (Table 2-2, Figure 2-2), ranging from 3.4 mm in stage 25 to 7.4 mm in stage 43. The ratio of mean BL to mean TL was conserved between 0.32 and 0.35 mm for all stages sampled (Table 2-2, Figure 2-2).

Eggs

Viable clutches of *A. geminisae* eggs of stage ≤ 3 were examined (n = 16). In our sample, the number of eggs per clutch was one (n = 14) or two (n = 2). While eggs from the same clutch were always in close contact, the translucent jelly capsules surrounding each egg (n = 18) were discrete. The embryos were either solid dark grey in color, or dark grey with varying amounts of light grey at the vegetal pole. Average egg diameter was 6.2 mm (n = 18, SD = 0.853), with a mean embryo diameter of 2.40 mm (n = 18, SD = 0.23) for embryos in Gosner stage ≤ 3.

Discussion

Systematics

A. geminisae and *A. minutus* larvae are the only described species of *Andinobates* that have a complete row of oral papillae along the posterior edge of the oral disc. This feature distinguishes them from *A. abditus, A. bombetes, A. opisthomelas, A. tolimense,* and *A. virolinensis,* all which have a large medial gap interrupting the posterior row of oral papillae. While indistinguishable as tadpoles, *A. geminisae* and *A. minutus* can still be distinguished from each other as adults based on their distinct

coloration and male advertisement calls (Batista et al. 2014). Myers and Daly (1980) first proposed the medial gap in the posterior oral papillae of tadpoles as a synapomorphy, uniting the species possessing it as a subgroup. The condition of the posterior oral papillae is still unknown for *A. claudiae, A. dorriswansonae* and *A. daleswansoni* (Table 2-1), but our description here is consistent with genetic studies based on mitochondrial and nuclear phylogenetic analyses (Batista *et al.* 2014; Brown *et al.* 2011; Márquez *et al.* 2017; Rueda-Almonacid *et al.* 2006; Ruiz-Carranza and Ramírez-Pinilla 1992) that place *A. abditus, A. bombetes, A. daleswansoni, A. dorisswansonae, A. opisthomelas, A. tolimense* and *A. virolinensis* together in the *Andinobates bombetes* species subgroup, and *A. claudiae, A. geminisae, A. minutus* and together in the *A. minutus* species group.

Ecologically and Morphologically Similar Species

A. geminisae is sympatric with three other species of dendrobatid frogs known to deposit their larvae in phytotelmata: *Andinobates minutus*, *Dendrobates auratus* and *Oophaga vicentei*. *A. geminisae* tadpoles are easily distinguished from those of *D. auratus*, because *D. auratus* tadpoles are larger in size, darker in color, and lack the lateral emargination of the oral disc characteristic of all species of *Andinobates* (Brown *et al.* 2011; Silverstone 1975). *A. geminisae* larvae are larger and have a rounder body shape than the much smaller and streamline-shaped tadpoles of *O. vicentei* (KH personal observations of *O. vicentei* tadpoles at PARCP's Gamboa facility). However, the tadpoles of *A. minutus* and *A. geminisae* are morphologically identical based on the characters examined (Table 2-1); therefore, we cannot yet distinguish free-living larvae of these species using morphology alone. The implication of this is that we still rely on the detection of adults to confirm the presence of *A. geminisae* during surveys or search efforts to find *A. geminisae* for ecological studies and conservation efforts.

Eggs

Andinobates minutus eggs taken from the ovary of a dissected female were 3 to 4 mm in diameter (Silverstone 1975), but these had not likely acquired their full size yet. Information on eggs from other congeneric species is missing, except for a brief note of "brown eggs", of unknown stage or state of preservation, from *A. daleswansoni, A. dorisswansoni,* and *A. tolimense* (Bernal *et al.* 2007; Rueda-Almonacid *et al.* 2006).

In conclusion, the morphology of *A. geminisae* tadpoles is consistent with its placement within the *A. minutus* subgroup of the genus *Andinobates*, based on the lateral indentation of the oral disc and uninterrupted row of oral papillae along its posterior edge. This description raises the number of tadpole descriptions in the genus *Andinobates* to seven species, four of which are complete. Descriptions of free-living dendrobatid larvae help make the detection of species based on tadpole presence possible. For example, free-living *A. geminisae* tadpoles are distinguishable from the larvae of other sympatric phytotelma-breeding species of dendrobatid frogs (*D. auratus* and *O. vicentei*) but not from the larvae of their closest relative, *Andinobates minutus*.

2.2. *Oophaga vicentei* Tadpole And Egg Description

Introduction

Species in the genus *Oophaga* (Bauer 1994) are known for their complex mating and parental care behaviours and bright color polymorphisms, and are the focus of many studies on dendrobatid behaviour and evolution (e.g., Richards-Zawacki *et al.* 2012; Stynoski 2012; Wang and Summers 2010). There are currently nine species of *Oophaga* found throughout Nicaragua, Costa Rica, Panama, Colombia and Ecuador (Frost 2018). Prior to the systematic revision of the family Dendrobatidae by Grant *et al.* (2006), *Oophaga* species were placed together as the *Dendrobates histrionicus* species group (Myers and Daly 1976), based on their shared “chirp” vocalization, female parental care, and nutritive egg feeding (Grant *et al.* 2006; Myers *et al.* 1984; Silverstone 1975; Van Wijngaarden and Bolaños 1992). This combination of distinct behavioural traits makes *Oophaga* one of the most well defined genera of dendrobatid existing today (Grant *et al.* 2006).

Tadpole oral morphology is also believed to unite all *Oophaga* species, who share an egg-based diet (Myers 1984, Grant 2006); however, tadpole descriptions are only available for 4 out of nine species of *Oophaga* (Table 2-3). All tadpoles examined to date have enlarged oral papillae and a single row of teeth on the anterior and posterior labia (Myers 1984, Savage 1968; Silverstone 1975; Van Wijngaarden and Bolaños 1992). When present, the anterior tooth row tends to be incomplete - the teeth are few and scattered unevenly, or there is a gap in the middle or either side of the row (Table 2-3). The posterior tooth row is generally more complete, but may have gap in the middle

or either side of the row as well (Table 2-3). These features are specialized for an egg-based diet (Weygoldt 1980, Grant *et al.* 2006, Ryan and Barry 2011), and we expect to observe them in all species of *Oophaga*. Jungfer et al. (1996) provide an account of an *O. vicentei* tadpole riding on the back of a nurse female, and report clutches of two to six eggs, along with colored photographs of eggs and the back-riding tadpole. Here, we add to these findings by describing the tadpole of *O. vicentei*, and also measure and describe the eggs of this species, to increase knowledge on this genus.

Materials and Methods

Tadpoles.

In an effort to improve the reproductive success of an assurance population of *O. vicentei* at the PARCP, we regularly checked various water-filled containers and bromeliads in breeding tanks for the appearance of tadpoles to determine where and what type of objects nurse females prefer to deposit tadpoles in. We found that females preferred to deposit the tadpoles in tank bromeliads, but some tadpoles were found dead. These dead tadpoles were stored in 10% formalin or ≥ 70% ethanol and were examined for this description. While the quality of the specimens was limited due to decomposition, n = 10 specimens were useable for description, including measurements of total length and body length. We examined specimens under a dissecting microscope, placed on top of 1 mm^2 graph paper for measurement. We examined three live specimens were also examined in water under a dissecting microscope to confirm the morphological traits observed in the ten dead specimens. For live specimen examination, we placed tadpoles in a small water-filled petri dish under a dissecting microscope and manipulated specimens with the aid of a plastic pipette. To examine the ventral side of the live specimens, we removed most of the water from the dish and flipped the individual so it lay ventral side up, adhered to the plate with surface tension, for a few seconds of examination. After examination, these tadpoles were returned to the same body of water in their parental breeding tank to complete their development. Terminology for tadpole morphology follows that of McDiarmid and Altig (1999), and developmental stages refer to Gosner (1960). For labial tooth row formulas, we used the “fractional designation” LTRF described in McDiarmid and Altig (1999) and exemplified by Altig (1970).

Eggs

Eggs were placed on 1 mm^2 graphical paper to be photographed, then returned to their original deposition sites to complete their development. We could only measure egg embryo diameter, and not individual egg diameter, because the separation between the jelly layer surrounding each egg and the rest of the egg mass was unclear and could not be separated without damaging them. To measure the embryo diameter, we took the mean of two cross-cutting diameter measurements for each embryo. We only observed eggs in Gosner stages ≤ 3. Embryo diameter was measured using Fiji digital imaging software (Schindelin *et al.* 2012), and summary statistics for tadpole and egg data were obtained out using R Statistical Software (R Core Team 2018).

Results

Tadpoles

The tadpole body is ovate and slightly tapered at the anterior end (Figure. 2-3). Eyes are located dorsally and oriented anterolaterally. Nares are located dorsally. The vent tube is median, and the spiracle is sinistral. The oral disc is round and not indented, with scarce large oral papillae surrounding the posterior labium and the lateral margins of the anterior labium (Figure. 2-3). Based on the sample we observed, the dental formula is 1[1]/1. However, the posterior labial tooth row was absent in all of our dead specimens (probably due to decomposition), and was observed in only one out of the three live specimens that were examined, so there may be variation in the number of posterior denticles or tooth rows. The labial teeth are scarce and large, and the beak is large and serrate. The mouth is oriented antero-ventrally. In ethanol, the specimens are gray in colour from above and below and translucent around the margins of the tail. Live specimens are dark grey to black in colour, with darker speckling on the body and tail, and translucent along the margins of the tail. A total 11 tadpoles of three developmental stages were measured for body length and total length (Figure 2-4, Table 2-4).

Eggs

13 eggs from 5 different breeding pairs were examined in detail. In our sample**,** there was 1 clutch of 4 eggs, 2 clutches of 3 eggs, 1 clutch of 2 eggs (only 1 egg was useable), and 2 clutches of 1 egg, but there may be anywhere between 1 and 12 eggs per clutch for this species, based on past observations. The clutches formed a cohesive transparent mass of eggs. The eggs (n = 13, embryo with jelly coat) measured approximately 3 mm each in diameter, but could not be measured precisely due to the unclear boundaries of the jelly coats. The embryos of eggs in Gosner stages 0-3 had varying amounts of light grey at the vegetal pole, and dark grey at the animal pole, and had an average diameter of 1.6 mm (n = 13, SD = 0.1 mm).

Discussion

O. vicentei tadpoles are indistinguishable from *O. arborea, O. granulifera*, *O. histrionica* and *O. pumilio* for the observed traits. *O. lehmanni, O. occultator, O. speciosa* and *O. sylvatica* tadpoles remain to be described. Tadpoles of *Oophaga* spp. exhibit the typical morphological features of an egg-eating tadpole: an antero-ventrally positioned mouth, reduced tooth rows and tooth number, a large beak, and a long tail (Kishimoto and Hayashi 2017). These morphological features are considered adaptations to an egg-based diet in other groups of anuran larvae (Kishimoto and Hayashi 2017; Kuramoto and Wang 1987; Rowley *et al.* 2012; Vassilieva *et al.* 2013; Wassersug *et al.* 1981), and are believed to be well-conserved in the genus *Oophaga* because they hold a similar adaptive significance. Tadpoles from the genus *Oophaga* can be distinguished from tadpoles from other genera by their tapered egg-shaped body, reduced tooth rows (maximum one anterior and one posterior row) and single row of large, scarce oral papillae lining the entire posterior labium and the lateral regions of the anterior labium. However, we could not distinguish between described tadpoles of the genus *Oophaga* based on morphology alone.

The genus *Oophaga* is united by complex parental care behaviours, including the use of nutritive egg feeding of the tadpoles. All tadpoles of *Oophaga* spp. described to date are morphologically indistinguishable from one another, supporting the notion that this is a well-defined clade of closely related species. Nutritive egg feeding probably led to the evolution of distinct larval morphology in this group, because the tadpoles exhibit

features that are adaptations to an egg-based diet in other anurans. Describing the remaining tadpoles in this genus will further confirm whtether larval morphology is a derived character trait for this group, and will contribute to our overall understanding of dendrobatid frogs and their systematics.

Acknowledgements

This project would not have been possible if it were not for the efforts of the staff, interns, and volunteers of the PARCP (Jorge Guerrel, Nancy Fairchild, Elliot Lassiter, Nair Cabezón, Lanki Cheucarama, Jennifer Warren, Guadalupe Ureña, Orlando Ariel Garcés, and Valeria Franco), who work tirelessly to provide the best possible care for Panama's most vulnerable captive amphibian populations, and who were always willing to lend a hand with the tasks related to this paper. We especially thank Elliot Lassiter for his help photographing specimens, collecting data, and assisting KH with a variety of other tasks related to this project, as well as Jorge Guerrel for staying after hours to help plan data collection and animal care protocols that would facilitate this research. We also want to thank Dr. Arne Mooers for his endless support and encouragement over the entire course of this project and for helping to edit this paper, Dr. Brian Gratwicke for his financial support for this project and Dr. Wendy Palen for making herself available to discuss ideas with KH as she carried out her work in Panama.

Tables and Figures

Table 2-1 **Summary of tadpole descriptions available for genus *Andinobates*, including new data on *A. geminisae*. Tadpole descriptions are not available for *A. viridis, A. victimatus, A. cassidyhornae, A. daleswansoni* or *A. dorisswansoni*.**

*** Silverstone (1975) found free-living tadpoles and suspected them to be from *A. altobueyensis* based on their locality, but he could not confirm whether or not they were *A. minutus* tadpoles (Silverstone 1975).**

****The specimen was thought to be greater than stage 25 because of its size, although no limb buds were visible. This may be due to the poor quality of the specimen, which was dead prior to preservation (Myers and Daly 1976).**

PE = Oral papillae complete on the posterior edge of the oral disc.

AE = Oral papillae complete on the anterior edge of the oral disc.

AE-MG = Oral papillae on the anterior edge of the oral disc, with a medial gap.

PE-MG = Oral papillae on the posterior edge of the oral disc, with a medial gap.

Species	N	Stage	Nares position	Vent tube position	Spiracle position	Eyes position	Mouth orientation	Oral disc shape	Dental formula	Beak	Oral papillae	Source
A. abditus	1	≥25**	Dorso-lateral	-	Sinistral, low	Dorso-lateral	Antero-ventrally	Laterally indented	2(2)/3(1)	Serrate, massive	PE-MG	Myers & Daly 1976
A. altobueyensis	-	-	-	-	-	-	-	-	-	-	-	Silverstone 1975*
A. bombetes	7	25	Dorso-lateral	Dextral	Sinistral, low	Dorso-lateral	Antero-ventrally	Laterally indented	2(2)/3 [1 or 3]	Serrate, massive	PE-MG, lateral AE	Myers & Daly 1980
A. claudiae	-	-	-	-	-	-	-	-	2(2)/3(1)	-	PE-MG	Brown et al. 2011
A. fulguritus	-	-	-	-	-	-	-	-	-	-	-	Silverstone 1975*
A. geminisae	40	Various	Dorso-lateral	Dextral	Sinistral, low	Dorsal	Antero-ventrally	Laterally indented	2(2)/3[1]	Serrate, large	PE, lateral AE	Present study
A. minutus	22	Various	Dorsal	Dextral	Sinistral, low	Dorsal	Antero-ventrally	Laterally indented	2(2)/3	Serrate, massive	PE, lateral AE	Silverstone 1975
A. opisthomelas	25	Various	-	Dextral	-	Dorsal	-	Laterally indented	2(2)/3(1)	Serrate, massive	PE-MG, lateral AE	Silverstone 1975
A. tolimense	1	25	Dorso-lateral	Dextral	Sinistral, low	Dorso-lateral	Antero-ventrally	Laterally indented	2(2)/3	Serrate, massive	PE-MG, AE-MG	Bernal et al. 2007
A. virolinensis	121	Various	Dorso-lateral	Dextral	Sinistral, posterior, & ventral	Dorso-lateral	Antero-ventrally	Laterally indented	2/3	Serrate, large	PE-MG, lateral AE	Ruiz-Carranza & Ramírez-Pinilla 1992

Table 2-2 *A. geminisae* body length (BL) and total length (TL) measurements.

Stage	Body Length (mm)			Total Length (mm)			Mean BL/TL
	n	Range	Mean (SD)	n	Range	Mean (SD)	
25	8	3.4-4.6	3.95 (0.54)	6	9.6-15.4	11.37 (2.50)	0.35
27	3	4.6-6.0	5.33 (0.70)	3	15-17	16.07 (1.01)	0.33
28	3	5.6-7.0	6.20 (0.72)	3	16.8-19.2	18.33 (1.33)	0.34
30	2	6.0	6.00 (0.00)	2	18.2-18.4	18.30 (0.14)	0.32
34	1	7.0	7.00 (NA)	1	22.2	22.20 (NA)	0.33
35	2	7.0-7.6	7.30 (0.42)	2	21.6-23.0	22.30 (0.99)	0.33
36	1	7.4	7.4 (NA)	1	22.6	22.60 (NA)	0.33
37	4	6.0-8.0	7.00 (0.82)	3	20.2-25.0	21.93 (2.66)	0.32
41	8	6.6-8.0	7.23 (0.47)	4	19.2-26.0	21.55 (3.04)	0.34
42	5	6.8-8.0	7.44 (0.43)	3	20.6-24.4	23.07 (2.14)	0.32
43	2	6.4-7.4	6.90 (0.71)	-	-	-	-

Table 2-3 Review of tadpole descriptions available for genus *Oophaga*, including new data on *O. vicentei.* Descriptions are not available for *O. lehmanni, O. occultator, O. speciosa* or *O. sylvatica.*

***One specimen may have had a dental formula of 1[1]/0, but this may have been due to poor specimen quality (Myers *et al.* 1984).**

PE = Oral papillae border the entire posterior edge of the oral disc.

Lateral AE = Oral papillae only border the lowermost sides of the anterior edge of the oral disc.

Species	N	Stage	Nares position	Vent tube position	Spiracle position	Eyes position	Mouth orientation	Oral disc shape	Dental formula	Beak	Oral papillae	Source
O. arborea	>1	28-29	Dorsal	Median	Sinistral	Dorsal	Antero-ventral	Round	1[1]/1[1] or 0/1[1]*	Serrate, massive	PE, lateral AE	Myers, Daly & Martínez 1984
O. granulifera	1	28	Dorsal	-	Sinistral	Dorsal	Antero-ventral	Round	1[1]/1	Serrate	PE, lateral AE	Van-Wijngaarden & Bolaños 1992
O. histrionica	9	25, 33, 35-38, 41	Dorsal	Median	Sinistral	Dorsal	Antero-ventral	Round	1[1]/1	Serrate, massive	PE, lateral AE	Silverstone 1975
O. pumilio	10	25, 26, 28, 41, 42	Dorsal	Median	Sinistral	Dorsal	Antero-ventral	Round	1[1]/1[1]	Serrate, massive	PE, lateral AE	Silverstone 1975, Savage 1968
O. vicentei	11	25, 33, 41	Dorsal	Median	Sinistral	Dorsal	Antero-ventral	Round	1[1]/1	Serrate, large	PE, lateral AE	Present study

Table 2-4 *O. vicentei* body length (BL) and total length (TL) measurements.

Stage	Body Length (mm)		Total Length (mm)	
	n	Mean (SD)	n	Mean (SD)
25	9	2.5 (0.79)	9	8.3 (1.92)
33	1	6.5 (NA)	1	16.0 (NA)
41	1	7.0 (NA)	1	19.8 (NA)

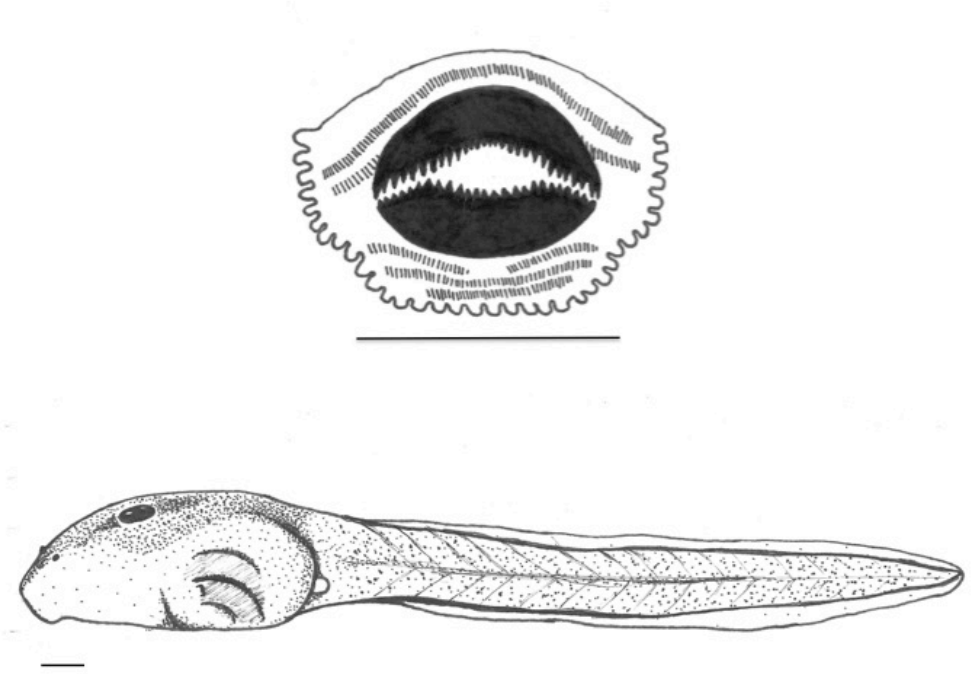

Figure 2-1 Stage 28 *Andinobates geminisae* tadpole mouthparts (above) and left lateral view (bottom). Lines represent 1 mm.

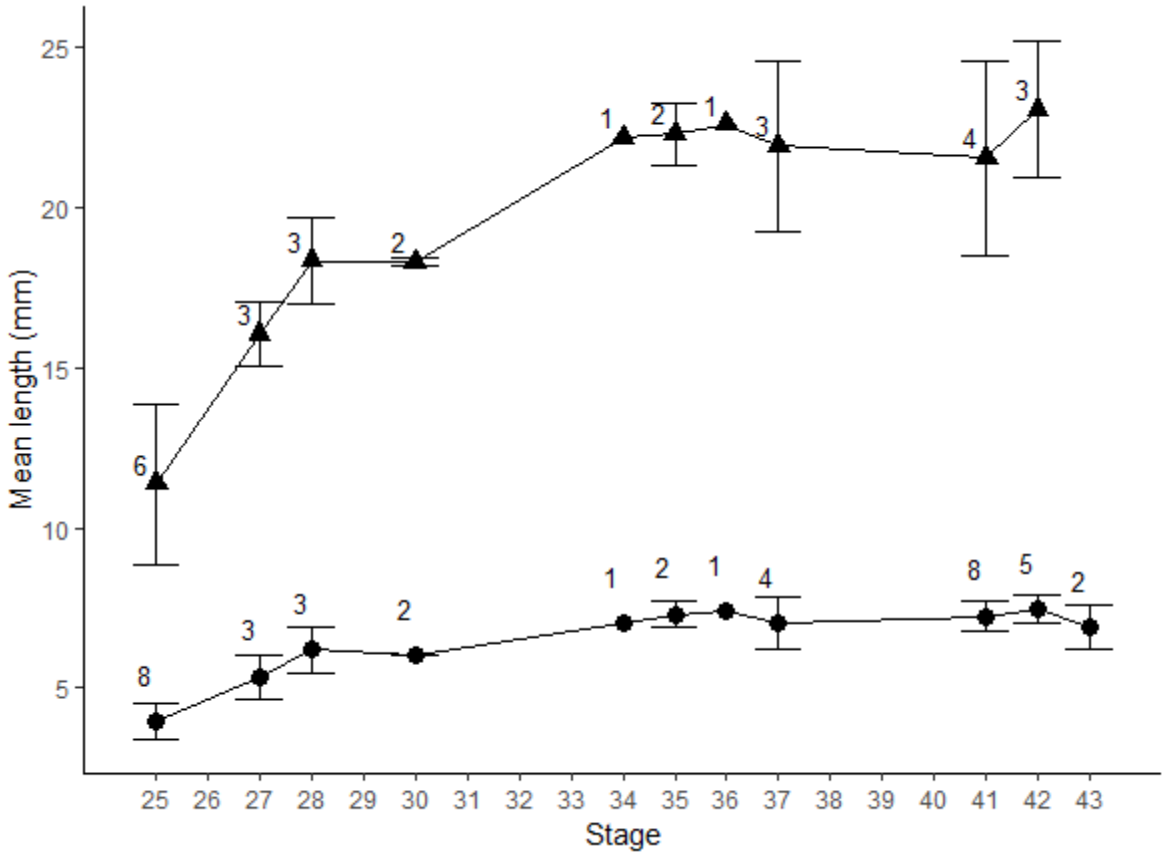

Figure 2-2 **Mean *Andinobates geminisae* total (▲) and body (●) lengths, by tadpole stage. The bars represent the mean ± standard deviation and the numbers next to each point denote sample size.**

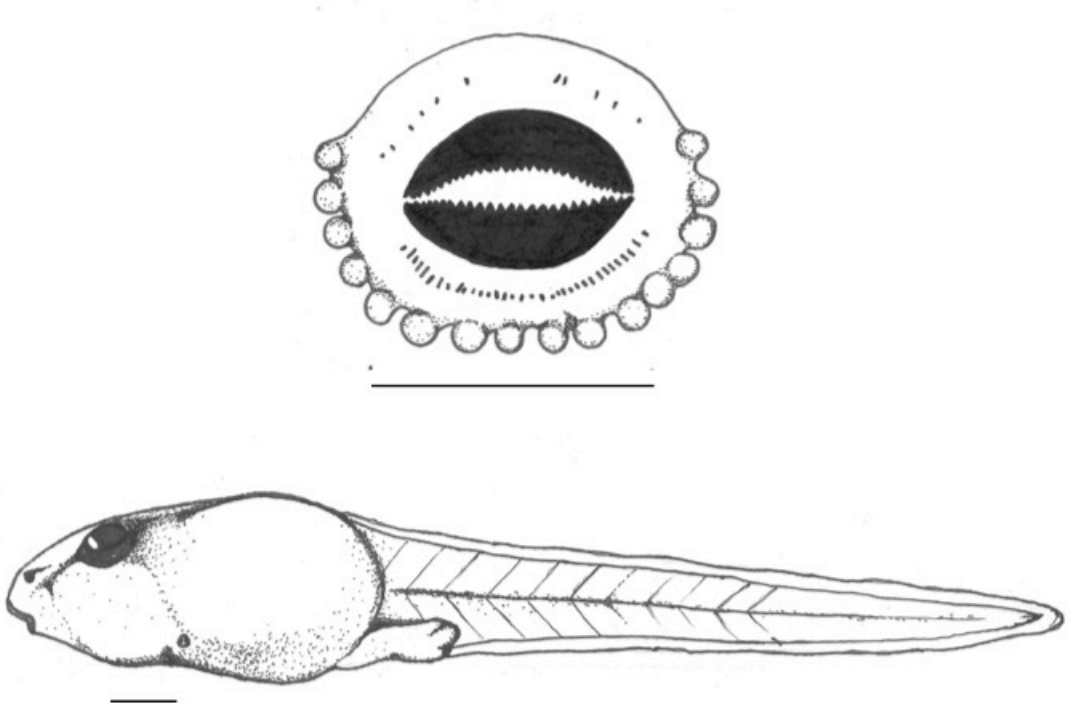

Figure 2-3 Tadpole stage 33 of *Oophaga vicentei*. Oral apparatus (above) and left lateral view (bottom) of tadpole. Lines represent 1 mm.

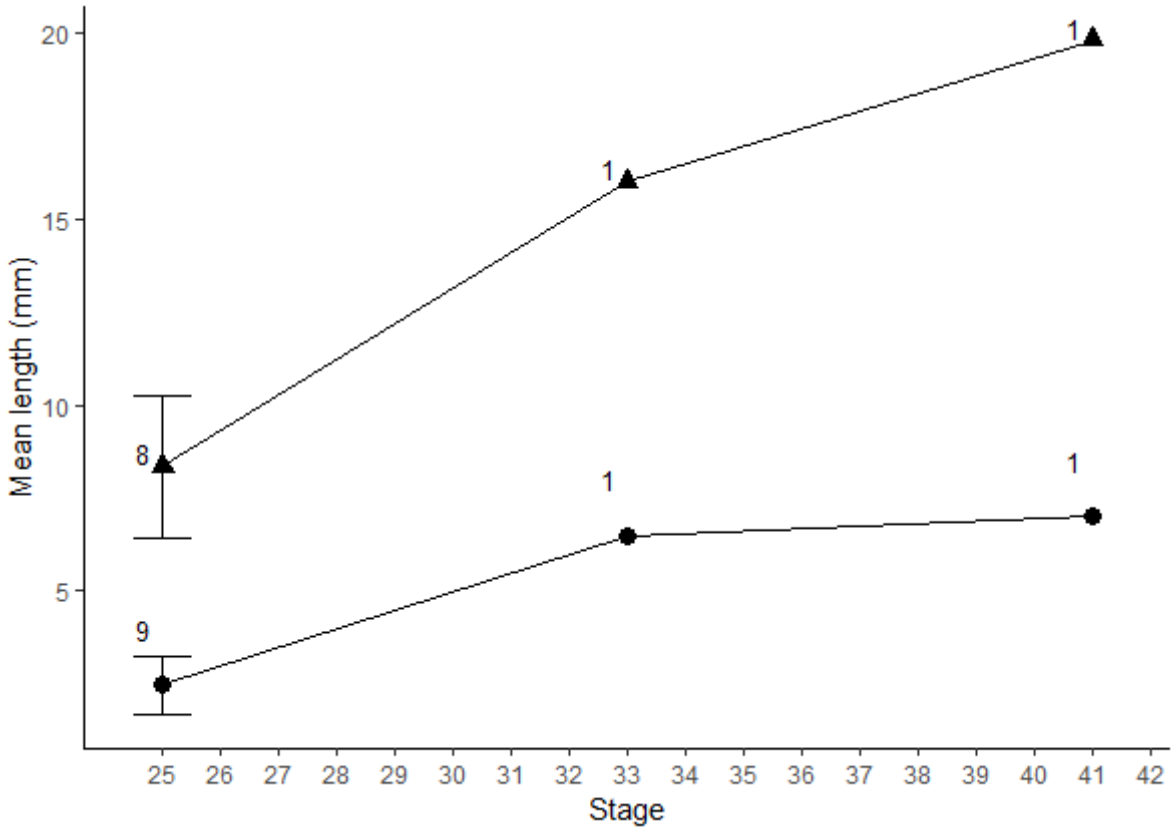

Figure 2-4 **Mean *Oophaga vicentei* total (▲) and body (●) lengths, by tadpole stage. The bars represent the standard deviation of the mean, and the numbers beside each point denote sample size.**

Chapter 3. Observations on Spindly Leg Syndrome in a Captive Population of *Andinobates geminisae*[2]

Abstract

Amphibian health problems of unknown cause may constrain the success of amphibian conservation captive breeding programs, and determining the causes of these health problems is critical as the number of amphibians brought into conservation captive breeding programs continues to rise. Spindly Leg Syndrome (SLS) is an amphibian limb developmental disease of unknown etiology. Affected individuals have underdeveloped limbs and oftentimes must be euthanized due to resulting immobility.. The following describes our work on isolating tadpole husbandry-related factors of SLS in a captive population of *Andinobates geminisae,* a tiny and critically endangered poison dart frog currently being bred at the Smithsonian's Panama Amphibian Rescue and Conservation Project (PARCP), Gamboa, Panama. The filtration method, vitamin B content, and quality of tadpole rearing water, as well as tadpole nutrition and husbandry, are considered factors in the occurrence of SLS according to hobbyist, zookeeper, and veterinary media, but few of these factors have been experimentally tested. We ran two experiments between July-December 2017 and March-August 2018 to test the effects of tadpole rearing water filtration and vitamin supplementation (2017) and tadpole husbandry protocol intensity (2018) on time to metamorphosis and SLS prevalence in emerging metamorphs*.* In our first experiment, we found that the vitamin content and filtration method of tadpole rearing water had no effect on the metamorphosis time of tadpoles, but our results regarding SLS were inconclusive. Our second experiment revealed that decreased tadpole husbandry (higher tadpole feeding and lower water change frequency) significantly delayed the metamorphosis time of tadpoles, but had no effect on SLS development or tadpole survival. A fortuitous accident resulted in the manipulation of the phosphate content of the tadpole rearing water and we were able to correlate the amount of time each tadpole had available to sequester calcium for ossification during metamorphosis and decreased incidence of SLS. This suggests that low calcium availability may lead to SLS.

[2] This chapter is a draft of a manuscript of the same name, authored by K. Higgins, J. Guerrel, E. Lassiter, A. Mooers, W. Palen, and R. Ibáñez, to be submitted to the journal *Zoobiology*.

Key words: Spindly Leg Syndrome/SLS, tadpole, metamorphosis, bone development, captive breeding, Anura, Dendrobatidae, *Andinobates geminisae,* Amphibian health, Amphibian captive husbandry, Amphibian calcium metabolism, phosphate contamination.

Introduction

40% of all amphibian species are threatened with extinction, one of the highest proportions across terrestrial vertebrate Classes (Scheele et al., 2019; IUCN 2018; Gratwicke et al., 2012; Hoffmann et al., 2010; Stuart et al., 2004). Amphibian population declines are caused by a variety of anthropogenic threats including habitat loss, non-native species introductions, and pollution. However, many of the rapidly declining species are undergoing declines (over 40% of cases in 2005: Stuart et al., 2004) in areas of protected or even pristine habitat. For these cases, it is suspected that disease and/or climate change are responsible for the observed declines (Scheele et al., 2019; Stuart et al., 2004). Amphibians are therefore in critical need of conservation action that goes beyond regular habitat protection measures, and conservation captive breeding is one important management component (Claunch & Augustine, 2015; Mendelson et al., 2006; Stuart et al., 2004). Amphibians are said to be amenable to captive breeding due to their small size and tolerance to the artificial conditions of captivity (Biega, 2017; Conde et al., 2015; Balmford et al., 1996; Bloxam & Tonge, 1995). Despite this alleged amenability, the success rate of amphibian captive breeding programs in producing viable offspring is only 52% (Biega, 2017). This low success rate is at least partially due to health problems resulting from our inadequate knowledge of the environmental and nutritional needs of species held in captivity (Ferrie et al*.,* 2014).

Spindly Leg Syndrome (SLS), also known as “match legs” or “skeletal and muscular underdevelopment (SMUD)” (Hakvoort et al., 1995), is a developmental disease of amphibians characterized by the deformation and/or underdevelopment of the limbs during the final stages of metamorphosis (Camperio Ciani et al., 2018; Claunch & Augustine, 2015; Hakvoort et al., 1995; Marlett et al., 1988). If not euthanized, metamorphs with SLS usually drown or die of starvation because they are immobile (Claunch & Augustine, 2015; Wright & Whitaker, 2001; Marlett et al., 1988). SLS is most heavily documented in poison dart frogs (Dendrobatidae), but has also been reported in a wide variety of other anuran taxa that also have free-swimming tadpoles as a developmental stage, e.g. *Bombina, Phyllomedusa, Discoglottus* (Hakvoort et al., 1995), *Mantella* (Claunch & Augustine, 2015), *Atelopus, Hyloscirtus* (Camperio Ciani et al.,

2018) and one salamander species (*Tylototriton shanjing*) (Wright & Whitaker, 2001). There are many putative cures for SLS circulating in zoo, veterinary, and hobbyist circles, but experimentally tested knowledge on the etiology of this disease is scarce (Table 3-1). SLS is a major problem for captive management of amphibians as it may cause the loss of up to 100% of captive born offspring in severe cases (Claunch & Augustine, 2015; Marlett, Eichler, & Fellows-Hagemeister, 1988). As the number of amphibian conservation captive breeding programmes increases (Harding, Griffiths, & Pavajeau, 2016), reducing the incidence of SLS is critical for improving the success of of ex-situ conservation.

Here we evaluated the efficacy of three environmental factors in reducing the incidence of SLS using the critically endangered frog, Andinobates geminisae, a species at the focus of ex-situ conservation effort at the Panama Amphibian Research and Conservation Project (PARCP) in Gamboa, Panama. We conducted a pilot-scale experiment in 2017, and a complete experiment in 2018 to identify factors associated with SLS incidence in newly metamorphosed frogs. During the pilot scale experiment, we tested the factors of tadpole water filtration method, vitamin B supplementation, and vitamin content. The only published treatment of SLS to date is by Wright & Whitaker (2001), who state that adding VEDCO® vitamin B supplement (containing vitamins B1 through B3, B5, B6, and B12) to tadpole rearing water at a dosage of 1mL per gallon will “drastically reduce” SLS in an afflicted population, based on anecdotal observations of the authors. Another popular theory is that SLS is caused specifically by a deficiency in folic acid (vitamin B9). While the link between folic acid and deformities has never been tested in frogs, the fact that folic acid deficiency in pregnant humans can lead to congenital limb deformities (Cleves et al. 2011) and the spread of anecdotal stories has led to somewhat widespread acceptance of this hypothesis. We tested the role of tadpole water vitamin supplementation on SLS during the first pilot scale experiment through vitamin B complex and folic acid treatments, with the hypotheses that (1) SLS is caused by a deficiency in any or all of the B vitamins contained in vitamin B complex and (2) that SLS is caused specifically by a deficiency in folic acid.

A recent study by Camperio Ciani et al. (2018) on SLS in *Atelopus glyphus* tadpoles found that the use of reconstituted reverse osmosis water, rather than carbon filtered water, for tadpole rearing significantly reduced the prevalence of SLS in emerging metamorphs, suggesting that the chemical composition of the tadpole rearing water may be an important factor in the occurrence of SLS. We included water filtration method as a second factor during our pilot experiment to see if Camperio Ciani et al.’s (2018) findings were applicable to *A.*

geminisae, with the hypothesis that raising tadpoles in reconstituted reverse osmosis water instead of carbon filtered water reduces the prevalence of SLS in *Andinobates geminsiae*.

Our second full-scale experiment tested the novel hypothesis that tadpole husbandry intensity (feeding frequency, food allotment, and cleaning frequency) is a key factor in the occurrence of SLS. This hypothesis was based on the observation that SLS prevalence decreased from 68% in *A. geminisae* metamorphs at the PARCP in 2016 and early 2017 to just 3% when more intensive tadpole husbandry protocols were implemented for the first pilot scale experiment.

Materials and Methods

Study Species

Andinobates geminisae is a critically endangered, tiny (<14mm adult snout-to-vent length) species of dendrobatid frog endemic to a small area in the Donoso region of the province of Colón, Panama currently adjacent to a copper mining concession area (IUCN SCC, in press; Batista et al., 2014). The Panama Amphibian Research and Conservation Project (PARCP) initiated captive assurance populations of *A. geminisae* in 2013 in response to concerns about impacts of chytridiomycosis and the potential loss of habitat.

General Husbandry

Tadpoles originating from six different breeding pairs were used in this experiment. Each breeding pair and their tadpoles were housed in their own 20 gallon breeding tank, where the adults were fed a standardized diet of cricket nymphs that were gutloaded on an alternating schedule with Mazuri® Hi Calcium Gut Loading Diet. Breeding tanks were maintained at (24-25)°C**,** 12:12 hours dark:light, and (89-100)% relative humidity, and were misted with carbon-filtered water for 5 minutes every 3 hours. Our cleaning schedule ensured that the amount of time between cleanings was the same for all tanks. We maintained tadpoles within each of their parental breeding tanks, instead of in a common garden, as a cautionary measure to prevent the loss of the entire tadpole population should an accident occur where they were kept.

To facilitate breeding behaviour, each breeding tank was fitted with a standardized set of objects to serve as oviposition or tadpole deposition sites (Figure 3-1). Between July 6th and October 2end 2017 (experiment 1) and March 4th and May 16th 2018 (experiment 2), *A. geminisae* females laid clutches of 1-3 eggs in capped PVC pipe (a preferred terrestrial egg-deposition sites (Figure 3-1) and the males transported hatched tadpoles to one of several water-filled petri dishes or bromeliad wells for development (Figure 3-1). We surveyed the breeding tanks three times weekly for clutches and tracked their development. Using this system, we could predict when an egg was ready to hatch and find the tadpole - either on a nurse male's back or in one of the tadpole deposition locations. If we saw a tadpole being transported by a male, we waited for the male to deposit the tadpole into a body of water before collecting it. This method of collection allowed us to collect all tadpoles within 3 days of hatching. We used a new disposable plastic pipette to move each tadpole from their original pool to enclosed petri dishes for further development. We raised tadpoles individually in closed-lid petri dishes filled with 12mL of water, placed in the front area of the breeding tank from which they originated (Figure 3-1). We kept 22 numbered petri dishes for tadpole rearing at the front of each breeding tank at all times, regardless of whether or not they were used in the experiment (Figure 3-1). In the first experiment, tadpoles were fed 0.005 ± 0.002g crushed Tetramin® fish flakes and given 50% water changes three times weekly. In the second experiment, feeding and water change protocol varied by treatment.

Experiment 1

To determine if filtration method or vitamin B content of tadpole-rearing water affected SLS prevalence, survival to metamorphosis, or development, we raised n = 97 *A. geminisae* tadpoles in one of four different water treatments: (1) reconstituted reverse osmosis water, (2) carbon filtered water, (3) carbon filtered water with Vedco® vitamin B complex added at a concentration of 300 ppm, or (4) carbon filtered water with folic acid added at a concentration of 417 ppm. We used a partial, rather than full factorial design because our sample size was limited to the number of tadpoles we could collect from six breeding pairs in four months. The concentrations of vitamin B complex and folic acid were calculated using Wright and Whitaker's (2001) recommended dosage of vitamin B complex for the prevention of SLS. For each treatment group, we compared the survival to metamorphosis, metamorphosis time (measured as the number of days from birth to the emergence of both forelimbs ± 2 days), and SLS prevalence in young metamorphs.

We assigned treatments to the tadpoles by a random draw, but without replacement, so that once a treatment was assigned to a tadpole, it was removed from the draw and could not be drawn again until all the other treatments had been assigned to a tadpole once in that tank. This method ensured there were approximately equal numbers of tadpoles in each treatment group and decreased the likelihood that tadpoles from the same clutch would be placed into the same treatment group. We replaced the carbon-filtered water that the tadpoles were initially placed in with treatment-specific water gradually during 50% water changes three times weekly using a 10mL plastic syringe for each tank. The position of each tadpole's dish was randomized three times weekly to control for any effects of within-tank dish position.

Experiment 2

Design and Rationale

During our first experiment, we witnessed a dramatic decrease in the prevalence of SLS from 68% in 2016 and early 2017 to just 3% during the six month experiment (see Results), indicating that an important disease factor had changed. Two possible SLS factors were knowingly changed during the first experiment: tadpole feeding and water change frequency were increased, and the diet of the breeding adults was changed from cricket nymphs twice a week and springtails once a week, to cricket nymphs three times a week (this change was implemented due to a shortage of springtails). Following the results of the first experiment, we chose to focus on the effects of the changed tadpole husbandry protocol on SLS because tadpole feeding frequency and allocation have direct impacts on water quality, and water quality is suspected to play an important role on tadpole health and development (Álvarez & Nicieza, 2002 ; Browne et al., 2003), and to be implicated in the occurrence of SLS (Wright & Whitaker, 2001).

We therefore ran a second experiment between March 4th and August 16th 2018 to determine if tadpole husbandry (feeding frequency, food allotment, and cleaning frequency) was the key factor in the near-disappearance of SLS during the first experiment. We predicted that tadpoles raised in carbon filtered water (the water used prior to the first experiment), under the same intensive feeding and cleaning protocols instituted for the first experiment, would have a significantly lower SLS prevalence than the pre-experiment baseline, perhaps as low as 18%, based on results from the carbon filtered water treatment group from the first experiment (see Results). In contrast, experimentally reverting tadpole feeding and cleaning procedures to the

original, less intense PARCP protocol was expected to increase SLS prevalence significantly, based on the 58% historical levels of SLS in *A. geminisae* at the PARCP.

To test if changing tadpole husbandry affects tadpole health or development, we raised n = 62 *A. geminisae* tadpoles under one of two husbandry treatments: the intensive husbandry treatment and the medium husbandry treatment (Table 3-2). Our response variables were tadpole survival, metamorphosis time, and SLS prevalence among metamorphs. Metamorphosis time was defined as the number of days it took each tadpole to reach metamorphic climax, marked by the emergence of the first forelimb (Etkin, 1964; Pilkington & Simkiss, 1966).

We flipped a coin to assign a husbandry treatment (intensive or medium) to the first tadpole collected from each breeding tank, and treatments were then alternated for each subsequent tadpole. This method of treatment assignment created complementary treatment pairs of tadpoles with similar birth dates and ensured that there were approximately equal numbers of tadpoles in each treatment group. The position of each tadpole's dish in the front of the tank was randomized once weekly to control for any effects of within-tank dish position. Water changes followed the same protocol as experiment 1, using only carbon filtered water. The frequency of these changes was three times weekly (intensive husbandry protocol) or once every two weeks (medium husbandry protocol) (Table 3-2). Tadpoles in the medium husbandry treatment group had the same 6mL of dirty water removed and replaced three times weekly to control for disturbance effects. We checked each tadpole for mortality or the appearance of forelimbs during water changes. Tadpoles in the intense husbandry protocol group were fed 0.003 ± 0.002 g crushed Tetramin® fish flakes three times a week, and tadpoles in the medium husbandry protocol group were fed 0.005 ± 0.002 g crushed Tetramin® fish flakes once a week (Table 3-2).

Water Sampling Protocol and Water Chemistry Analysis:

Every 2 weeks we tested wastewater (6mL, removed during water changes) from the first 2-3 tadpoles added to each treatment group within each tank for ammonia (using a Hanna checker H1700), phosphates (using a Quantofix® 91320), pH (using a Macherey-Nagel 92110), total dissolved solids (using a Hanna® Instruments GroLine HI98131), conductivity (using a Hanna® Instruments GroLine HI98131), nitrites/nitrates (using a Hach® 2745425), and total hardness (net dissolved calcium and magnesium hardness, represented as ppm $CaCO_3$

- using a Hach® 1194T51EA). This method of sampling created complementary treatment pairs of tadpoles of approximately the same age within each tank. To control for effects of tadpole age on wastewater chemistry, we analyzed the water chemistry of tadpoles in four age class groups (0-25, 25-50,50-75, and 75-100 days old). We informally compared the wastewater chemistry of tadpoles from each husbandry treatment group by examining plots of the mean and 95% confidence intervals of each parameter. Differences in parameters were considered significant if the 95% confidence intervals from each treatment group did not overlap. A multi-parameter analysis (using a Hach® DR/890*,* YSI Professional Plus multiparameter instrument) of the source water used for tadpole rearing was run on April 26th, March 5th, May 24th, and July 26th 2018 to get an overview of the chemistry of the water used in the experiment (Figure 2). For the purposes of discussion, we also measured phosphate and calcium content of filtered tadpole-rearing water before and after it underwent phosphate removal in July 2018 (see Statistics and Data Visualization: accounting for phosphate exposure).

Statistics and Data Visualization.

We used generalized linear (experiment 1) or generalized linear mixed effects (experiment 2) models (GLM or GLMERs) to assess the effects of water type (experiment 1) and husbandry treatment (experiment 2) on metamorphosis time, survival, and SLS using the lme4 package (Bates *et al.* 2015) in R **(**R core team 2018**)**. We controlled for possible effects of parental genetics or tank environment by including the fixed (experiment 1) or random (experiment 2) effect “tank.Id” in our models (sample size was too small to include random effects in the first experiment) . Models were compared using Aikake’s Information Criterion, corrected for small sample sizes (AICc). We considered a predictor to be statistically significant if its coefficient did not include 0 in its 95% confidence interval. For experiment 2, we generated full model averaged coefficients using the MuMIn package (Bartón 2018) following the recommended methodology of Grueber et al. (2011). All figures were generated using base plot functions or the ggplot2 package (Wickham 2016) in R (R core team 2018).

Accounting for Phosphate Exposure:

Halfway through the second experiment, we discovered that the phosphate reactor used to remove excess phosphates from the tadpole rearing source water was malfunctioning, which

introduced the new variable of phosphate exposure that needed to be accounted for in our analyses.

Time periods of high phosphate exposure may represent time periods that tadpoles could not access dissolved calcium, thereby shortening the window of time tadpoles had to store the calcium they needed for limb bone development during metamorphosis. This could have influenced the occurrence of SLS (see Discussion – Phosphate Exposure).

We could not include measured phosphates in our models because only a subset of tadpoles had their water sampled for phosphates, but we were able to approximate phosphate-dependent calcium storage time of each tadpole by creating the calcium (Ca^{2+}) sequestering time variable. Because tadpoles are known to store calcium in specialized sacs during the growth period after hatching until just prior to metamorphic climax (Pilkington & Simkiss, 1966), Ca^{2+} sequestering time, measured as the time difference (in days) between the repair of the phosphate reactor and metamorphic climax (Equation 3-1) represents the amount of time tadpoles had to store calcium while uninhibited by potentially high phosphates in the water. We included Ca^{2+} sequestering time as a fixed effect in our models of SLS and of metamorphosis time in response to husbandry treatment, with the random effect of tank Id. We also modelled SLS in response to Ca^{2+} sequestering time with husbandry treatment and breeding tank Id included as random effects.

The biologically-relevant variable Ca^{2+} sequestering time could not be used in models of survival to metamorphosis because its' calculation depends on the emergence of forelimbs. To account for the effects of phosphate exposure in our analysis of the effects of husbandry treatment on tadpole survival to metamorphosis, we considered a closely related variable, % PO_4^{3-} exposure, measured as the percent of the total lifetime of each tadpole that was spent in potentially high phosphate water before the phosphate reactor was repaired (Equation 3-2). Ca^{2+}sequestering time and % PO_4^{3-} exposure are strongly correlated across tadpoles ($r = -0.86$, $n = 45$). % PO_4^{3-} exposure was treated in the same way as Ca^{2+} sequestering time: as a fixed effect in models of survival in response to husbandry treatment, with the random effect of tank id.

We centered and scaled both Ca^{2+} sequestering time and % PO_4^{3-} exposure by the mean and two standard deviations, respectively, using the recommended methodology of Schielzeth (2010) and Gelman (2008). This allowed us to compare binary, continuous, and interaction coefficients in the same space of the mean (equal to 0) ± two standard deviations.

Water quality data of the source water used during the second experiment was not designed to capture the effects of a broken phosphate reactor, so measurements of calcium and phosphate from this time period are scarce. However, we were able to directly test the phosphate reactor by measuring the phosphate and calcium of the source water used for tadpole rearing before and after passing through the repaired phosphate reactor during July 2018. Using these data, we could estimate the impact the broken phosphate reactor would have had on phosphate and calcium levels in the source water during the experiment, with the assumption that the water chemistry effects of the phosphate reactor do not change over time. Mean calcium and phosphate values before and after phosphate removal were considered to be significantly different if the 95% confidence intervals of their means did not overlap.

Results

Experiment 1

A total of 97 tadpoles were born during experiment 1, and 10 died before metamorphosis. There was no effect of water treatment or breeding tank on survival to metamorphosis (Table 3-4). This is reflected in the results of our AICc analysis, which showed that the inclusion of breeding tank and/or treatment did not improve the fit of models compared to the null model (Table 3-3). Mean metamorphosis time for surviving tadpoles was 58.30 days across all treatments ($n = 87$, $\sigma = 4.6$), and there was no effect of either water treatment or breeding tank on the number of days it took for tadpoles to reach metamorphosis (Figure. 3-2); All models that include tank and/or treatment ranked lower than the null model by $\Delta AIC > 3$ (Table 3-3). Only five out of out of the 87 surviving tadpoles exhibited SLS. Four out of these five SLS tadpoles were raised in carbon-filtered water and one was raised in reconstituted reverse osmosis water, and all five SLS tadpoles came from three of the six breeding pairs (Figure 3-3). The number of SLS cases was too low to statistically disentangle the effects of breeding tank and water treatment. While the null SLS model did not have the lowest AICc score (Table 3-3), neither breeding tank nor water treatment were statistically significant predictors of SLS, with their coefficient confidence intervals including zero across all models.

Experiment 2

Wastewater from tadpoles reared using the medium husbandry treatment had significantly higher phosphates, nitrates, and nitrites in two out of three age class comparisons,

and significantly higher total dissolved solids in all age classes, based on the discrete 95% confidence intervals of each treatment group's mean (Figure 3-4). The medium husbandry treatment had a significant positive effect on tadpole metamorphosis time (Figure 3-5: medium treatment 95% CI: 0.35, 0.50, Table 3-5); Tadpoles in the medium treatment group took ~30 days longer to metamorphose ($\bar{x}$ = 93, n = 21, σ = 15) than tadpoles in the intensive husbandry treatment group ($\bar{x}$ = 60, n = 24, σ = 4, Figure 3-7, Figure 3.15). There was no apparent difference in the metamorphosis time of tadpoles that developed SLS compared to tadpoles that developed healthy limbs (Figure 3-8) and tadpole survival in the two husbandry treatments was similar (Figure 3-6, Table 3-6).

Neither Ca^{2+} sequestering time nor husbandry treatment had a significant effect on the development of SLS when SLS was modelled in response to fixed effects of Ca^{2+} sequestering time and husbandry treatment (Figure 3-5, Table 3-5). However, Ca^{2+} sequestering time had a relative importance value of 1 in the full-averaged model (Figure 3-5). When we analyzed the effects of Ca^{2+} sequestering time on SLS with husbandry treatment and breeding tank included as random effects, Ca^{2+} sequestering time had a sizeable negative effect on SLS development (Table 3-7, Figure 3-9: 95% CI of the log-odds ratio SLS given a one-unit increase in Ca^{2+} seq. time : -137.03, -8.40). A one-unit change in Ca^{2+} sequestering time is 50 days, and, as can be seen from Figure 3-10, the likelihood of a tadpole developing SLS sharply decreased with increasing calcium sequestering time when tadpoles had ≥ 10 calcium sequestering days.

Figures 3-11 through 3-14 present an overview of the chemistry of the water used in the second experiment. Tadpole rearing water tested before the phosphate reactor was known to be broken had a phosphate level of ~0.4ppm PO_4^{3-}, which decreased to 0 - 0.04 ppm PO_4^{3-} after the phosphate reactor was repaired (Figure 3-12). Dissolved calcium levels seemed to be unaffected by the phosphate reactor function, varying between 15.6 and 20.8ppm $CaCO_3$ (Figure 3-12) over the course of the experiment. When we directly tested the phosphate reactor in July, we found that it significantly reduced the phosphate concentration of filtered source water from 0.54ppm PO_4^{3-} (n = 9, σ = 0.45) to 0.04ppm PO_4^{3-} (n = 6, σ = 0.03, Figure 3-15: non-overlapping CIs), while the calcium concentration remained constant (Figure 3-13). The estimates of the phosphate to calcium ratio before and after phosphate removal are consistent with estimates taken before and after the repair of the phosphate reactor during the experiment (Figure 3-12). Phosphate measured from individual tadpole wastewater fluctuated between 0

and 100 mg/L over the course of the experiment, while calcium hardness stayed at 120mg/L (Figure 3-14).

Discussion

Combined, both experiments reveal that tadpole husbandry practices affect different aspects of tadpole health and development to varying degrees, and that these effects may be mediated through changes in the chemistry of tadpole rearing water. First, I discuss the effects of vitamin supplementation and water filtration method tested in experiment 1, followed by a discussion of the effects of husbandry intensity tested in experiment 2. Lastly, I discuss our *post hoc* analysis of the effects of phosphate exposure/calcium sequestering time on SLS and introduce a new candidate hypothesis on the etiology of SLS.

Experiment 1

Neither water filtration method nor vitamin supplementation had an effect on tadpole metamorphosis time, which was between 47 and 77 days across all treatments (Figure 3-2). This result is consistent with preliminary PARCP data noting an average metamorphosis time of about 58 days for *A. geminisae*. Both the five tadpoles that had SLS and the 87 tadpoles that were healthy had a mean metamorphosis time of 58 days; there is no suggestion of a link between SLS and the development time of tadpoles from this experiment.

Of the five SLS cases, four were from carbon-filtered water and one was from reconstituted reverse osmosis water (Figure 3-3). This pattern is consistent with Camperio Ciani et al. (2018), who found that SLS was drastically reduced in *Atelopus glypus* tadpoles that were raised in reconstituted reverse osmosis water instead of carbon-filtered water. Another similarity between Camperio Ciani et al. (2018) and the present study is that SLS was reduced to very low levels, but not eliminated, in reconstituted reverse osmosis water. In our experiment, only one (~4% of all RO tadpoles) had SLS, compared to < 2% of all reconstituted reverse osmosis tadpoles in Camperio Ciani et al. (2018). The similarity between our results and those of Camperio Ciani et al. (2018) suggests that there may be a consistent pattern between SLS and water filtration method indicating that water composition is likely an underlying factor in the occurrence of SLS. However, the specific elements involved are still not known (Camperio Ciani et al. 2018). None of the 42 tadpoles raised in carbon-filtered water supplemented with vitamin B complex or folic acid exhibited SLS, whereas four out of the 21 tadpoles raised in carbon

filtered water had SLS. This suggests that folic acid or other B vitamins may have offset any negative effects carbon-filtered water on tadpole development. The role of vitamins on tadpole development and SLS merits further experimental work with increased statistical power.

Experiment 2

Main Results

Metamorphosis time was significantly longer for tadpoles in the medium husbandry treatment group (Figure 3-5; Figure 3-7). We cannot conclude if this effect was due to differences in feeding frequency or water change frequency since both factors were manipulated, but decreased tadpole feeding has been shown to decrease tadpole metamorphosis time across multiple studies (Griffiths et al., 1993; Hota & Dash, 1981; Murray, 1990), whereas the water quality parameters that differed between our treatment groups have no known clear effect on tadpole metamorphosis time (Egea-Serrano et al., 2012; Marco & Blaustein, 1999). Regardless of the mechanism, this finding provides vital information to project managers who are responsible for allocating limited staff hours to the husbandry of amphibian populations in conservation breeding facilities; in the case of *A. geminisae*, increasing husbandry intensity decreases the metamorphosis time of tadpoles by approximately one month.

42% of tadpoles in the medium husbandry treatment died before metamorphosis vs. 25% of tadpoles in the intensive husbandry treatment, and 14% of tadpoles from the carbon-filtered treatment of experiment 1. Tadpole water in the medium husbandry treatment also had elevated levels of nitrite and ammonia (Figure 3-4), which are known to negatively impact amphibian larvae survival (Egea-Serrano et al., 2012; Huey & Beitinger, 1980; Odum & Zippel, 2011). However, the difference in survival we observed was not significant (Table 3-6, Figure 3-6: medium treatment 95% CI: -1.77, 0.69). One explanation for this is possible ecological adaptation of dendrobatid tadpoles to withstand elevated levels of and nitrites and ammonia. The toxicity of nitrogenous compounds to amphibians has been shown to vary greatly between species (Odum & Zippel 2011). For example, typical nitrite concentration of bromeliad water containing wild *Ranitomeya amazonica* tadpoles is about 10ppm (Poelman et al., 2013), while general amphibian husbandry guidelines warn against nitrite levels exceeding 0ppm (Wright & Whitaker 2001). Five tadpoles in the intensive husbandry treatment developed SLS vs. three tadpoles in the medium husbandry treatment. This ran contrary to our expectation that SLS would be significantly higher in the medium husbandry treatment group, but can be explained as

due to the delayed metamorphosis date of tadpoles in the medium husbandry treatment group relative to the date the phosphate reactor was repaired: tadpoles in the medium husbandry treatment group had longer calcium sequestering times (see Discussion: Phosphate Exposure).

Phosphate Exposure

Because husbandry treatment affected metamorphosis time (see Results), Ca2+ sequestering time is weakly correlated with husbandry treatment ($r = 0.52$, $n = 45$) such that tadpoles in the medium husbandry treatment had longer calcium sequestering times than tadpoles in the intensive husbandry treatment. Consequently, the effects of Calcium sequestering time on SLS or metamorphosis time may be confounded by husbandry treatment. However, for the SLS analysis, the expected husbandry treatment effect was that tadpoles in the medium husbandry treatment would have higher SLS prevalence than tadpoles in the intensive husbandry treatment, while the observed effect of Ca2+ sequestering time on SLS was negative; tadpoles in the medium treatment with higher Ca2+ sequestering time had less SLS. It Is therefore unlikely that the negative effect of Ca2+ sequestering time on SLS was confounded by husbandry treatment. We therefore assert the use of the Ca2+ sequestering time in our analyses of SLS based on the biological relevance of the Ca2+ sequestering time effect and the opposite direction of the expected treatment effect and the observed Ca2+ sequestering time effect. Ca2+ sequestering time is a direct function of metamorphosis time (equation 3-2), so Ca2+ sequestering time and metamorphosis time are strongly correlated ($r = 0.69$, $n = 45$). We included Ca2+ sequestering time in our analysis of the effect of husbandry treatment on metamorphosis time to control for the effects of the broken phosphate reactor but acknowledge that any effect of Ca2+ sequestering time on metamorphosis time detected in this analysis is likely the result of this relationship.

We now turn to our most surprising results. Ca2+ sequestering time had a relative importance value of 1 in our full averaged model (Figure 3-5) and had a significant negative effect on SLS when we analyzed the effect of Ca2+ sequestering time on SLS treating husbandry treatment and breeding tank as random effects (Figure 3-9). This suggests that change(s) in water chemistry associated with the failure and repair of the phosphate reactor had an important effect on SLS. Specifically, we hypothesize that the excess phosphates introduced during the time that the phosphate reactor was broken indirectly caused SLS by reducing the amount of dissolved calcium available to developing tadpoles. We base this hypothesis on the original findings and discussion of Camperio Ciani et al. (2018), our post-hoc analysis of water

chemistry, the effect of Ca^{2+} sequestering time on SLS, and the physiology of tadpoles during metamorphosis. Importantly, the same phosphate reactor that failed during our second experiment was installed at the PARCP between 2016 and 2017, i.e. it was a third (and unknown) factor that changed between the pre-experimental and 2017 pilot experiment. The installation of a working phosphate reactor may have led to the surprising low levels of SLS in experiment 1 by freeing up dissolved calcium.

Calcium plays an important role in amphibian metamorphosis (Baldwin & Bentley, 1980; Oguro et al., 1975; Pilkington & Simkiss, 1966; Stiffler, 1993) and previous studies have shown an increased incidence of skeletal deformities for tadpoles raised in low calcium water (Marshall, Amborski, & Culley, 1980; Pollack & Liebig, 1989). Camperio Ciani *et al.* (2018) found that SLS prevalence was reduced in *Atelopus glypus* if tadpoles were raised in reconstituted reverse osmosis water instead of carbon-filtered water, and we found a similar pattern with *Andinobates geminisae* tadpoles during our first experiment. Reconstituted reverse osmosis and carbon filtered water differ drastically in their chemical composition: in Camperio et al.'s (2018) experiment, reconstituted reverse osmosis water was over twice as hard as the hardness of carbon-filtered water (56mg/L $CaCO_3$ vs. 26mg/L $CaCO_3$). These researchers also noted that the ratio of phosphate to calcium was 1:189 in carbon-filtered water, compared to 1:986 in reconstituted reverse osmosis water. Phosphate reacts with calcium in aqueous solution to form insoluble calcium phosphate salt (NCBI, 2019), thereby sequestering dissolved calcium needed by developing amphibian larvae. The correlation between an increased phosphate to calcium ratio and high SLS prevalence noted by Camperio Ciani *et al.* (2018) provides the foundation of our hypothesis that SLS may be a metamorphosis-related symptom of phosphate-induced calcium deficiency.

We initially hypothesized that SLS decreased from 58 to 3% during the first experiment because of changes to tadpole husbandry. During the first experiment, 4 out of 22 tadpoles raised in carbon-filtered water developed SLS at staggered time points over a six-month period. In the second experiment, four tadpoles raised in carbon-filtered water under nearly identical care conditions developed SLS in succession within the first four months (Figure 3-12). This latter prevalence of SLS was much higher than predicted based on our initial hypothesis and indicated that another important SLS factor had changed. Genetics is an unlikely factor because five out of six of the breeding pairs used in the second experiment were unchanged from the first experiment. All of the tadpoles that developed SLS during the first three months of our study were from the intensive husbandry treatment group, but we cannot say if this is due to a

treatment effect or to the date of tadpole metamorphosis, because of the delayed metamorphosis time of tadpoles in the medium husbandry treatment group. However, the high prevalence of SLS at the beginning of our experiment coincided with the period of time just before the repair of the phosphate reactor (Figure 3-12), suggesting that the timing of metamorphosis relative to the repair of the reactor may be more directly related to SLS than tadpole husbandry treatment.

Given the purpose of phosphate reactors, one would assume that excess phosphates were present in the water used for tadpole rearing over the time period that the phosphate reactor was broken, and that phosphate levels would decrease following its' repair. Data on the phosphate to calcium content of the tadpole source water taken during the experiment confirms that phosphates decreased from ~0.4ppm to 0-0.04ppm PO_4^{3-} during the repair of the reactor while calcium levels remained relatively constant (Figure 3-12), resulting in an elevated calcium to phosphate ratio that sharply decreased following the repair of the phosphate reactor; this decrease in the phosphate to calcium ratio was accompanied by a drop in the prevalence of SLS (Figure 3-12). This pattern is consistent with the water filtration data we collected in July 2018, which shows that phosphate levels in the tadpole rearing water would remain at about 0.54 ppm PO_4^{3-} without a functioning phosphate reactor, vs. 0.04ppm PO_4^{3-} with a functioning phosphate reactor, while calcium levels would remain between 15 and 20ppm $CaCO_3$ regardless (Figure 3-12). Our *ad hoc* and *post hoc* water analyses both consistently show that the failure of the phosphate reactor resulted in an elevated ratio of phosphate to calcium in the tadpole rearing water and that this ratio decreased following the repair of the reactor. However, our phosphate and calcium data from individual tadpole wastewater does not reflect this pattern (Figure 3-14). This is likely due to the cyclic introduction and removal of phosphates through food waste and feces and/or the limited accuracy of the test strips we used for these measurements. Nonetheless, we can still say with some degree of confidence that the broken reactor increased the phosphate to calcium ratio of the water used for tadpole rearing, and that this was correlated with an increased incidence of SLS, based on the data presented in figures 3-12 and 3-13.

So far, we have discussed two instances of correlation between the phosphate to calcium ratio and SLS prevalence: one observed by Camperio Ciani *et al.* (2018) due filtration method choice and one due to an equipment malfunction in the present study. It was not until this phenomenon became a focus of conversation at the PARCP that we learned the phosphate reactor units had been installed for the first time at the PARCP just prior to running experiment 1

(July-December 2017), i.e. coincident with the 55% decrease in the incidence of SLS in Experiment 1.

Calcium is vital for the ossification bones during amphibian metamorphosis (Baldwin & Bentley, 1980; Oguro et al., 1975; Pilkington & Simkiss, 1966; Stiffler, 1993). In anurans, the most dramatic metamorphic changes — including metamorphosis of the mouth, reabsorption of the tail and much of bone ossification — take place during a stage known as metamorphic climax, marked by the emergence of forelimbs and ending with the completion of tail reabsorption (Etkin 1964; Pilkington and Simkiss 1966, Stiffler 1993; Gosner 1960). Tadpoles can acquire environmental calcium across the skin (Guirguis et al., 1997; Stiffler, 1995, 1996; Stiffler et al., 1997; Zerella & Stiffler, 1999) and gills (Baldwin & Bentley, 1980; Stiffler, 1993, 1996), and via dietary calcium through the wall of the small intestine (Robertson, 1976, 1975; Stiffler, 1993, 1996). However, during peak metamorphosis, tadpoles are unable to feed due to the restructuring of the mouth (Gosner, 1960; Pilkington & Simkiss, 1966) and external gills are no longer present at this stage (Gosner, 1960), limiting the capacity to acquire calcium from the environment (Pilkington & Simkiss, 1966). However, tadpoles store calcium as calcium carbonate in specialized regions of the inner ear, called endolymphatic sacs, and this calcium is mobilized for use during metamorphic climax (Pilkington and Simkiss, 1966). In *Rana temporaria*, the calcium concentration of tadpole rearing water is directly correlated with the amount of calcium carbonate in the endolymphatic sacs and the degree of skeleton mineralization in metamorphs (Pilkington and Simkiss, 1966).

Calcium accumulates in the endolymphatic sacs of tadpoles during the growth period prior to bone development, which occurs up until Gosner stage 38-41 (right before metamorphic climax). Once bone development begins, there is a plateau in endolymphatic calcium storage until it is mobilized for use during metamorphic climax (Pilkington and Simkiss, 1966). The availability of Calcium, and the window of time tadpoles have to accumulate calcium in the endolymphatic sacs should therefore be directly reflected in the health of bones that develop during metamorphic climax. We may have quantified this phenomena in our model of the effect of Ca^{2+} sequestering time on SLS. Ca^{2+} sequestering time directly quantifies the amount of time that each tadpole had to sequester calcium during the growth period prior to metamorphic climax. As such, Ca^{2+} sequestering time was a statistically significant predictor of SLS (Figure 3-9); According to our statistical model, tadpoles must have a minimum of 10 days to accumulate calcium prior to peak metamorphosis in order to lower their probability of developing SLS below 50% (Figure 3-10) in this system.

Conclusion

Our first experiment revealed that neither filtration method nor vitamin supplementation of tadpole rearing water effect the time it takes for *Andinobates geminisae* tadpoles to metamorphose. We found no significant effect of water supplementation or filtration on survival or SLS, however, our study had limited statistical power. The distribution of the 5 SLS cases across treatment groups is consistent with the pattern reported by Camperio Ciani et al. (2018) and there were no SLS cases from either vitamin-supplemented treatment groups. Both of these patterns merit further study with increased statistical power. In our second experiment we found that less intensive tadpole husbandry increased the amount of time it took for tadpoles to reach metamorphosis by approximately 30 days; this provides important information for the cost-benefit analyses of tadpole husbandry staff hours at the PARCP and similar projects. The effects of husbandry intensity treatment on tadpole survival and SLS were unclear. However, we observed an association between elevated phosphate-to-calcium levels in the water used to rear tadpoles, and an elevated incidence of SLS. Further investigation revealed a second association between elevated dissolved phosphate and SLS at the PARCP, and our post-hoc analysis of the ability of tadpoles to sequester calcium on SLS development suggests that SLS is a symptom of hypocalcemia during metamorphic climax, induced specifically by high phosphate content of the tadpole rearing water in this system. Our manipulation of the phosphate to calcium ratio of the tadpole rearing water was unintentional, and as such, changes in the phosphate to calcium ratio were confounded by the variable of time, and the exact phosphate and calcium concentrations involved in these changes could only be approximated. We do not intend for our findings to result in the circulation of another putative SLS factor. Rather, the hypocalcemia hypothesis for the etiology of SLS, which is based on our observations, known tadpole physiology, and the work by Camperio Ciani *et al.* (2018), should be considered a top candidate for future directed testing.

Acknowledgements

In addition to the people mentioned under the thesis acknowledgements, I would like to thank my labmates Dan Greenberg, Phillip Fernandez, Jayme Lewthwaite, and Gavia Lertzman-Lepofsky for their advice and feedback on the analysis and presentation of this chapter.

Tables and Figures

Table 3-1 Summary of experimentally tested husbandry-related causes of causes of SLS.

Experimental variable	Model Species (tadpoles)	Result	Source
Different types of commercial diets (dried brine shrimp, rabbit chow, baby food, fish food, dried tubifex worms, *Xenopus* tadpole diet)	*Dendrobates auratus*	There was no effect of commercial diet type on SLS.	Marlett, Eichler, & Fellows-Hagemeister (1988)
Dietary Calcium	*Rana catesbeiana*	Lower dietary calcium significantly increased skeletal deformities, including "crooked limbs", for tadpoles ≤ stage 18.	Culley (1980)
Environmental Calcium	*Epipedobates tricolor*	Inconclusive: tadpoles died or developed poorly at all calcium concentrations.	Hakvoort & Gouda (1990)
Overfeeding	*Atelopus glyphus*	Overfeeding significantly increased SLS prevalence, especially if tadpoles were raised in carbon filtered water instead of reconstituted reverse osmosis water	Camperio Ciani et al. (2018)
Experimental vs. Commercial diet	*Atelopus glyphus*	There was no difference between experimental and commercial diet on SLS.	Camperio Ciani et al. (2018)
Water filtration method (reconstituted reverse osmosis water vs. carbon filtered water)	*Atelopus glyphus*	Raising tadpoles in reconstituted reverse osmosis water significantly reduced SLS prevalence.	Camperio Ciani et al. (2018)
Diet Ration	*Atelopus glyphus*	Ration had a significant positive effect on SLS in carbon filtered water and increased the effect of filtration method on SLS.	Camperio Ciani et al. (2018)
Dietary Protein	*Atelopus certus*	There was no effect of dietary protein on SLS.	Camperio Ciani et al. (2018)
Time into the breeding season*	*Dendrobates auratus*	SLS prevalence increased with increased time into the breeding season.	Marlett, Eichler, & Fellows-Hagemeister (1988)

Table 3-2 Summary of the differences in tadpole care protocol between the two husbandry intensity treatment groups.

	Husbandry Treatment	
Care parameter	Intensive	Medium
Feeding dose	0.003 ± 0.002 g crushed fish flakes per feeding.	0.005 ± 0.002 g crushed fish flakes per feeding.
Feeding frequency	Tadpoles fed three times weekly.	Tadpoles fed once weekly.
Water change frequency	50% water change 3 times weekly.	50% water change once every 14 days.

Table 3-3 Generalized linear models of tadpole metamorphosis time (in days), survival, and SLS status in response to water vitamin/filtration treatment and breeding tank ID in Experiment 1. Breeding tank ID represents the parental tank origin and tank Id of each tadpole.

Response Variable	Model	K	AICc	**ΔAICc**	**AICcω**	Cum. **AICω**	LL
Met. Time ~ n = 87	1	1	547.00	0.00	0.85	0.85	-272.48
	tmt	4	550.78	3.78	0.13	0.97	-271.15
	tmt + tank.Id	6	554.08	7.08	0.02	1.00	-270.51
	tmt*tank.Id	9	558.59	11.59	0.00	1.00	-269.13
	tank.Id	24	600.59	53.59	0.00	1.00	-266.62
Survival ~ n = 97	1	1	66.42	0.00	0.69	0.69	-32.19
	tank.Id	6	69.05	2.63	0.19	0.88	-28.06
	tmt	4	70.31	3.90	0.10	0.97	-30.94
	tmt + tank.Id	9	73.00	6.58	0.03	1.00	-26.46
	tmt*tank.Id	24	108.39	41.98	0.00	1.00	-21.86
SLS ~ n = 87	tmt + tank.Id	9	32.11	0.00	0.87	0.87	-5.88
	tmt	4	37.25	5.14	0.07	0.93	-14.38
	tank.Id	6	37.72	5.62	0.05	0.99	-12.34
	1	1	40.32	8.21	0.01	1.00	-19.14
	tmt*tank.Id	24	79.12	47.02	0.00	1.00	-5.88

Table 3-4 The number of tadpoles to die or survive to metamorphosis, by (a) water treatment group or (b) breeding tank, for Experiment 1. Total N=97

(a) Water treatment	CB	CF	FA	RO
# died	2	3	4	1
# survived	22	21	20	24

(b) Breeding Tank	t24	t25	t58	t70	t72	t77
# died	1	2	0	1	4	2
# survived	6	24	19	13	10	15

Table 3-5 Generalized linear mixed effects models of *Andinobates geminisae* tadpole time to metamorphosis (Met.time) and SLS , and survival as a function of husbandry treatment (tmt) and Ca^{2+} sequestering time with the random effect of breeding tank (tank.Id) for Experiment 2. Ca^{2+} sequestering time was centered and scaled by the mean and 2 standard deviations.

Response Variable	Model	K	AICc	**ΔAICc**	**AICcω**	Cum. **AICω**	LL
Met.time ~ (n = 45)	tmt * Ca^{2+} Seq. Time + (1|tank.Id)	6	335.81	0.00	0.72	0.72	-160.80
	tmt + Ca^{2+} Seq. Time + (1|tank.Id)	5	337.66	1.85	0.28	1.00	-163.06
	tmt + (1|tank.Id)	4	350.96	15.15	0.00	1.00	-170.98
	Ca^{2+} Seq. Time + (1|tank.Id)	4	362.78	26.98	0.00	1.00	-176.89
	1 + (1|tank.Id)	3	391.99	56.18	0.00	1.00	-192.70
SLS ~ (n = 45)	tmt* Ca^{2+} Seq. Time + (1|tank.Id)	5	31.05	0.00	0.52	0.52	-9.75
	Ca^{2+} Seq. Time + (1|tank.Id)	3	31.69	0.64	0.38	0.91	-12.55
	tmt+ Ca^{2+} Seq. Time + (1|tank.Id)	4	34.49	3.44	0.09	1.00	-12.75
	1 + (1|tank.Id)	2	44.74	13.70	0.00	1.00	-20.23
	tmt + (1|tank.Id)	3	46.08	15.03	0.00	1.00	-19.75

Response Variable	Model	K	AICc	**ΔAICc**	**AICcω**	Cum. **AICω**	LL
Survival ~ (n = 60)	tmt*PO_4^{3-} exp. + (1|tank.Id)	5	68.25	0.00	0.27	0.27	-28.57
	PO_4^{3-} exp. + (1|tank.Id)	3	68.33	0.08	0.26	0.53	-30.95
	tmt + PO_4^{3-} exp. + (1|tank.Id)	4	68.38	0.13	0.25	0.78	-29.83
	1 + (1|tank.Id)	2	69.46	1.21	0.15	0.93	-32.63
	tmt + (1|tank.Id)	3	70.82	2.57	0.07	1.00	-32.20

Table 3-6 Generalized linear mixed effects models of Andinobates geminisae tadpole survival to metamorphosis as a function of husbandry intensity treatment (tmt) and % PO_4^{3-} exposure with the random effect of breeding tank (tank.Id) for Experiment 2. % PO_4^{3-} exposure was centered by the mean and scaled by 2 standard deviations.

Table 3-7 AICc comparison of null and alternate generalized linear mixed effects models of SLS in response to Ca2+ sequestering time in Experiment 2. Breeding tank (tank.Id) and husbandry intensity treatment (tmt) were included as random effects. Ca^{2+} sequestering time was centered by the mean and scaled by 2 standard deviations.

Response Variable	Model	K	AICc	**ΔAICc**	**AICcω**	LL
SLS ~	Ca^{2+} Seq. Time + (1|tank.Id) + (1|tmt)	4	30.97	0	1	-10.97
	1+ (1|tank.Id) + (1|tmt)	3	46.68	15.71	0	-20.04

Figure 3-1 **Habitat design of parental breeding tanks with tadpoles in the front area. Each breeding tank had an identical set of objects: 10 capped pieces of PVC, 6 water-filled petri dishes, a pot hide, a small bromeliad, a clear shelter, and 22 numbered petri dishes for tadpoles. Objects were positioned as seen above in all tanks. Emerging metamorphs were placed under the clear shelter after forelimb emergence to complete metamorphosis.**

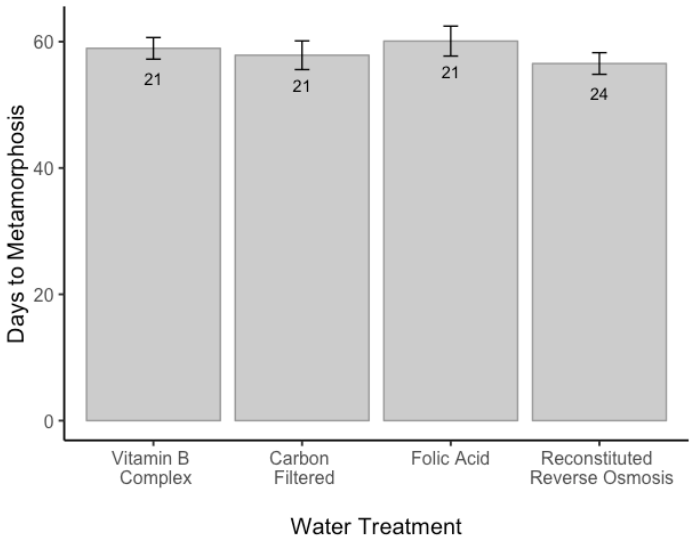

Figure 3-2 Mean and standard error of metamorphosis time for tadpoles raised in one of four water filtration and vitamin supplementation treatments in Experiment 1. Numbers at the top of each bar represent the number of observations in each treatment group. N = 87 tadpoles.

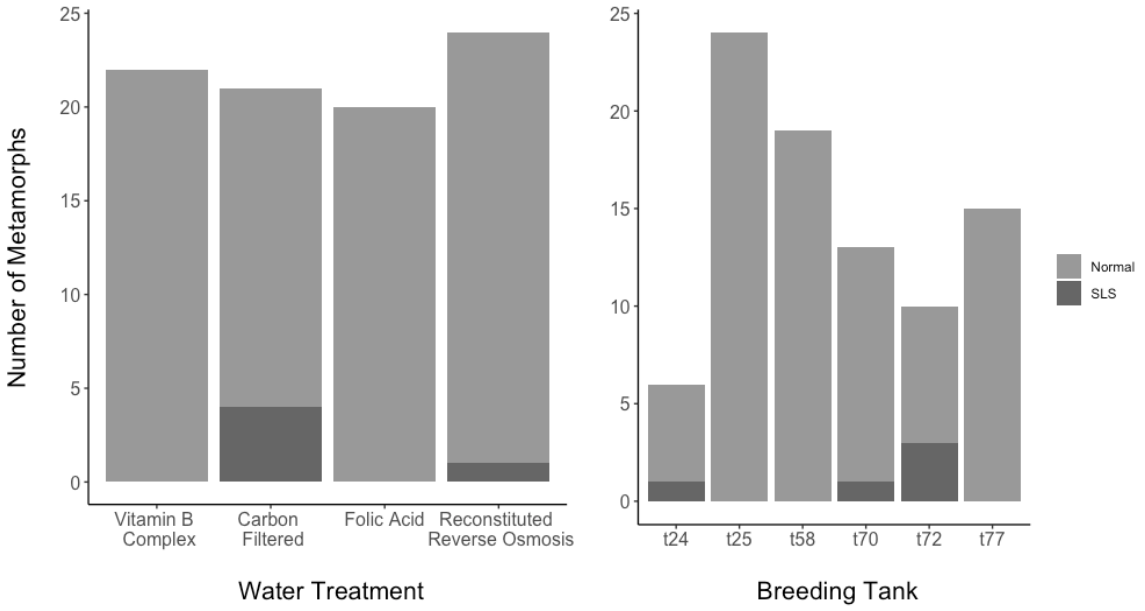

Figure 3-3 The number of tadpoles to metamorphose with SLS (dark grey) or healthy (light grey) from each water treatment group (left) and for each breeding tank (right) in Experiment 1. N = 87 tadpoles.

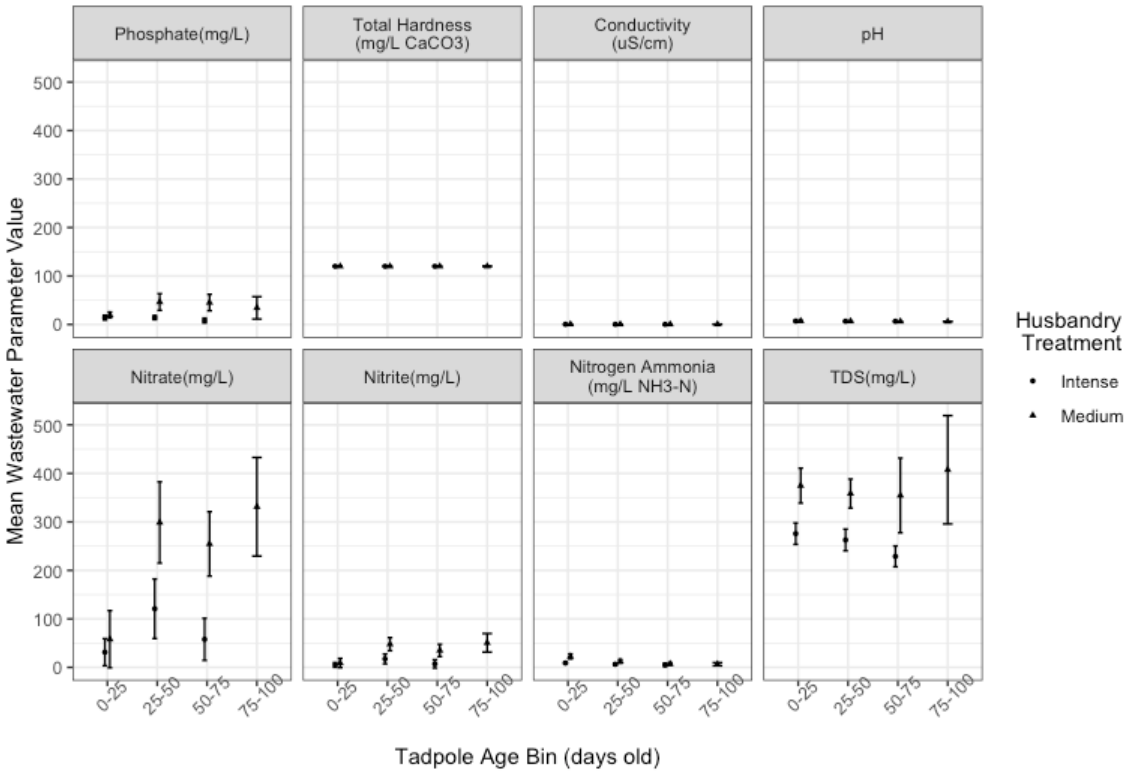

Figure 3-4 Comparison of mean water quality parameters measured from the wastewater of individual tadpoles of four age classes in Experiment 2. Water quality data is only available for tadpoles in the medium husbandry group for the oldest age class because tadpoles in the high husbandry group had all metamorphosed prior to 75 days old. Error bars represent 95% confidence intervals.

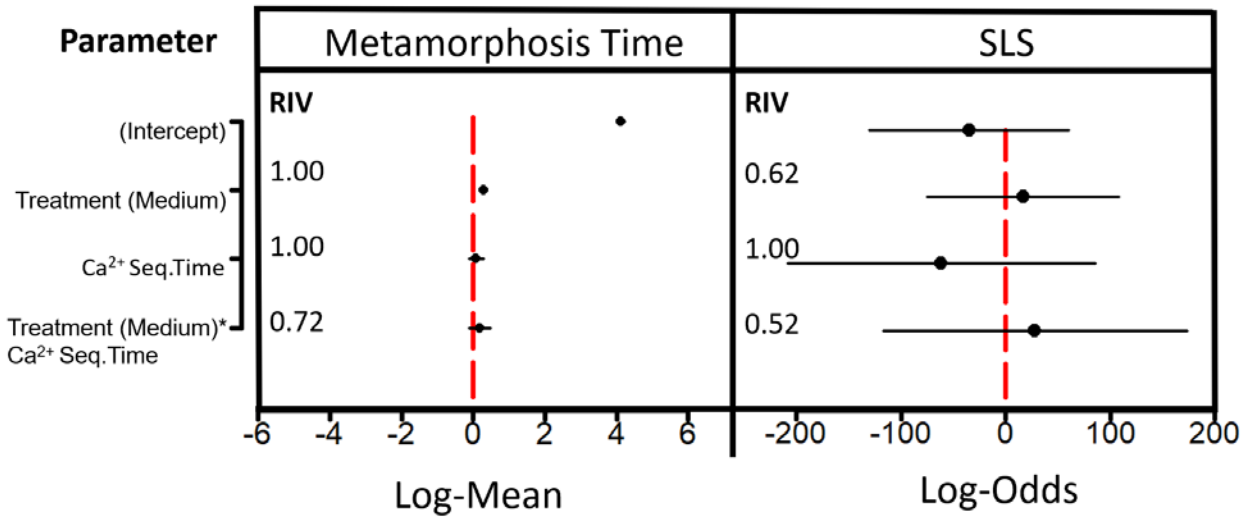

Figure 3-5 **Full averaged model coefficients for models of Metamorphosis time and SLS in response to husbandry treatment, Ca^{2+} sequestering time, and their interaction in Experiment 2. Models with a cumulative AICω ≥ 0.95 were selected for averaging, and Ca^{2+} sequestering time was centered by the mean and scaled by 2 standard deviations. RIV = Relative importance value.**

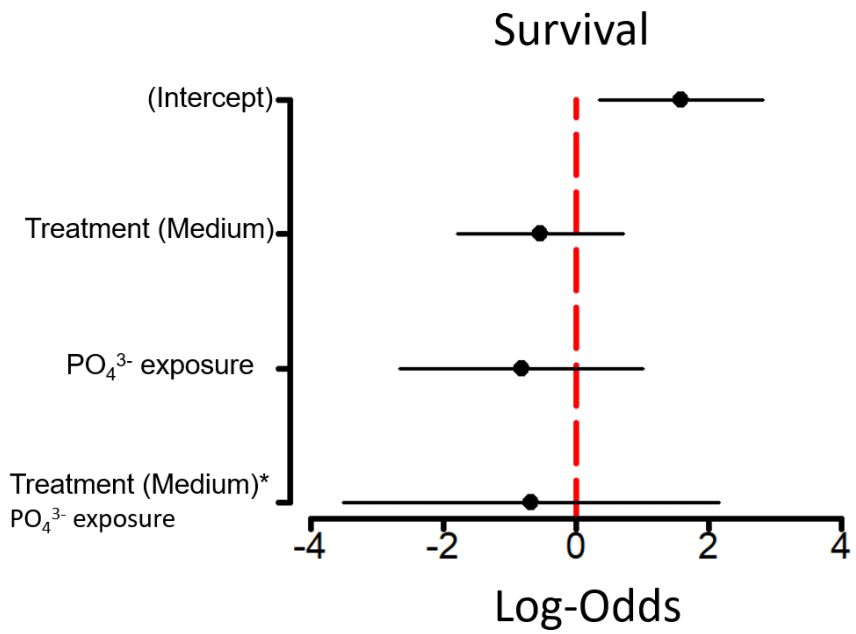

Figure 3-6 **Coefficient estimates from a model of tadpole survival to metamorphosis in response to husbandry treatment, % PO_4^{3-} exposure, and their interaction, with breeding tank included as a random effect in Experiment 2. Error bars represent the 95% confidence intervals of the coefficient estimates. % PO_4^{3-} exposure was centered by the mean and scaled by 2 standard deviations.**

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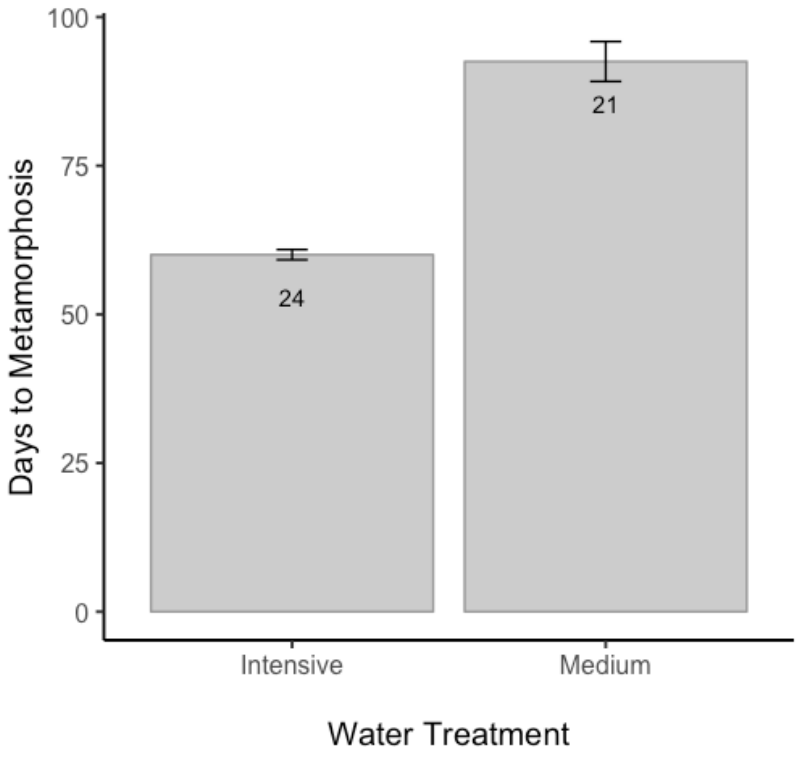

Figure 3-7 **Mean ± standard error of the metamorphosis time of tadpoles raised under medium and high intensity husbandry treatments in Experiment 2. Numbers at the top of the bars represent the number of observations in each treatment group. N=45 tadpoles.**

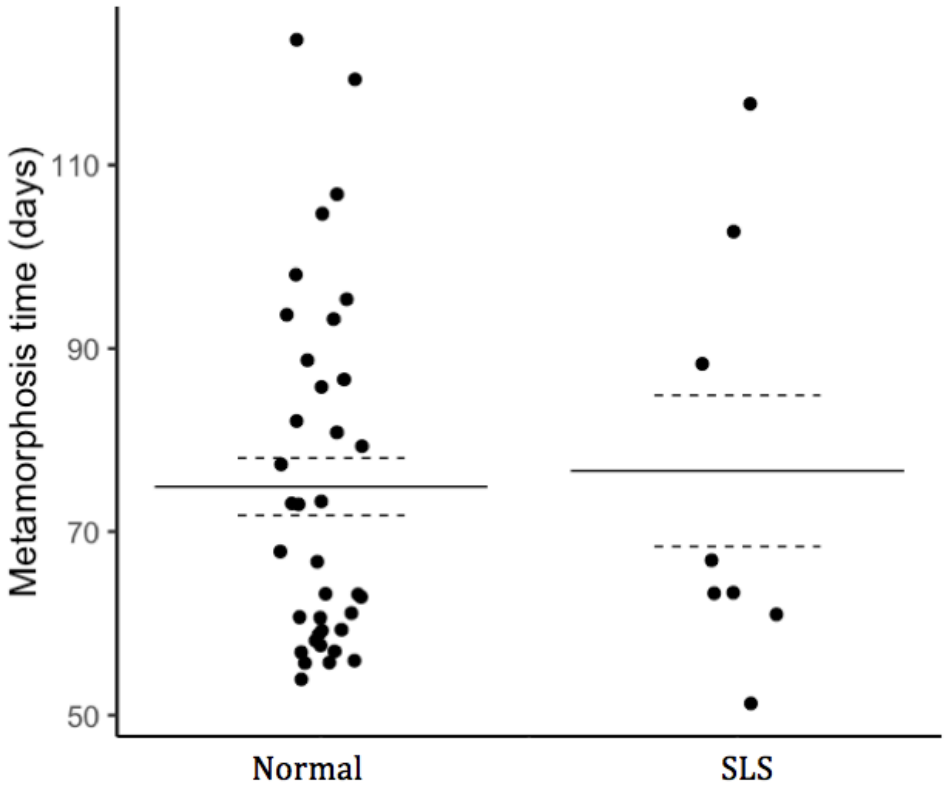

Figure 3-8 **Comparison of the metamorphosis times of n = 8 tadpoles that developed SLS to n = 37 tadpoles that developed healthy limbs in Experiment 2. Each point represents a single tadpoles's metamorphosis time in days. Solid lines represent the group means and dashed lines trepresent the mean ± standard error.**

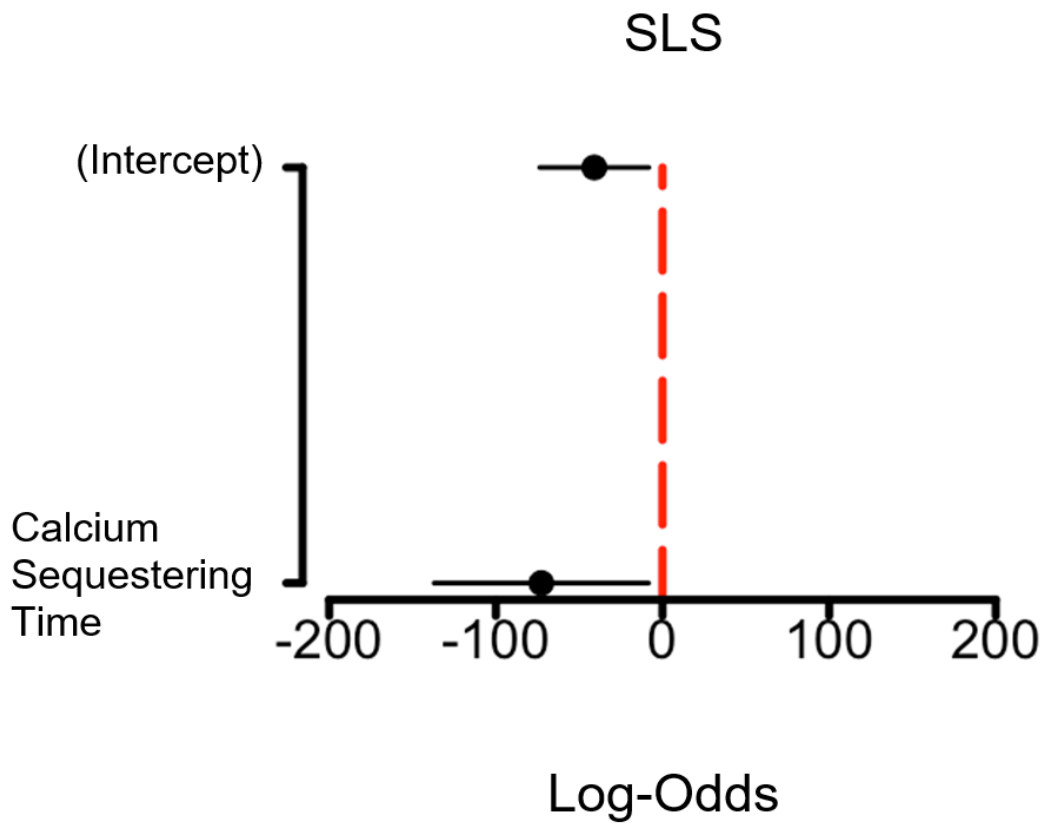

Figure 3-9 **Coefficient estimates from a model of SLS in response to Ca^{2+}sequestering time, with breeding tank and husbandry treatment included as random effects in Experiment 2. Error bars represent the 95% confidence intervals of the coefficient estimates. N=45. Ca^{2+}sequestering time was centered by the mean and scaled by 2 standard deviations.**

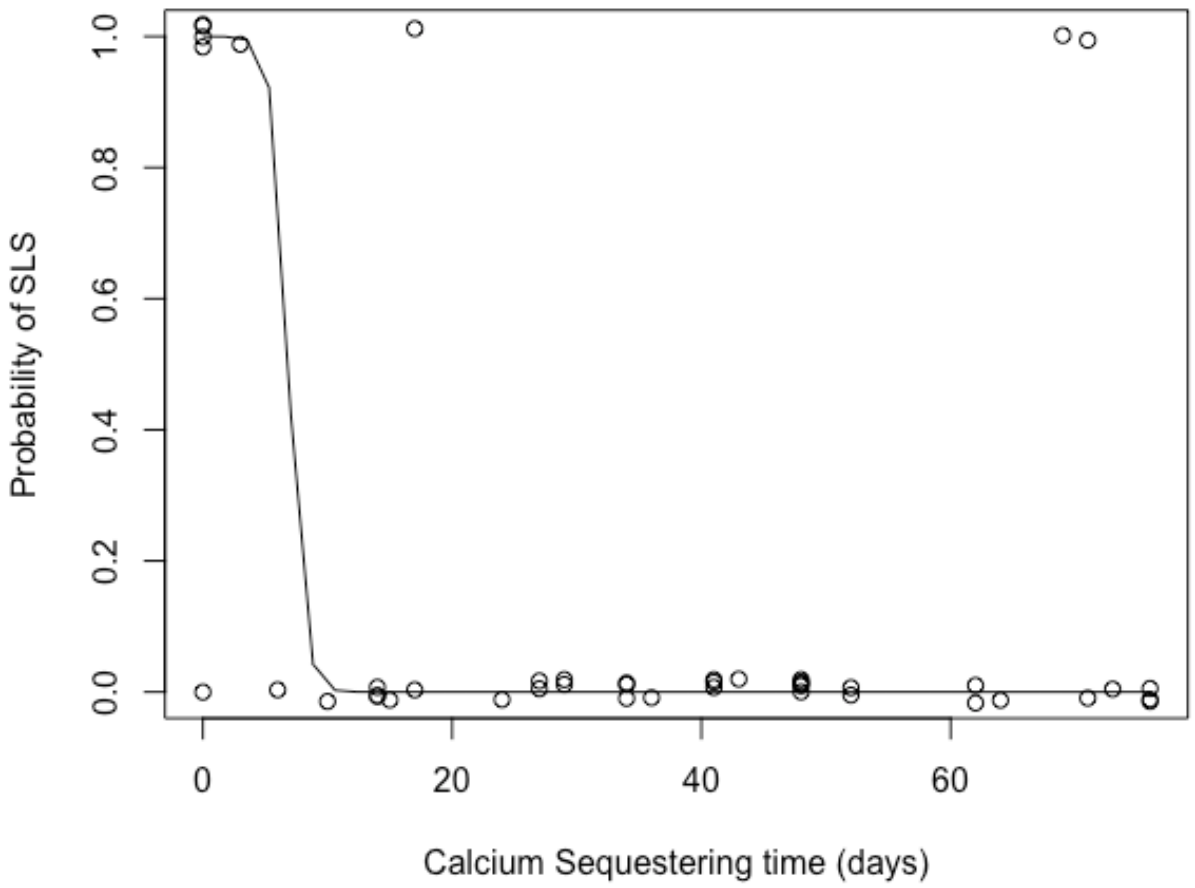

Figure 3-10 **The probability of SLS (represented by the fitted line) over increasing calcium-sequestering time, generated from our model of SLS in response to increasing calcium exposure (AICω = 1), with husbandry treatment and breeding tank included as a random effects for Experiment 2. The circles represent the SLS status (1 = SLS, 0 = healthy) and calcium sequestering time of each experimental tadpoles (N = 45).**

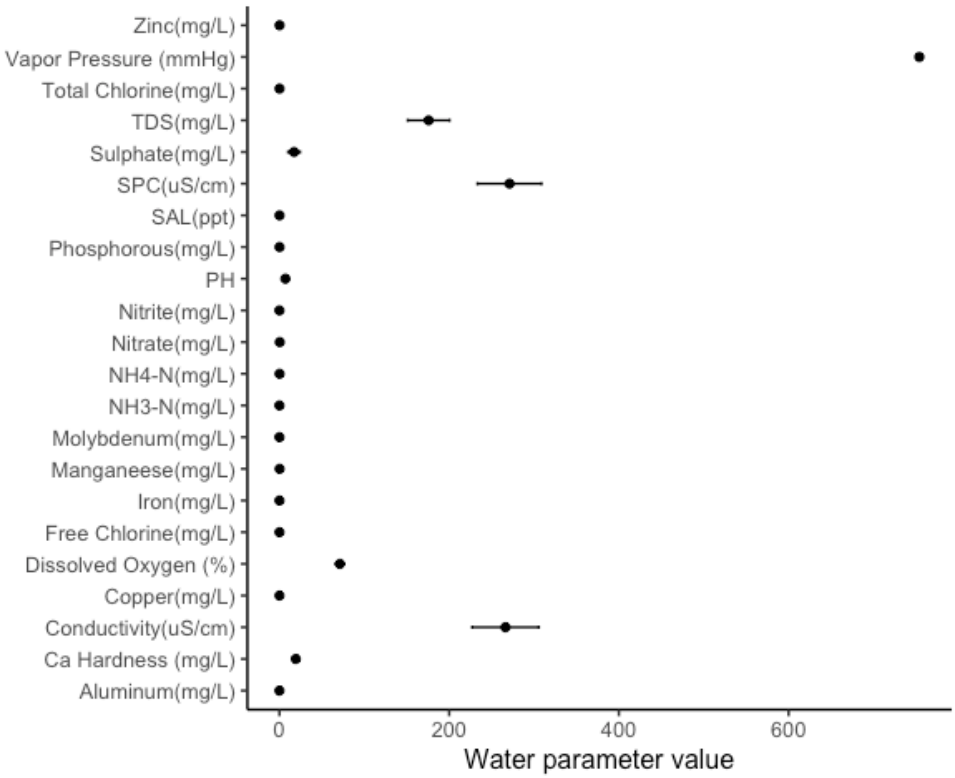

Figure 3-11 Mean values of various water parameters covered during full spectrum analyses of PARCP *A. geminisae* source water. The mean for each parameter is taken from n = 3 or n = 4 measurements taken over the 6 month period of the experiment. Whiskers represent the standard error of the mean.

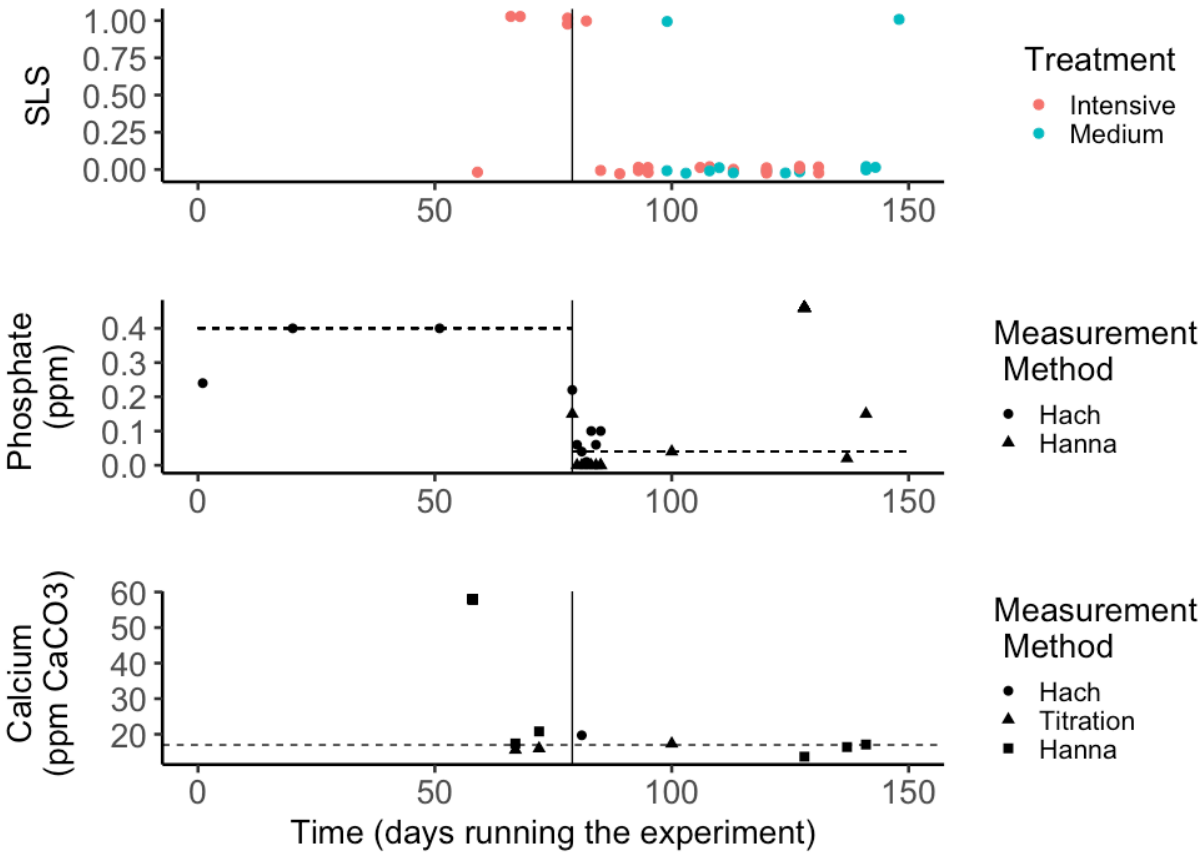

Figure 3-12 **Comparison of SLS at metamorphosis, PO_4^{3-} concentration, and calcium concentration of the source water used for tadpoles, over the duration of Experiment 2. The vertical line at day 79 represents the day that the phosphate reactor was repaired. In the top graph, each (jittered) point represents the metamorphosis of a single tadpole, with 1 indicating that the emerging metamorph had SLS and 0 indicating they were healthy. We used three methods to measure PO_4^{3-} and calcium: a "Hanna" colorimeter, a "Hach" colorimeter, and manual titration. The dashed lines represent the median phosphate (middle panel) or calcium (bottom pannel) concentration of the water before and after the phosphate reactor was repaired (indicated by the vertical line).**

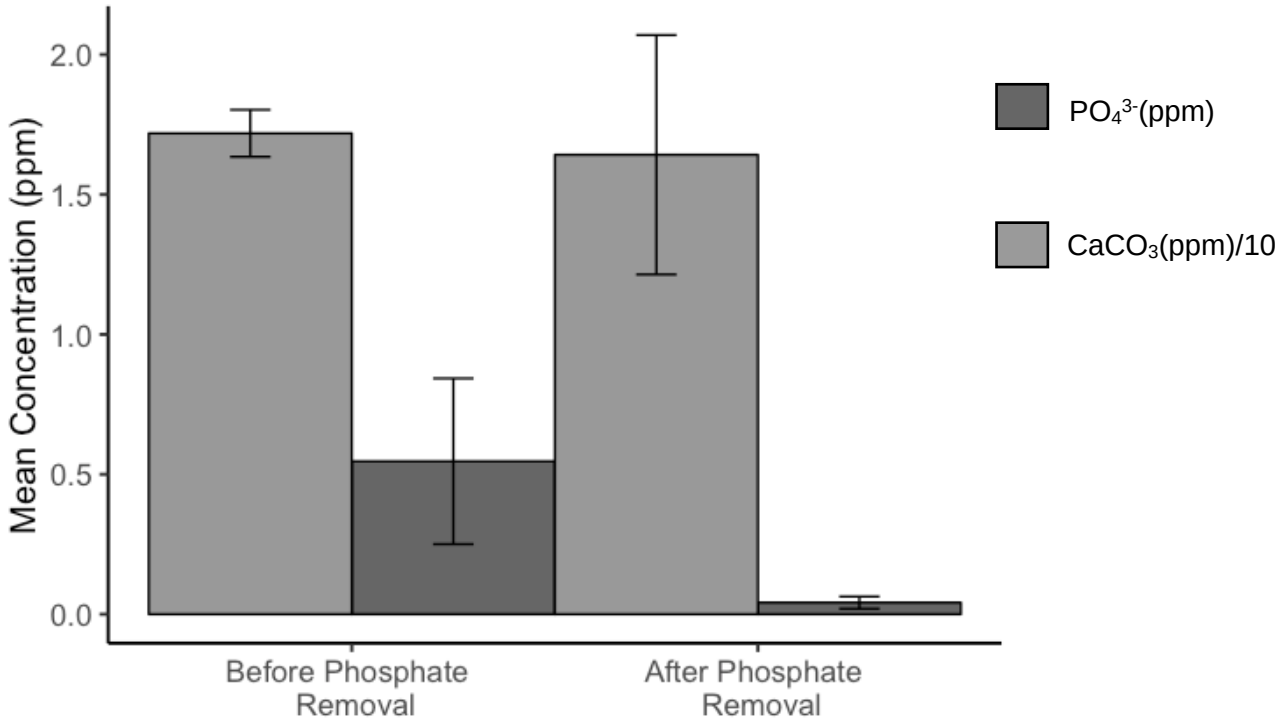

Figure 3-13 Mean phosphate (PO_4^{3-}) and calcium concentration ($CaCO_3$) of the tadpole source water before and after passing through the repaired phosphate reactor in July 2018. Error bars represent 95% confidence intervals. Concentrations are in mg/L, and calcium concentration is divided by a factor of ten for visualization on the same scale as phosphate data.

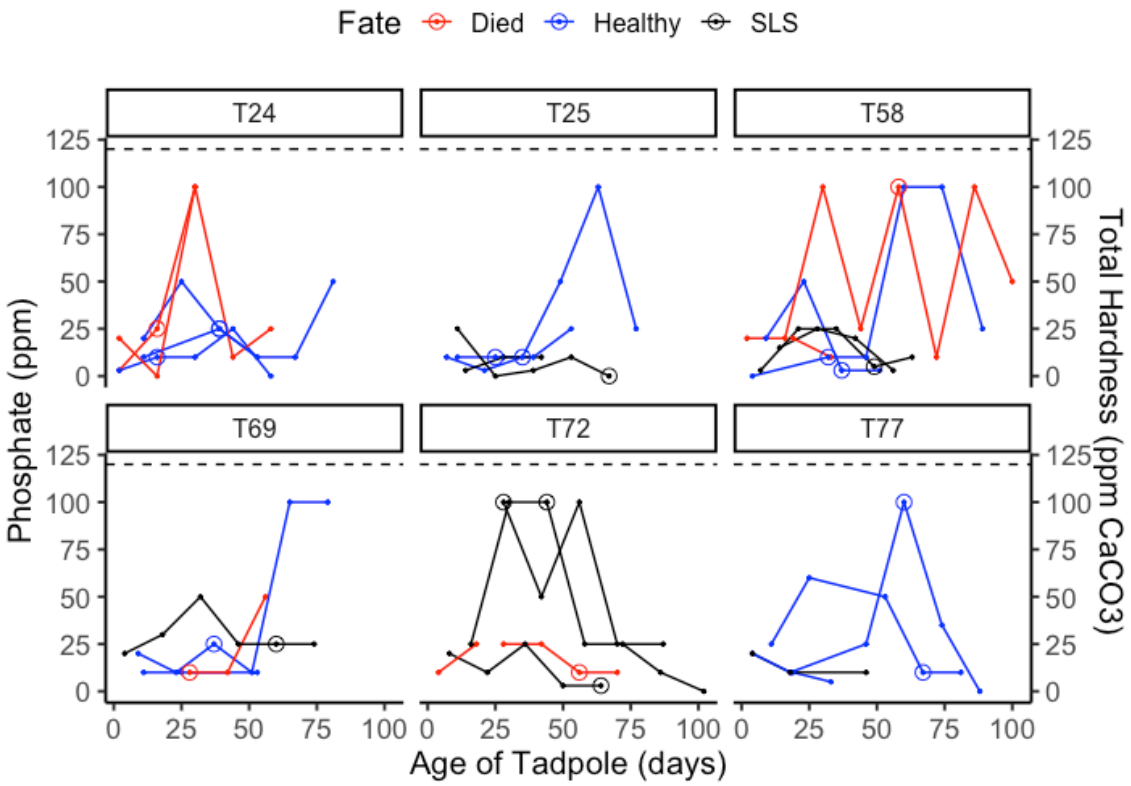

Figure 3-14 **Phosphate and hardness readings taken from the wastewater of individual tadpoles in the six breeding tanks. Each solid line connects chronological phosphate measurements (represented by the solid points) taken from a single tadpole. The dashed black line represents the total water hardness, which was the same in all measurements. The circled points highlight the age of each tadpole when the phosphate reactor was repaired, and are absent on the lines of tadpoles that metamorphosed prior this time. Fate refers to the fate of each tadpole regarding survival to metamorphosis or SLS development.**

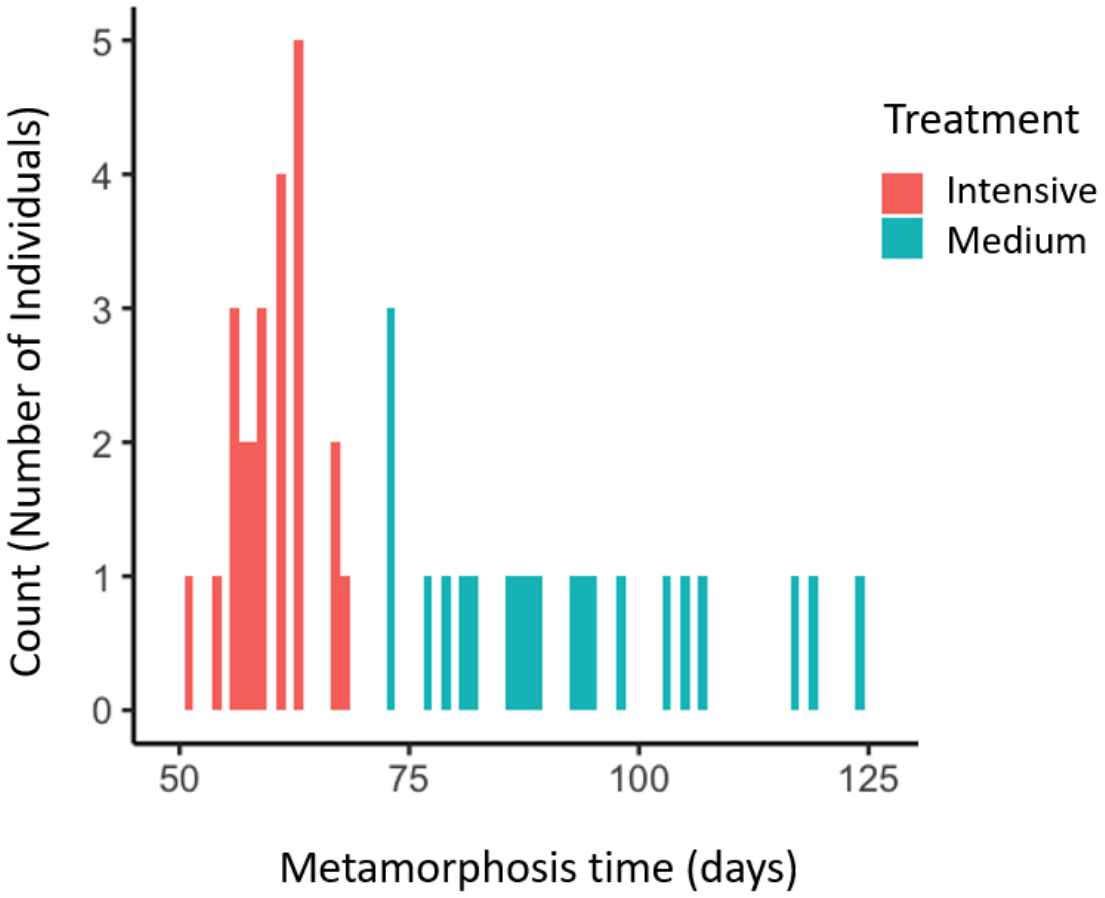

Figure 3-15 Distribution of individuals from experiment 2 by metamorphosis time in each husbandry treatment group. N = 45

Equation 3-1 **Ca^{2+} sequestering time. The difference between the two dates is represented in days since the start of the experiment (day 0). If a tadpole metamorphosed prior to the repair of the phosphate reactor, Ca^{2+} sequestering time is set = 0.**

$$Ca^{2+} sequestering\ time = (Date\ of\ forelimb\ emergence - Date\ of\ phosphate\ reactor\ repair)$$

Equation 3-2 **$\%PO_4^{3-}$ exposure. Negative numerators indicate tadpoles that metamorphosed prior to the repair of the phosphate reactor and are set to equal zero.**

$$\%PO_4^{3-} exposure = \frac{\%PO_4^{3-} reactor\ repair\ date - tadpole\ birth\ date}{date\ of\ metamorphos - tadpole\ birth\ date} \times 100$$

Chapter 4. Conclusion

The thesis presents my work at the PARCP as part of a broader discussion of the health problems that limit the success of CBPs and the contributions that CBPs can make to conservation and science. First, I described the tadpoles and eggs of *Oophaga vicentei* and *Andinobates geminisae* using data that was collected from conservation breeding populations at the PARCP. I found that the tadpoles of both species are indistinguishable from the tadpoles of congeneric species, consistent with the hypothesis that tadpole morphology is a shared derived trait within the *Oophaga* genus and within the *Andinobates minutus* species subgroup (of which *A. geminisae* is a part) (Myers 1984; Myers & Daly 1980). However, this cannot be confirmed until descriptions are complete for the remaining species in each genus.

Generally, descriptions of dendrobatid eggs are limited to a few notes on the color or size of eggs taken from dissected females, while dendrobatid tadpole descriptions are scarce and available descriptions are generally incomplete because they are based on a small sample of wild, early stage individuals. More abundant and complete egg and tadpole data might provide insight into the highly debated phylogenetic relationships between dendrobatid species. In conjunction with biogeographical and ecological data, tadpole descriptive data can be used to identify free-living tadpoles in the field. For example, described *Andinobates* tadpoles are morphologically distinct from the tadpoles of other genera, and *A. geminisae* tadpoles do not overlap ecologically or geographically with any congeneric species except for *A. minutus,* allowing tadpoles of this morphology to be identified to genus, and more specifically, as either *A. minutus* or *A. geminisae*. Likewise, eggs could also be used to detect species presence if descriptive data were available for more species. The ability to identify free-living tadpoles and eggs in the field could potentially increase the sensitivity of biological surveys and improve the accuracy of population estimates and range data relevant to conservation. In addition to the descriptions I provide, this chapter also demonstrates that useful morphological data on eggs and tadpoles can be collected non-invasively at conservation breeding facilities. The collection of tadpole and egg data from conservation breeding facilities is superior to field data collection because (1) a larger sample size can be attained, (2) no animals must be sacrificed, (3) tadpoles are available from a variety of stages, and (4) freshly laid eggs are available (rather than immature eggs from dissected females). CBPs have sole access to these valuable data, all

of which could potentially contribute to the conservation of species in the wild. This is one way to further increase the conservation and scientific value of CBPs.

Next, I carried out two experiments to investigate potential husbandry-related causes of Spindly Leg Syndrome (SLS), a captivity-specific disease of unknown etiology that was severely limiting the survival of the PARCP's population of poison dart frogs. My results suggest that raising tadpoles in reconstituted reverse osmosis water instead of carbon-filtered water has no effect on the time it takes tadpoles to reach metamorphosis or on tadpole survival, but may decrease the prevalence of SLS in *Andinobates geminisae*. None of the tadpoles raised in vitamin B or folic acid- supplemented carbon-filtered water had SLS. Since there is anecdotal evidence that this manner of supplementing tadpole rearing water drastically reduces SLS prevalence, future studies with increased statistical power should continue investigating this potential treatment.

My second experiment revealed that decreasing the intensity of the tadpole husbandry protocols delayed the time it took for tadpoles to reach metamorphosis but did not affect SLS prevalence or tadpole survival; This information is important to consider when allocating limited staff resources to animal care at CBPs.

During experiment 2, a fortuitous accident led to the manipulation of the phosphate content of tadpole rearing water, which in turn affected the amount of time tadpoles had to sequester calcium for use during metamorphic climax. When I accounted for these factors in my analysis, I found that calcium sequestering time was the most important predictors of SLS. In light of these findings and available information on tadpole physiology, it appears likely that SLS is a symptom of phosphate-induced hypocalcaemia leading to metamorphic climax in this system. A third experiment that manipulates the phosphate and calcium content of tadpole-rearing water and compares the resulting prevalence of SLS between treatment groups is needed to confirm this hypothesis, however. This experiment is now (May 2019) underway at PARCP (Roberto Ibáñez, pers. comm.).

SLS prevalence drastically decreased from 68% to 3% for *Andinobates geminisae* metamorphs following the installation of phosphate reactor units at the PARCP. Phosphate reactors are often used in zoos, aquaria, and CBPs, based on general knowledge that excess phosphates are bad for amphibian health, but phosphate reactors may be critical in specifically preventing SLS. Understanding the importance of husbandry practices can have long-lasting

positive effects: Since completing my work, SLS prevalence has stayed below 3% in the PARCP's population of *A. geminsae,* and in light of our findings, we believe this to be due to diligent maintenance of a functioning phosphate reactor.

During my work, we discovered that the experimental collection protocol proved to be highly efficient in locating tadpoles consistently from actively breeding pairs. This methodology has since been adapted by the PARCP as a regular protocol resultantly; the number of metamorphs produced per month has tripled for this population.

Knowledge gaps in amphibian husbandry have led to the discovery of numerous idiopathic husbandry-related diseases that can severely limit the success of CBPs. Using an experimental approach, it is feasible to identify the causes of these diseases and revise husbandry accordingly with drastic positive effects. In the interest of saving time, husbandry staff may be tempted to use a shotgun approach to treat disease, whereby several treatments are simultaneously applied to the patient until they are cured. However, this approach provides no information on the etiology of the disease. If we are to continue increasing the number of amphibian species in CBPs (Harding et al. 2016), then our knowledge on husbandry-related diseases must also increase proportionally; therefore sufficient staff resources should be put towards hypothetico-deductive problem-solving protocols that generate focussed new knowledge. In addition to studying husbandry-related diseases, this approach can be used to solve other problems that can constrain the success of CBPs, such as reproductive or behavioural problems. For example, at the Vancouver Aquarium, captive populations of endangered Northern Leopard Frogs (*Lithobates pipiens*) and Oregon Spotted Frogs (*Rana pretiosa*) are breeding, but their reproductive output is currently too low to produce the number of tadpoles and metamorphs for release necessary to prevent their extirpation in the wild according to existing PVA models (Kissel *et al.* 2017; pers comm. Kendra Morgan). This problem sets the stage for a study to test hypotheses on (a) which parameters (eg. egg mass size, clutch fertility, hatching success) are primarily responsible for the overall low reproductive output of these species in captivity, and (b) what factors (eg. gamete quality, behaviour, hormones) are causing the identified parameters to be low. Low reproductive output is a common problem in amphibian CPBs (Browne & Zippel 2007), so this study could contribute valuable knowledge that could help elevate the success rate of amphibian CBPs and increase our understanding of amphibian reproductive biology in general.

As mentioned in my thesis introduction, habitat loss, pollution, and overexploitation are driving amphibian declines on a global scale. These threats can be mitigated through habitat protection, legislation, and public outreach, but declines are still expected to continue due to, inter alia, disease in protected areas where *Bd* and *Bsal* have already been introduced, leaving captive breeding as the last hope for the survival of many species. Despite the capacity of CBPs to succeed and contribute to science, the question remains as to whether or not it is ethically correct to maintain species in captivity with so much uncertainty about when and how they will return to the wild due to the unresolved threat of disease. Furthermore, if the ecological risks and benefits of captive breeding and release programs are not carefully weighted, they have the potential to do more harm than good through the depletion of remaining populations and outbreeding depression (Araki et al. 2007; McCleery et al. 2014; Heinrichs et al. 2019). I believe that it is ethically justifiable to keep these species in captivity because we simply do not have enough information to assume that they cannot be successfully released in the future, and so discussion of pulling the plug at this point is premature. For example, research initiatives are currently underway to investigate the feasibility of release of chytridiomycosis-sensitive species into their native range; the first trial release of captive-bred *Atelopus limosus* frogs from the PARCP into the Mamoní valley preserve in Panama began in 2017, and will provide important data on the ecology, health, and survival of individuals post-release. Likewise, studies quantifying the effects of collecting amphibians for conservation CBPs on wild population health and the genetic fitness risks of interbreeding between captive-bred and wild amphibians must be carried out before deciding if captive breeding and release is an appropriate conservation action for a species. The need for the release of captive-bred individuals is still largely unknown: though the recovery capacity of species to chytrid is poorly understood (Newell et al. 2013), if remnant or recovering populations of species previously thought to be extinct in the wild are found, then perhaps there is no need to reintroduce individuals from captivity. But we must be ready to not find any survivors. Until we find out more about the feasibility of releasing captive-bred individuals and their subsequent success, and until we know more about the recovery capacity of individuals in the wild, I argue that CBPs have a clear purpose to efficiently safeguard amphibian biodiversity from human-caused extinction while providing new scientific information. Shepherding these species is the least we can do.

References

Alroy, John. (2015) Current Extinction Rates of Reptiles and Amphibians. *Proceedings of the National Academy of Sciences of the United States of America, 112*(42), 13003–8.

Altig R. (1970) A key to the tadpoles of the continental United States and Canada. *Herpetologica,* 26, 180–207.

Alvarez, D., & Nicieza, A. G. (2002). Effects of temperature and food quality on anuran larval growth and metamorphosis. *Functional Ecology*, *16*(5), 640–648. http://doi.org/10.1046/j.1365-2435.2002.00658.x

Anganoy-Criollo, M. (2013). Tadpoles of the high-Andean *Hyloxalus subpunctatus* (Anura: Dendrobatidae) with description of larval variation and species distinction by larval morphology. *Papéis Avulsos de Zoologia*, 53, 221-224. https://dx.doi.org/10.1590/S0031-10492013001500001

Araki, H., Cooper, B. and Blouin, M. (2007). Genetic Effects of Captive Breeding Cause a Rapid, Cumulative Fitness Decline in the Wild. *Science*, 318(5847), 100-103.

Araki, H., Cooper, B. and Blouin, M. (2009). Carry-over effect of captive breeding reduces reproductive fitness of wild-born descendants in the wild. *Biology Letters*, 5(5), 621-624.

Baldwin, G. F., & Bentley, P. J. (1980). Calcium metabolism in Bullfrog tadpoles (*Rana catesbeiana*). *Journal of Experimental Biology*, *88*, 357–365.

Balmford, A., Mace, G. M., & Leader-Williams, N. (1996). Designing the ark: Setting priorities for captive breeding. *Conservation Biology,* 10, 719–727.

Bartoń, K. (2018). MuMIn: Multi-Model Inference. R package version 1.42.1. https://CRAN.R-project.org/package=MuMIn

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1-48.

Batista, A., Jaramillo, C., Ponce, M., and Crawford, A. (2014) A new species of Andinobates (Amphibia: Anura: Dendrobatidae) from west central Panama. *Zootaxa*, 3866, 333–52. https://doi.org/10.11646/zootaxa.3866.3.2

Bauer, L. (1994) New names in the family Dendrobatidae (Anura, Amphibia). *Ripa*, Fall, 1-6.

Bernal, M., Luna, V., Gallego, O., and Quevedo, A. (2007) A new species of poison frog (Amphibia: Dendrobatidae) from the Andean mountains of Tolima, Colombia. *Zootaxa*, 1638, 59-68. https://doi.org/10.5281/zenodo.179597.

Biega, Alannah. 2017. “Evaluating the Role of Zoos and Ex Situ Conservation in Global Amphibian Recovery.” Simon Fraser University.

Bloxam, Q. M. C., & Tonge, S. J. (1995). Amphibians: suitable candidates for breeding-release programmes. *Review of Industrial Organization,* 4, 636–644.

Brown, J., Twomey, E., Amezquita, A., De Sousa, M., Caldwell, J.L., Lötters, S., Von May, R., Melo-Sampaio, P., Mejía-Vargas, D., Perez-Peña, P., Pepper, M., Poelman, E., Sanchez-Rodriguez, M., and Summers, K. (2011) A taxonomic revision of the Neotropical poison frog genus *Ranitomeya* (Amphibia: Dendrobatidae). *Zootaxa*, 3083, 1-120. https://doi.org/10.1055/sos-SD-201-00174

Brown, J, Evan, T, Morales, V., and Summers, K. (2008) Phytotelm size in relation to parental care and mating strategies in two species of Peruvian poison frogs. *Behaviour*, 145, 1139-1165. https://doi.org/10.1163/156853908785387647

Browne, R. K., & Zippel, K. (2007). Reproduction and Larval Rearing of Amphibians. *ILAR Journal,48*(3), 214-234. doi:10.1093/ilar.48.3.214

Browne, R., Pomering, M., & Hamer, A. (2003) High density effects on the growth, development and survival of *Litoria aurea* tadpoles. *Aquaculture,* 215, 109-121.

Caldwell, J., & de Oliveira, R. L. (1999) Determinants of biparental care in the spotted poison frog, *Dendrobates vanzolinii* (Anura: Dendrobatidae). *Copeia*, 3, 565-575.

Camperio Ciani, J. F., Guerrel, J., Baitchman, E., Diaz, R., Evans, M., Ibáñez, R., Ross, H., Klaphake, E., Nissen, B., Pessier, A. P., Power, M. L., Arlotta, C., Snellgrove, D., Wilson, B., & Gratwicke, B. (2018). The relationship between spindly leg syndrome incidence and water composition, overfeeding, and diet in newly metamorphosed harlequin frogs (*Atelopus* spp.). *PLOS ONE*, *11*, 445–453. http://doi.org/10.1371/journdal.pone.0204314

Claunch, N., & Augustine, L. (2015). Morphological description of spindly leg syndrome in Golden Mantella (*Mantella aurantiaca*) at the Smithsonian National Zoological Park. *Journal of Herpetological Medicine and Surgery*, *25*(3–4), 72–77. http://doi.org/10.5818/1529-9651-25.3.72

Conde, D. A., F. Colchero, B. Güneralp, M. Gusset, B. Skolnik, M. Parr, O. Byers, K. Johnson, G. Young, N. Flesness, H. Possingham, and J. E. Fa (2015). Opportunities and costs for preventing vertebrate extinctions. *Current Biology*, 25, 219–221.

Connelly, S., Pringle, C. M., Bixby, R. J., Brenes, R., Whiles, M. R., Lips, K. R., Kilham, S. & Huryn, A. D. (2008). Changes in Stream Primary Producer Communities Resulting from Large-Scale Catastrophic Amphibian Declines: Can Small-Scale Experiments Predict Effects of Tadpole Loss?. *Ecosystems,* 11, 1262-1276.

Crawford, Andrew J, Karen R Lips, and Eldredge Bermingham. 2010. “Epidemic Disease Decimates Amphibian Abundance, Species Diversity, and Evolutionary History in the Highlands of Central Panama.” *Proceedings of the National Academy of Sciences of the United States of America* 107(31): 13777–82. http://www.ncbi.nlm.nih.gov/pubmed/20643927 (April 19, 2019).

Crump, Martha L. 2015. *Eye of Newt and Toe of Frog, Adder’s Fork and Lizard’s Leg : The Lore and Mythology of Amphibians and Reptiles*. eds. Martha L. Crump and Danté B Fenolio. Chicago: University of Chicago Press.

Daly, J. W., Garraffo, H. M., Spande, T. F., Decker, M. W., Sullivan, J. P., & Williams, M. (2000). Alkaloids from frog skin: The discovery of epibatidine and the potential for developing novel non-opioid analgesics. *Natural Product Reports, 17*(2), 131-135. doi:10.1039/a900728h

Dodd, C. K. (2010). *Amphibian ecology and conservation: A handbook of techniques*. Oxford: Oxford University Press.

Egea-Serrano, A., Relyea, R. A., Tejedo, M., & Torralva, M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and Evolution*, *2*(7), 1382–1397. http://doi.org/10.1002/ece3.249

Etkin, W. (1964). Metamorphosis. In J. Moore (Eds.), *Physiology of the Amphibian*. (pp. 427-468) New York, NY: Academic Press.

Ferrie, G.M., Vance, C., Alford, V., Atkinson, J., Baitchman, E., Barber, D., Blaner, W. S. Crawshaw, G., Deneault, A., Finke, M., Fleming, G., Gagliardo, R., Hoffman, E. A., Karasov, W., Klassing, K., Koutsos, El., Lankton, J., Lavin, S. R., Lentini, A., Livingston, S., Mason, T., McComb, A., Morrtis, C., Pessier, A. P., Olea-Popelka, F., Probst, T., Rodriguez, C., Schad, K., Semmen, K., Sincage, J., Stamper, A. M., Steinmetz, J., Sullivan, K., Terrell, S., Wertan, N., Wheaton, C., Wilson, B., & Valdes, E.V. (2014) Nutrition and Health in Amphibian Husbandry. *Zoo biolog, 33*(6): 485–501.

Frost, Darrel R. (2018) Amphibian Species of the World: An Online Reference. Version 6.0. Dendrobatidae. Available from http://research.amnh.org/herpetology/amphibia/index.html (accessed March 6, 2018).

Gelman, A. (2008) Scaling regression inputs by dividing by two standard deviations. *Statistics in medicine*, *27*, 2865–2873. http://doi.org/10.1002/sim.3107

Gielens, A. (2008) Unknown Pathogen or Parasite Decimates Head - Start Rana Pretiosa Tadpoles in Captive Husbandry Program. Unpublished report poster for the Oregon Spotted Frog Recovery Team.

Gosner, K. (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16, 183-190.

Grant, T., Frost, D., Caldwell, J., Gagliardo, R., Haddad, C., Kok, P., Means, D., Noonan, B., Schargel, W., and Wheeler, W. (2006) Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History*, 299, 1-262.

Grant, T., Rada, M., Anganoy-Criollo, M., Batista, A., Dias, P., Jeckel, A., Machado, D., and Rueda-Almonacid, J. (2017) Phylogenetic systematics of dart-poison frogs and their relatives revisited (Anura: Dendrobatoidea). *South American Journal of Herpetology*, 12, S1-S90.

Gratwicke, B., Lovejoy, T. E., & Wildt, D. E. (2012). Will amphibians croak under the Endangered Species Act? *BioScience*, *62*(2), 197–202. http://doi.org/10.1525/bio.2012.62.2.13

Griffiths, R. A., Denton, J., & Wong, A. L.-C. (1993). The effect of food level on competition in tadpoles: interference mediated by protothecan algae? *The Journal of Animal Ecology*, *62*(2), 274. http://doi.org/10.2307/5358

Grueber, C. E., Nakagawa, S., Laws, R. J., & Jamieson, I. G. (2011). Multimodel inference in ecology and evolution: challenges and solutions. *Journal of Evolutionary Biology*, *24*(4), 699–711. http://doi.org/10.1111/j.1420-9101.2010.02210.x

Guirguis, S. M. E., Yee, J. C., & Stiffler, D. F. (1997). Active calcium ion transport in *Xenopus laevis* skin. *Journal of Comparative Physiology B*, *167*(5), 328–334. http://doi.org/10.1007/s003600050081

Hagman, Mattias, and Richard Shine. 2007. “Effects of Invasive Cane Toads on Australian Mosquitoes: Does the Dark Cloud Have a Silver Lining?” *Biological Invasions,* 9(4): 445–52. http://link.springer.com/10.1007/s10530-006-9051-3 (April 20, 2019).

Hakvoort, H., Gouda, E., Zwart, P., & Matz, G. (1995). Skeletal and muscular underdevelopment (SMUD) in Dendrobatidae. *Proceedings of the 5th International Symposium of Pathology of Reptiles and Amphibians in Alphen Aan Den Rijn, The Netherlands*, 271–276.

Harding, G., Griffiths, R. A., & Pavajeau, L. (2016). Developments in amphibian captive breeding and reintroduction programs. *Conservation Biology*, *30*(2), 340–349. http://doi.org/10.1111/cobi.12612

Hoffmann, M., Hilton-Taylor, C., Angulo, A., Böhm, M., Brooks, T. M., Butchart, S. H., Carpenter, K. E., Chanson, J., Collen, B., Cox, N. A., Darwall, W. R., Dulvy, N. K., Harrison, L. R., Katariya, V., Pollock, C. M., Quader, S., Richman, N. I., Rodrigues, A. S., Tognelli, M. F., Vié, J. C., Aguiar, J. M., Allen, D. J., Allen, G. R., Amori, G., Ananjeva, N. B., Andreone, F., Andrew, P., Aquino, Ortiz, A. L., Baillie, J. E., Baldi, R., Bell ,B. D., Biju, S. D., Bird, J. P., Black-Decima, P., Blanc, J. J., Bolaños, F., Bolivar-G., W., Burfield, I. J., Burton, J. A., Capper, D. R., Castro, F., Catullo, G., Cavanagh, R. D., Channing, A., Chao, N. L., Chenery, A. M., Chiozza, F., Clausnitzer, V., Collar, N. J., Collett, L. C., Collette, B. B., Cortez, Fernandez, C. F., Craig, M. T., Crosby, M. J., Cumberlidge, N., Cuttelod, A., Derocher, A. E., Diesmos, A.C., Donaldson, J.S., Duckworth, J.W., Dutson, G., Dutta, S.K., Emslie, R. H., Farjon, A., Fowler, S., Freyhof, J., Garshelis, D. L., Gerlach, J., Gower, D. J., Grant, T. D., Hammerson, G. A., Harris, R. B., Heaney, L. R., Hedges, S. B., Hero, J. M., Hughes, B., Hussain, S. A., Icochea, M. J., Inger, R. F., Ishii, N., Iskandar, D. T., Jenkins, R. K., Kaneko, Y., Kottelat, M., Kovacs, K. M., Kuzmin, S. L., La Marca E., Lamoreux, J. F., Lau, M. W., Lavilla, E. O., Leus, K, Lewison, R. L., Lichtenstein, G., Livingstone, S. R., Lukoschek, V., Mallon, D. P., McGowan, P. J., McIvor, A., Moehlman, P. D., Molur S., Muñoz Alonso, A., Musick, J. A., Nowell, K., Nussbaum, R. A., Olech, W., Orlov, N. L., Papenfuss, T. J., Parra-Olea, G., Perrin, W. F., Polidoro, B. A., Pourkazemi, M., Racey, P. A, Ragle, J. S., Ram, M., Rathbun, G., Reynolds, R. P., Rhodin, A. G., Richards, S. J., Rodríguez, L. O., Ron, S. R., Rondinini, C., Rylands, A. B., Sadovy de Mitcheson, Y., Sanciangco, J. C., Sanders, K. L., Santos-Barrera, G., Schipper, J., Self-Sullivan, C., Shi, Y., Shoemaker, A., Short, F. T., Sillero-Zubiri, C., Silvano, D. L., Smith, K. G., Smith, A. T., Snoeks, J., Stattersfield, A. J., Symes, A. J., Taber, A. B., Talukdar, B. K., Temple, H. J., Timmins, R., Tobias, J. A., Tsytsulina, K., Tweddle, D., Ubeda, C., Valenti, S. V., van Dijk, P. P., Veiga, L. M., Veloso, A., Wege, D. C., Wilkinson, M., Williamson, E. A., Xie, F., Young, B. E., Akçakaya, H. R., Bennun, L., Blackburn, T. M., Boitani, L., Dublin, H. T., da Fonseca, G. A., Gascon, C., Lacher, T. E. Jr, Mace, G. M., Mainka, S. A., McNeely, J. A., Mittermeier, R. A., Reid, G. M., Rodriguez, J. P., Rosenberg, A. A., Samways, M. J., Smart J., Stein, B. A., Stuart, S. N. (2010). The impact of conservation on the status of the world's vertebrates. *Science, 330*(6010), 1503-1509.

Hota, A. K., & Dash, M. C. (1981). Growth and metamorphosis of *Rana tigrina* larvae: effects of food level and larval density. *Oikos*, *37*(3), 349-352.

Huey, D. W., & Beitinger, T. L. (1980). Toxicity of nitrite to larvae of the salamander *Ambystoma texanum*. *Bulletin of Environmental Contamination and Toxicology, 25*(6), 909-912.

IUCN (2018) The IUCN Red List of Threatened Species. Version 2018-1. Available from http://www.iucnredlist.org (Accessed 23 Oct 2018)

IUCN SSC Amphibian Specialist Group. In press. *Andinobates geminisae*. The IUCN Red List of Threatened Species.

Jungfer, K.H., Weygoldt, P., and Juraske, N. (1996) Dendrobates vicentei, ein neuer pfeilgiftfrosch aus zentral-Panama. *Herpetofauna*, 18, 17-26.

Kagemann, Catherine, and Barbara Han. (2015) "The Effects of Amphibian Presence and Predation On Mosquitoes." Cary Institute.

Khatiwada, J. R., Ghimire, S., Khatiwada, S. P., Paudel, B., Bischof, R., Jiang, J., & Haugaasen, T. (2016). Frogs as potential biological control agents in the rice fields of Chitwan, Nepal. *Agriculture, Ecosystems & Environment, 230*, 307-314. doi:10.1016/j.agee.2016.06.025

Kishimoto, K., and Hayashi, F. (2017) The complete embryonic and larval stages of the oophagous frog *Kurixalus eiffingeri* (Rhacophoridae). *Current Herpetology*, 36, 37-45.

Kissel, Palen, & Govindarajulu. 2017. A Decision-theory Approach to Cost-effective Population Supplementation for Imperiled Species. *Ecological Economics* 142:194-202

Krishnamurthy, P., and Frances, R. (2012) A critical review on the utility of DNA barcoding in biodiversity conservation. *Biodiversity and Conservation*, 21, 1901-1919. https://doi.org/10.1007/s10531-012-0306-2

Kuramoto, M, and Wang, C-S. (1987) A new rhacophorid treefrog from Taiwan, with comparisons to *Chirixalus eiffingeri* (Anura, Rhacophoridae). *Copeia*, 1987, 931-42.
https://doi.org/10.2307/1445556

Marco, A., & Blaustein, A. R. (1999). The effects of nitrite on behavior and metamorphosis in Cascades Frogs (*Rana cascadae*). *Environmental Toxicology and Chemistry*, *18*(5), 946-949.

Marlett, J., Eichler, V., & Fellows-Hagemeister, M. (1988). Spindly leg syndrome in frogs: present knowledge and future directions to elucidate its causes. Proceedings of the *International Herpetological Symposium: Captive Propagtion and Husbandry*, 11, 145–150.

Marshall, G. A., Amborski, R. L., & Culley, D. D. (1980). Calcium and ph requirements in the culture of Bullfrog (*Rana catesbeiana*) larvae. *Proceedings of the World Mariculture Society*, 453(11), 445–453. http://doi.org/https://doi.org/10.1111/j.1749-7345.1980.tb00139.x 19.

Mendelson, J. R., Lips, K. R., Gagliardo, R. W., Rabb, G. B., Collins, J. P., Diffendorfer, J. E., Daszak, P., Ibáñez D., R., Zippel, K. C., Lawson, D. P., Wright, K. M., Stuart, S. N., Gascon, C., da Silva, H. R., Burrowes, P. A., Joglar, R. L, La Marca, E., Lötters, S., du Preez, L. H., Weldon, C., Hyatt, A., Rodriguez-Mahecha, J. V., Hunt, S., Robertson, H., Lock, B., Raxworthy, C. J., Frost, D. R., Lacy, R. C., Alford, R. A., Campbell, J. A., Parra-Olea, G., Bolaños, F., Calvo Domingo, J. J., Halliday, T., Murphy, J. B., Wake, M. H., Coloma, L. A., Kuzmin, S. L., Price, M. S., Howell, K. M., Lau, M., Pethiyagoda, R., Boone, M., Lannoo, J. M., Blaustein, A. R., Dobson, A., Griffiths, R. A., Crump, M. L, Wake, D. B., & Brodie, Jr., E. D. 2006. Confronting amphibian declines and extinctions. *Science,* 313(5783), pp.48.

Márquez, R., Mejía-Vargas, D., Palacios-Rodríguez, P., Ramírez-Castañeda, V., & Amézquita, A. (2017) A new species of *Andinobates* (Anura: Dendrobatidae) from the Urabá region of Colombia. *Zootaxa*, 4290, 531-46.

McCleery, R., Hostetler, J. and Oli, M. (2014). Better off in the wild? Evaluating a captive breeding and release program for the recovery of an endangered rodent. *Biological Conservation*, 169, 198-205.

McDiarmid, R., and Altig, R. (1999) Tadpoles: The Biology of Anuran Larvae. University of Chicago Press. Chicago, Illinois, 458 pp.

Murray, D. L. (1990). The effects of food and density on growth and metamorphosis in larval Wood Frogs (*Rana sylvatica*) from central Labrador. *Canadian Journal of Zoology*, 68(6), 1221–1226.

Myers, C., and Daly J. (1976) Preliminary evaluation of skin toxins and vocalizations in taxonomic and evolutionary studies of poison-dart frogs (Dendrobatidae). *Bulletin of the American Museum of Natural History*, 157, 173-262.

Myers C. and Daly J. (1980) Taxonomy and ecology of *Dendrobates bombetes*, a new Andean poison frog with new skin toxins. *American Museum Novitates*, 2692, 1-23.

Myers C., Daly J., and Martinez V. (1984) An arboreal poison frog (*Dendrobates*) from western Panama. *American Museum Novitates*, 2783, 1-20.

National Center for Biotechnology Information (2019). PubChem Database. Calcium phosphate, CID=24456, https://pubchem.ncbi.nlm.nih.gov/compound/24456 (accessed on Apr. 12, 2019)

Newell, D. A., Goldingay, R. L., & Brooks, L. O. (2013). Population Recovery following Decline in an Endangered Stream-Breeding Frog (*Mixophyes fleayi*) from Subtropical Australia. *PLoS ONE*, *8*(3), 58559. http://doi.org/10.1371/journal.pone.0058559

Odum, R. A., & Zippel, K. (2011). "Water quality" Amphibian Ark. Retrieved from http://www.amphibianark.org/wp-content/uploads/2018/08/Water-Quality-Odum-Zippel-2011.pdf

Oguro, C., Sasayama, Y., Ikadai, H., & Yoshizawa, H. (1975). Changes in calcium concentrations of serum and coelomic fluid of the Bullfrog, *Rana catesbeiana*, during larval development and metamorphosis. *Development, Growth & Differentiation*, 17(1), 71–76. http://doi.org/10.1111/J.1440-169X.1975.00071.X

Pilkington, J. B., & Simkiss, K. (1966). The mobilization of the calcium carbonate deposits in the endolymphatic sacs of metamorphosing frogs. *Journal of Experimental Biology*, 45(2), 329-341. http://jeb.biologists.org/content/jexbio/45/2/329.full.pdf

Pollack, E., & Liebig, V. (1989). Abstract 724: An induced developmental disorder of limbs and motor neurons in *Xenopus*. *American Zoology*, *29*(163A).

Poole, V. a, & Grow, S. (2012). Amphibian Husbandry Resource Guide Edition, 2.0. *Association of Zoos and Aquariums*

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raghavendra, K., P. Sharma, and A.P. Dash. (2008) Biological Control of Mosquito Populations through Frogs: Opportunities & Constrains. *Indian Journal of Medical Research,* 128: 22–25.

Ranvestel, A. W., Lips, K. R., Pringle, C. M., Whiles, M. R., & Bixby, R. J. (2004). Neotropical tadpoles influence stream benthos: Evidence for the ecological consequences of decline in amphibian populations. *Freshwater Biology, 49*(3), 274-285. doi:10.1111/j.1365-2427.2004.01184.x

Richards-Zawacki, C., Wang, I., and Summers K. (2012) Mate choice and the genetic basis for colour variation in a polymorphic dart frog: inferences from a wild pedigree. *Molecular Ecology*, 21, 3879-3892.

Robertson, D. R. (1976). Diurnal and lunar periodicity of intestinal calcium transport and plasma calcium in the frog, *Rana pipiens*. *Comparative Biochemistry and Physiology Part A: Physiology*, *54*(2), 225–231. http://doi.org/10.1016/S0300-9629(76)80102-8

Robertson, D. R. (1975). Effects of the ultimobranchial and parathyroid glands and vitamins D_2 , D_3 and dihydrotachysterol$_2$ on blood calcium and intestinal calcium transport in the frog. *Endocrinology*, 96(4), 934–940. http://doi.org/10.1210/endo-96-4-934

Rodríguez, Candelario et al. 2017. “Toxins and Pharmacologically Active Compounds from Species of the Family Bufonidae (Amphibia, Anura).” *Journal of Ethnopharmacology,* 198: 235–54.

Rowley, J.J.L., Tran, D.T.A., Le, D.T.T., Hoang, H.D., and Altig, R. (2012) The strangest tadpole: the oophagous, tree-hole dwelling tadpole of *Rhacophorus vampyrus* (Anura: Rhacophoridae) from Vietnam. *Journal of Natural History*, 46, 2969-2978. https://doi.org/10.1080/00222933.2012.732622

Rudh, A., Rogell, B., and Höglund, J. (2007) Non-gradual variation in colour morphs of the strawberry poison frog *Dendrobates pumilio*: genetic and geographical isolation suggest a role for selection in maintaining polymorphism. *Molecular Ecology*, 16, 4284-4294.https://doi.org/10.1111/j.1365-294X.2007.03479.x

Rueda-Almonacid, J., Rada, M., Sánchez-Pacheco, S., Velásquez-Álvarez, A., and Quevedo, A. (2006) Two new and exceptional poison dart frogs of the genus *Dendrobates* (Anura: Dendrobatidae) from the northeastern flank of the Cordillera Central of Colombia. *Zootaxa*, 1259, 39-54.

Ruiz-Carranza, P.M., and Ramírez-Pinilla, M.P. (1992) Una nueva especie de *Minyobates* (Anura: Dendrobatidae) de Colombia. *Lozania*, 61, 1-16.

Ryan, Mason J., Jay M. Savage, Karen R. Lips, & J. Tomasz Giermakowski. (2010) A New Species of the *Craugastor Rugulosus* Series (Anura: Craugastoridae) from West-Central Panama." *Copeia,* 2010(3): 405–9.

Ryan, M., and Barry, D. (2011) Competitive interactions in phytotelmata-breeding pools of two poison-dart frogs (Anura: Dendrobatidae) in Costa Rica. *Journal of Herpetology*, 45, 438-443. https://doi.org/10.2307/41415313.

Sánchez, D. (2013) Larval morphology of dart-poison frogs (Anura: Dendrobatoidea: Aromobatidae and Dendrobatidae). *Zootaxa*, 3637, 569-91.https://doi.org/10.11646/zootaxa.3637.5.5

Savage, J. (1968) The dendrobatid frogs of Central America. *Copeia*, 1968, 745-776. https://doi.org/10.2307/1441845

Scheele, B.C, Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., Burrowes, P.A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M.C., Flechas, S.V., Foster, C.N., Frías-Álvarez, P., Garner, T. W.J., Gratwicke, B., Guayasamin, J.M., Hirschfeld, M., Kolby, J.E., Kosch, T.A., La Marca, E., Lindenmayer, D.B., Lips, K.R., Longo, AV., Maneyro, R., McDonald, C.A., Mendelson III, J., Palacios-Rodriguez, P., Parra-Olea, G., Richards-Zawacki, C.L., Rödel, M.O., Rovito, S.M., Soto-Azat, C., Toledo, L.F., Voyles, J., Weldon, C., Whitfield, S.M., Wilkinson, M., Zamudio, K.R., & Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science,* 363 (6434), 1459-146.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D., Hartenstein, V., Eliceiri, K.,Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9, 676-682. https://doi.org/10.1038/nmeth.2019.

Silverstone, P. (1975) A revision of the poison-arrow frogs of the genus *Dendrobates* Wagler. *Bulletin of Los Angeles County Museum of Natural History*, 21, 1-53.

Stiffler, D. F. (1993). Amphibian calcium metabolism. *Journal of experimental Biology*, 184, 47-61.

Stiffler, D. F. (1995). Active calcium transport it the skin of the frog *Rana pipiens*: kinetics and seasonal rythms. *Journal of Experimental Biology*, *198*, 967–974.

Stiffler, D. F. (1996). Exchanges of calcium with the environment and between body compartments in amphibia. *Physiological Zoology*, 69(2), 418–434.

Stiffler, D. F., Eskandari, S., & Dejbakhsh, S. (1997). Cutaneous transport in Ca^{2+} in the frog *Rana pipiens*: significance and specificity. *The Journal of Experimental Zoology*, 277(5), 371–381.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science, 306*(5702), 1783–1786.

Stynoski, J. (2012) Behavioral ecology of parental care in a dendrobatid frog (*Oophaga pumilio*). University of Miami Open Access Dissertations. 755.

Tapley, B., Michaels, C. J., Gumbs, R., Böhm, M., Luedtke, J., Pearce-Kelly, P., & Rowley, J. J. (2018). The disparity between species description and conservation assessment: A case study in taxa with high rates of species discovery. *Biological Conservation, 220*, 209-214. doi:10.1016/j.biocon.2018.01.022

Tapley, Benjamin, Kay S. Bradfield, Christopher Michaels, and Mike Bungard. (2015) Amphibians and Conservation Breeding Programmes: Do All Threatened Amphibians Belong on the Ark? *Biodiversity and Conservation,* 24(11): 2625–46.

Twomey, E., Brown, J. (2008) Spotted poison frogs: rediscovery of a lost species and a new genus (Anura: Dendrobatidae) from northwestern Peru. *Herpetologica*, 64, 121-137. https://doi.org/10.1655/07-009.1.

Van Wijngaarden, R, and Bolaños, F. (1992) Parental care in *Dendrobates granuliferus* (Anura: Dendrobatidae), with a description of the tadpole. *Journal of Herpetology*, 26, 102-105. https://doi.org/10.2307/1565037

Vassilieva, A., Galoyan, E., and Poyarkov, N. (2013) *Rhacophorus vampyrus* (Anura: Rhacophoridae) reproductive biology: a new type of oophagous tadpole in Asian treefrogs. *Journal of Herpetology,* 47, 607-614. https://doi.org/10.1670/12-180

Wang, I., and Summers, K. (2010) Genetic structure is correlated with phenotypic divergence rather than geographic isolation in the highly polymorphic strawberry poison-dart frog. *Molecular Ecology*, 19, 447-458. https://doi.org/10.1111/j.1365-294X.2009.04465.x

Wassersug, R., Frogner, K., and Inger, R. (1981) Adaptations for life in tree holes by rhacophorid tadpoles from Thailand. *Journal of Herpetology*, 15, 41-52. https://doi.org/10.2307/1563645

Wells, K. D. (2010). *The Ecology and Behavior of Amphibians*. Chicago, IL: University of Chicago Press.

Weygoldt, P. (1987) Evolution of parental care in dart poison frogs (Amphibia: Anura: Dendrobatidae). *Zeitschrift für zoologische Systematik und Evolutionsforschung*, 25, 51-67. https://doi.org/10.1111/j.1439-0469.1987.tb00913.x

Whiles, M. R., Lips, K. R., Pringle, C. M., Kilham, S. S., Bixby, R. J., Brenes, R., . . . Peterson, S. (2006). The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. *Frontiers in Ecology and the Environment, 4*(1), 27-34. doi:10.1890/1540-9295(2006)004[0027:teoapd]2.0.co;2

Wickham H (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4

Willoughby, J. R., & Christie, M. R. (2018). Long-term demographic and genetic effects of releasing captive-born individuals into the wild. *Conservation Biology, 33*(2), 377-388. doi:10.1111/cobi.13217

Woodford, M. H., & Rossiter, P. B. (1994). Disease risks associated with wildlife translocation projects. *Creative Conservation,* 178-200. doi:10.1007/978-94-011-0721-1_9

Wright, Kevin M., and Brent R. Whitaker. 2001. *Amphibian Medicine and Captive Husbandry*. Krieger Pub. Co.

Zerella, M. J., & Stiffler, D. F. (1999). Active transport of calcium ions in the skin of the salamander *Ambystoma tigrinum*. *Journal of Comparative Physiology*, 169(7), 481–486. https://link.springer.com/content/pdf/10.1007%2Fs003600050245.pdf