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Addressing Amphibian Decline Through the Amphibian Conservation Action Plan

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UNIVERSITY OF MIAMI

ADDRESSING AMPHIBIAN DECLINE THROUGH THE AMPHIBIAN
CONSERVATION ACTION PLAN

By

Danté B. Fenolio

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2009

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ADDRESSING AMPHIBIAN DECLINE THROUGH THE AMPHIBIAN
CONSERVATION ACTION PLAN

Danté B. Fenolio

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The amphibian decline phenomenon now involves in excess of a third of the roughly 6000 species of amphibians on the planet. The problems that drive the declines are diverse with no end in sight. The Amphibian Conservation Action Plan (ACAP) aims to stem amphibian decline through four recommended actions by researchers and conservation biologists: (1) Expand scientific understanding of amphibian declines and extinctions; (2) continue to document amphibian diversity and ecology and how they are changing; (3) develop and implement long-term conservation programs; (4) prepare emergency response actions for eminent crises. This Dissertation focused on two of those recommendations: expanding scientific understanding of amphibian declines and extinctions and continuing to document amphibian diversity and ecology and how they are changing. The first chapter is a review of the amphibian decline phenomenon. The second, third, and fourth chapters focus on expanding scientific understanding of amphibian diversity and ecology with the description of a formerly unknown species (chapter 2), and ecological papers on two poorly known species (chapters 3 and 4). Chapter five focuses on the first ACAP recommendation in improving scientific understanding of the causes behind amphibian decline. The chapter is an experimental examination of two related species and their developmental reactions to common heavy

metal contaminants. The goal of this Dissertation is to contribute toward the general amphibian knowledge base relative to the recommendations of ACAP.

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CHAPTER 1:

An Introduction to the Amphibian Decline Phenomenon and the Impetus for this Dissertation

Introduction

Recent reports suggest that as much as a third of all known amphibians are in decline, many inhabiting areas far from obvious human disturbances (Stuart et al., 2004). To put amphibian decline into perspective numerically, about 2000 of roughly 6300 described species are seriously threatened (GAA, 2007). Such dramatic declines in amphibian populations have elicited numerous hypotheses for the ongoing phenomenon. The hypotheses are varied and recent hypotheses have become increasingly complicated in nature. Recent arguments are complex but can be clarified by first explaining the history behind the problem.

Mass amphibian declines have been apparent to the scientific community since the early 1980s. The first informal reports were made at meetings that suggested a global decline might be unfolding (Collins and Storer, 2003). Several particularly high profile declines in the 1980s were initially discussed at the First World Congress of Herpetology (1989). Costa Rica's golden toad, *Bufo periglenes*, and one of the country's harlequin frogs, *Atelopus varius*, had both declined sharply from within Monte Verde Cloud Forest Preserve between 1988 and 1989 (Crump et al., 1992; Pounds and Crump, 1994). Just earlier, a gastric brooding frog from Australia (*Rheobatrachus vitellinus*) had also disappeared between 1985 and 1986 (Retallick et al., 2004). These declines were not immediately apparent. Rather, one or two years persisted where a few individuals were observed but far fewer than were normally recorded. Several more years with no observations passed, culminating in the apparent extinctions of *Bufo periglenes* (Pounds

and Crump, 1994) and of *Rheobatrachus vitellinus* (Retallick et al., 2004). At that point, unusually warm and dry weather at the cloud forest site, where the disappearances in Central America had been recorded, was suspect.

Amphibian decline was formally discussed at the National Research Council Workshop in 1990. The general consensus among scientists was that declines were occurring, but it was not clear exactly why or if they all stemmed from a similar problem (Collins and Storfer, 2003). By this time, papers were being published covering the various hypotheses for the declines (e.g., Blaustein and Wake, 1990; Phillips, 1990; Vitt et al., 1990; Wake, 1991). By the Third World Congress of Herpetology (1997), the reports of declines were far more numerous and compelling (Collins and Storfer, 2003). Wake (1998) then asked the question “Is the threat of amphibian extinctions increasing?” This basic question had no clear answer at that point, although evidence was mounting. Some declines may have begun before the 1980s but they were either unnoticed or were not formally documented. Since that time, several problems have been identified that are involved with specific declines. That amphibian declines are occurring is no longer in question. Problems, and their scope, driving amphibian declines are not universally agreed upon to this day; however, no single cause can account for all declines (Pechmann and Wilbur, 1994; Blaustein, 1994; Pounds et al., 2006; Mendelson et al., 2006). Likewise, dealing with the declines is controversial. One viewpoint holds that we can’t stop the declines and simply need to document them as they unfold (Halliday, 2006). Conversely, an entire program to combat amphibian declines has been developed by scientists on the opposite side of that argument who want to try to intervene (Gascon et al., 2007).

Many problems have driven global amphibian declines, but the details between specific species' declines are unique mixes of circumstance and problems. Different amphibian species, even different populations of the same species, may react to the same environmental problem in dissimilar ways (Stenseth, et al., 2002). Forces driving the declines can therefore be different both on spatial and temporal levels. Issue complexity continues when considering that global changes may directly affect a region and its entire amphibian fauna. Conversely, global changes may induce chain reactions that indirectly affect a region's amphibian fauna (Stenseth, et al., 2002).

Science continues to clarify issues underlying amphibian decline and it is becoming clear that forces behind the declines can combine to form synergies. These synergies can be far more devastating to amphibian populations than any single problem. For this reason, I will expand on this concept of synergistic problems or multiple stressors at the end of my coverage of mechanisms driving amphibian decline (see #8). It is some of these hypotheses that have grown increasingly complex in nature and scope.

The proposed mechanisms for amphibian declines follow:

- (1) Habitat alteration has significantly affected amphibian populations. Long before amphibian decline was a familiar phrase among herpetologists, habitat alteration, including deforestation and wetland removal, was a concern for biologists and forest managers with regard to specific amphibians (e.g. Schuierer, 1961; Crook et al., 1971; Bury and Ruth, 1972). There is no doubt that deforestation has impacted amphibian species and amphibian communities (e.g., Hedges, 1993; Hecnar, 1997; Collins and Strofer, 1993; Silvano and Segalla, 2005). Wetlands removal has also impacted

amphibian populations (e.g., Hecnar, 1997; Kolozsvary and Swihart, 1999; Delis et al., 2004). Further, significant damage can be done to an amphibian population without completely removing a habitat. For example, fragmentation of a habitat, such that movement of an amphibian population between a living area and a breeding area is restricted or halted all together, can have devastating effects. Many studies have documented the damage done by habitat fragmentation to amphibian communities, including road construction (e.g., Hecnar and M'Closkey, 1996; Vos and Chardon, 1998; Kolozsvary and Swihart, 1999). In fact, roads serve as a major mortality factor for local amphibian populations (Carr and Fahrig, 2001; Puky, 2005). Roads and timber harvesting also serve as a source for fine sediments to wash into aquatic habitats (Trombulak and Frissell, 2000; Ashton et al., 2006), degrading them relative to the needs of amphibians living there (Welsh and Oliver, 1998; Ashton et al., 2006). However, at least some amphibian species appear capable of withstanding sediment runoff from roads while maintaining natural population densities (Lowe et al., 2005). Global warming and climate change have been implicated as forces likely to drive amphibian declines by significantly changing a habitat through time (e.g., Ovaska, 1997; Pounds, 2001; Carey and Alexander, 2003; Raffel et al., 2006). Global warming has also been suggested as working synergistically with other forces and is discussed below as a multiple stressor. Without a doubt, habitat alteration has affected amphibian declines. In fact, the Global Amphibian Assessment has indicated that 90% of all amphibian species identified as “declining” are suffering from habitat alteration (Stuart et al., 2004).

(2) The term “harvest” relates to human activities that involve taking amphibians from wild populations. Take can include removing wild amphibians as food for humans (e.g., Jennings and Hayes, 1985; Kaiser, 1994; Humraskar and Velho, 2007), removing amphibians for alleged medicinal purposes (e.g., Daily et al., 1992; Goreman, 1993; Oxford, 2003; Costa-Neto, 2004; Josephs, 2007), removing amphibians for scientific purposes (e.g., Schlaepfer et al., 2005; Nickerson and Briggler, 2007), and removing individuals for commercial reasons and the pet trade (e.g., Wisnieski et al., 1997; Schlaepfer et al., 2005; Nickerson and Briggler, 2007). In some cases, harvest has been recorded across decades (Lannoo et al., 1994) and has involved significantly depleting the target species from a given region (Emmons, 1973; Jennings and Hayes, 1985; Lannoo et al., 1994).

(3) Adding an exotic species to a habitat can adversely affect native amphibians (e.g., Moyle, 1973; Bradford, 1989; Fisher and Shaffer, 1996; Crossland, 1998). For example, introducing exotic sport fish (particularly salmonid fishes) has been detrimental to native species’ larvae because many sport fish prey upon amphibian larvae (e.g., Rudolph, 1980; Hayes and Jennings, 1986; Bradford, 1989; Bradford et al., 1993; Fisher and Shaffer, 1996; Knapp et al., 2001; Pilliod and Peterson, 2001; Carey et al., 2003; Lowe et al., 2005). Bullfrog (*Rana catesbeiana*) introductions have been harmful to native amphibians, particularly west of the Sierra Nevada mountain range in North America. Bullfrogs are believed to prey on native western ranid frogs and to compete with them for prey (e.g., Moyle, 1973; Hammerson, 1982; Jennings and Hayes, 1985; Hayes and Jennings, 1986; Adams, 1999). Another example involves pantropical

introductions of marine toads, *Bufo marinus*. These amphibians have had negative affects on native amphibians by preying upon them and on naive predators that try to consume the toads, succumbing to their formidable skin toxins (Tyler, 1989; Crossland, 1998, 2000). Further, introduced species can hybridize with related native species. Hybridization has the potential to drive a loss in native genetic diversity, swamp the native genes, and drive native endangered species to extinction; such a process has been documented with salamanders of the genus *Ambystoma* in North America (Riley et al., 2003).

(4) The Earth's thinning ozone layer may have increased ultraviolet light exposure to amphibians and their larvae, causing irreparable damage and driving population declines (e.g., Blaustein et al., 1994; Licht and Grant, 1997; Middleton et al., 2001; Hatch and Blaustein, 2003). Increased ultraviolet radiation has been documented in some areas experiencing amphibian declines (Middleton et al., 2001). However, the degree to which amphibians are affected and the actual potential for increased UV exposure driving amphibian decline is not yet conclusive (Palen et al., 2002; Corn and Muths, 2002).

(5) Acidifying both terrestrial waters and terrestrial soils via human industrial activities has been linked to amphibian declines (e.g., Freda and Dunson, 1986; Freda et al., 1991; Dunson et al., 1992; Preest, 1993). Acidification is typically delivered through rain containing acidic industrial biproducts. Aquatic larval amphibians in particular seem sensitive to changes in the pH of their aquatic habitats (Dunson et al.,

1992; Preest, 1993). There is evidence that acidifying the environment can also weaken the immune system of amphibians (Vatnick et al., 2006).

(6) Contaminating the environment with a host of chemical substances has had devastating effects on amphibian populations. For example, nitrate fertilizer use has increased in recent decades with harmful effects. The fertilizers affect local amphibian populations once field runoff reaches wetlands and aquatic habitats (Hecnar, 1995; Hatch and Blaustein, 2003; de Wijer et al., 2004). Heavy metal contamination is often associated with human industrial activities. Negative impacts by heavy metals on amphibians has been well demonstrated (Fort and Stover, 1997; Linder and Grillitsch, 2000; Chen et al., 2006, 2007; Gross et al., 2007). Energy plant byproducts, like coal and fly ash, have saturated local environments with trace elements and harmed resident amphibian populations (Birge, 1978). Similarly, pesticide runoff and contamination has impacted a wide range of amphibians through chemical poisoning, including chemicals that disrupt the endocrine systems of amphibians (e.g., Cole and Casida, 1983; Paulov, 1990; Salibián, 1992; Calumpang et al., 1995; Sparling et al., 2001; Greulich and Pflugmacher, 2003; Datta et al., 2004). Because many pesticides are highly volatile, a portion of each volatile application becomes gaseous vapor in the atmosphere. The pollutants can settle back into the terrestrial environment through rain. For example, Atrazine can be detected in rainwater, even in regions where it is not in use (Hayes et al., 2003). The extensive damage to the amphibian endocrine and reproductive systems by Atrazine is well documented (Allran and Karasov, 2000; Tevera-Mendoza et al., 2002; Hayes et al. 2002; Hayes et al., 2003). One key result of Atrazine exposure is

feminization of male frogs (Hayes et al., 2002, 2006). Another volatile herbicide, Glyphosate, can alter amphibian physiological systems, causing a variety of disorders and death (e.g., Mann et al., 1999; Perkins et al., 2000; Smith, 2001; Relyea, 2005a, 2005b, 2005c). Indirect effects of pesticides have also been documented. Pesticide exposure at low doses may not kill amphibians but instead weakens their immune systems and renders them more susceptible to microbial infection (Christin et al., 2003; Christin, 2004; Hayes et al., 2006).

(7) Emergent infectious diseases have impacted amphibian populations around the globe with devastating consequences. The precipitous declines of countless montane amphibians in Central America received a potential explanation when in 1999 a pathogenic amphibian fungus was described (Longcore et al., 1999). This chytrid fungus, *Batrachochytrium dendrobatidis*, has subsequently been implicated in a wide variety of declines, not just in Central America but also in places like North America, South America, and Australia (Berger et al., 1998; Daszak et al., 2003; Muthsa et al., 2003; Mendelson et al., 2004; Rachowicz et al., 2006; Schloegel et al., 2006). Attention immediately turned to deciphering where the fungus may have originated. There is some genetic evidence that the chytrid fungus affecting amphibian populations has spread recently (Daszak et al., 1999, 2000; Morehouse et al., 2003). The origin of the pathogen has been suggested as Africa and specimens dating back to the 1930s have evidence of the fungus in their skin (Weldon et al., 2004); however, the evidence is not yet conclusive (Rachowicz et al., 2005). If Africa is a point of origin, movement of this pathogen and other pathogens may have taken place via wild collected frogs of the genus *Xenopus* and

their export through tropical aquarium fish and scientific supply companies (Weldon et al., 2004; Reed et al., 2006; Blaustein and Dobson, 2006). *Xenopus* is widely used in studies dealing with genetics, endocrinology, biochemistry, and environmental toxicology.

Fungal infections are not the only emergent infectious diseases that amphibian populations have faced. Bacterial infections have been implicated in some declines (Carey, 1993). *Ranavirus* (iridovirus) have been implicated in large scale mortality of amphibian populations as well (Jancovich et al., 1997; Green et al., 2002; Pearman et al., 2004). Recently, these viruses were identified in salamander larvae sold as bait and from salamanders in a university run *Ambystoma mexicanum* colony. Strains from both Colorado and Arizona demonstrated polyphyly, which suggests a recent spread of the virus and potential spread through commercial transport of salamander larvae (Jancovitch et al., 2005).

(8) Synergy among the above-mentioned forces can create a greater lethal effect than a single force (Vitt et al., 1990; Blaustein, 1994; Pounds, 2001; Sih et al., 2004; Pounds et al., 2006). These interactions are also recognized as multiple stressor effects. For example, ultraviolet radiation may be more lethal to amphibians when toxic chemicals are present in the environment (Blaustein et al., 2003). Increased UV exposure may also have stronger lethality when nitrate based fertilizers are present in the aquatic ecosystem (Hatch and Blaustein, 2003). In the presence of iridovirus, high Atrazine (a herbicide) concentrations can accelerate metamorphosis in *Ambystoma macrodactylum*, forcing a change into smaller metamorphic salamanders with lower fitness (Forson and

Storfer, 2006a). A subsequent study found that nitrate fertilizers and Atrazine suppress the salamander immune system and render them more vulnerable to viral infection (Forson and Storfer, 2006b). Mixtures of pesticides may also have more lethal effects on amphibian populations (Hayes et al., 2006). Another example involves introduced species and disease transmission. Widely distributed through the pet trade and for scientific research, African aquatic frogs of the genus *Xenopus*, bullfrogs (*Rana catesbeiana*), and salmonid fishes may be carriers of amphibian diseases which they transport to native amphibian species when distributed by humans (Reed et al., 2000; Knapp et al., 2001; Carey et al., 2003; Daszak et al., 2004). These introduced species already harm native amphibians by preying on them and in some cases, competing for prey items (Moyle, 1973; Bradford, 1989; Fisher and Shaffer, 1996; Crossland, 1998). Acidification of the environment together with contamination by heavy metals may have a far more lethal effect on amphibian populations than either would have individually (Bradford et al., 1992; Horne and Dunson, 1994, 1995). Pounds et al. (2006) has argued that global warming has harmed amphibian populations across Central America and that the environmental change has promoted chytrid fungus spread across amphibian populations. It should be noted that this explanation is controversial and not the consensus (Mendelson et al., 2006). Temperature change in habitats has also been implicated in weakening the amphibian immune system (Raffel et al., 2006).

Dealing with the Amphibian Decline Phenomenon

In 2005, the Amphibian Conservation Summit (ACS) was held in Washington DC. This meeting included amphibian biologists, conservation biologists, conservation

agencies, and government agencies overseeing environmental issues. The intent of the meeting was to organize stakeholders and form an action plan to stem amphibian decline. The ACS document outlines some strategies that were devised in that meeting. The document suggests that the Amphibian Action Fund should be developed to fund the measures suggested by the Amphibian Conservation Action Plan (ACAP). ACAP is a list of steps outlined to mitigate amphibian decline. The estimated cost to do the work suggested by ACAP is 400 million US dollars between the years 2006 and 2010. The Amphibian Action Fund will be developed within the program to manage finances and fund programs. The initiative/program describes itself in the following paragraph:

“The Amphibian Conservation Action Plan is the most ambitious program ever developed to combat the extinction of species. This response is necessary because the amphibian extinction crisis is unlike anything that the modern world has previously experienced, and a large proportion of amphibian diversity remains undocumented. The ACAP requires the international community to enter uncharted territory and to take great risks. But the risks of inaction are even greater. The Amphibian Conservation Summit calls on all governments, corporations, civil society and the scientific community to respond to this unprecedented crisis. There needs to be unprecedented commitment to implementing the Amphibian Conservation Action Plan with accompanying changes in international and local environmental policies that affect this class of vertebrate animals. They are indeed canaries in the global coalmine.”

The summarized ACAP suggestions include:

- (1) Expand scientific understanding of amphibian declines and extinctions

- (2) Continue to document amphibian diversity and how it is changing
- (3) Develop and implement long-term conservation programs
- (4) Prepare emergency response actions for eminent crises

The four broad categories can be developed further from the Amphibian Conservation Action Plan (Gascon et al., 2007):

(1) By improving our understanding of amphibian declines and extinctions, we might be able to develop better strategies to deal with the specific forces driving the declines in the field. Supporting research that looks into containing or limiting the spread of disease in the field is paramount. These studies should focus on understanding persistence of pathogens in the field, identifying any species that are serving as reservoirs for disease in the field, identifying the interactions that these pathogens might be having with climate change or other anthropogenic causes of amphibian declines (environmental contamination, habitat alteration, etc.), comparing diseased sites with non-diseased sites, investigating how pathogens cause death, clarifying how amphibians respond to emerging infectious diseases (particularly if development of immunity or resistance is noted or behavioral changes mediate the effects of the disease(s)), understanding the spread and distribution of emerging infectious amphibian diseases, documenting how responses differ between amphibian populations exposed to the same pathogen, and recording any interactions between pathogens. Further, studies should focus on how human activities, like captive breeding programs and reintroduction programs, might stem amphibian declines. Regional Centers for Disease Diagnostics should be

established around the world to handle and run free testing to field research groups working on amphibian decline. These centers are to be funded through the Amphibian Action Fund. In addition, rapid response teams should be established so that disease outbreak can be rapidly managed early off during an episode.

(2) Further understanding amphibian diversity and biogeography are essential elements to conservation. Emphasis should be placed on species descriptions and studies of their ecological requirements. ACAP plans on funding an initiative to describe 2500 new amphibian species in ten years, understand their distributions, clarify species complexes, and carry out amphibian faunal surveys in specific regions. The effort will be accomplished by developing taxonomic capabilities in tropical countries. Priority should be set in poorly known areas, areas of high endemism, and areas of high diversity, focusing on the biology and ecology of unknown and poorly known species (“data deficient species”), unique and ancient evolutionary lineages, threats and their differences between related lineages, and genetic diversity in relation to a capacity to deal with emergent infectious disease. Species identification should be improved through the production of field guides and internet resources.

The Global Amphibian Assessment (GAA) was developed to clarify amphibian biodiversity, amphibian decline, and ecological information for species which are considered data deficient. The GAA should have a team dedicated to its continual update, to making that data widely available, in maintaining a website, in making data reporting more reliable in difficult areas, in performing continuous analyses of updated

data sets, in deciphering between real declines and improper sampling of difficult to observe species, and in communicating those findings.

(3) Habitat alteration affects nearly 4000 extant amphibian species (Stuart et al., 2004) and threatens 90% of the declining amphibians for which we have sufficient data regarding their declines (Gascon et al., 2007). Identifying and preserving critical habitat is critically important. ACAP will establish a program with the following objectives: to identify the top 120 priority sites for amphibian conservation, affect a conservation action at each including management plans, monitoring, regular assessment, and funding plans. The region around each site should be covered in a management plan so that external forces are dealt with if possible. Once disease management in the field is possible, reintroduction programs should be used to re-establish viable amphibian populations. Experience with reintroduction programs should be supported to develop the skills necessary to perform these tasks. Reintroduction populations will come from both captive bred colonies and wild populations, circumstances permitting.

The harvest of wild amphibians for food and other activities has impacted amphibians. ACAP will develop a harvest management program. The program will attempt to stem harvest and regulate or mitigate amphibian take via: developing sustainable harvest programs, promoting wildlife law enforcement, monitoring amphibian harvest and trade, promoting and implementing recovery programs for impacted species, certifying captive breeding programs, and improving amphibian decline awareness of harvest in the general public.

(4) An emergency response plan should be developed with regional response teams. These teams will react to circumstances like emergent disease outbreak. Tools used to stem the emergency situation are: developing emergency captive breeding colonies within the countries of origin, developing short and long term captive management plans, training and capacity building within the range country, supporting and developing captive breeding sciences, supporting research into disease management, and support for education and outreach programs.

The Amphibian Emergency Fund will support conserving remaining habitats in top priority sites. These immediate conservation measures may conserve remaining habitat fragments that accommodate communities of species facing extinction. Species facing similar extinction owing to over-harvest should also be addressed through these emergency funds.

Goals and Expected Results of My Dissertation Projects

My Dissertation work is focused to contribute toward the areas identified by the Amphibian Conservation Action Plan as necessary to stem amphibian declines. The summarized ACAP goals are:

- (1) Expand scientific understanding of amphibian declines and extinctions
- (2) Continue to document amphibian diversity and how it is changing
- (3) Develop and implement long-term conservation programs
- (4) Prepare emergency response actions for eminent crises

I felt that my abilities were best suited to contribute toward ACAP objectives one and two. My first Dissertation chapter serves as an amphibian decline summary, to provide a context and to explain the problem's scope. Chapters two and three address ACAP objective #2. I wanted to contribute to the biology and ecology relating to "data deficient" species. I did so through the following publication and manuscript (chapters 2, and 3):

Fenolio, D.B., H.L.R. Silva, and Da Silva, N.G. Jr. 2006. *Leptodactylus pustulatus* Peters (Amphibia: Leptodactylidae) with notes on habitat, ecology, and color in life. *Herpetological Review* **37**(2): 140–142.

Fenolio, D.B., J.R. III Mendelson, and W.W. Lamar. Redescription of *Rhinella ceratophrys* (Boulenger) (Amphibia:Bufonidae), with notes on its ecology and distribution. In Preparation.

My fourth Dissertation chapter also addresses ACAP objective #2. Continuing to document amphibian biodiversity, and to publish on new species' ecology, the following manuscript was published:

Fenolio, D.B., M.E. Walvoord, J.F. Stout, J.E. Randrianirina, and F. Andreone. 2007. A new tree hole breeding *Anodonthyla* (Chordata: Anura: Microhylidae: Cophylinae) from low-altitude rainforests of the Masoala Peninsula, northeastern Madagascar. *Proceedings of the Biological Society of Washington* **120**(1): 86–98.

The fifth Dissertation chapter is an effort to contribute toward ACAP objective #1. Identifying forces driving amphibian decline has been identified as a crucial component to combating the phenomenon (Gascon et al., 2007). Specifically, I am interested in heavy metal accumulation in the environment. Heavy metals have been shown to be a considerable problem for amphibians (Linder and Grillitsch, 2000; Chen et al. 2006, 2007; Gross, 2007). For example, heavy metal pollution is now ubiquitous

around the planet and these metals are predominantly concentrated through anthropogenic processes (Linder and Grillitsch, 2000). Often heavy metal pollution runs hand in hand with pesticide use, as pesticides can contain heavy metals (Gross et al., 2007). These contaminants can have adverse effects on amphibian larval development (Linder and Grillitsch, 2000; Chen et al. 2006, 2007; Gross, 2007).

Sensitivity to contaminants may vary among frog species (Birge, 1978; Birge et al., 1979). Preliminary evidence suggests that each species, or closely related species group, may have different sensitivities to heavy metals (Birge, 1978; Birge et al., 1979; Linder and Grillitsch, 2000). Because previous studies have employed incomparable methods, measurable differences in sensitivity have not been drawn in a significant number of species (Linder and Grillitsch, 2000). A common methodology applied to different species would produce comparisons not previously possible.

The United States Environmental Protection Agency (USEPA) has widely used *Xenopus laevis* as a model species in scientific studies. Related species to *X. laevis* have become available, providing more options for future studies. For example, *X. laevis* is triploid, some of its close relatives are diploid. The triploid genetic state in *X. laevis* has complicated some studies. However, the closely related *X. tropicalis* is diploid and would be much better suited to future studies. *X. tropicalis* is a species that the USEPA has decided to use on an increasing basis owing to its genetic state. Use of *X. tropicalis* also includes environmental toxicity studies. However, data has not been produced to clarify how *X. laevis* responds to metals in comparison to *X. tropicalis*. Chapter 5 here examines the response to 6 heavy metals between *X. laevis* and *X. tropicalis*. My study compares effects between related species of frogs to heavy metal exposures using

standard methodologies. Standardized methodology will clarify differences between the species in teratogenic, survival, and developmental responses to the heavy metals. These comparisons will provide a framework to further understand how aquatic African frogs respond to heavy metal contamination and how they might be used by the USEPA.

CHAPTER 2:

A New Tree Hole Breeding *Anodonthyla* (Chordata: Anura: Microhylidae: Cophylinae) from Low-Altitude Rainforests of the Masoala Peninsula, Northeastern Madagascar

The complex of protected areas in Madagascar is growing, and the recent creation of several new reserves may enhance the chances for safeguarding some of the island's most natural and pristine biota and communities. Regardless of the SLOSS debate, "single large or several small" reserves (Diamond 1975, Simberloff & Abele 1976, Terborgh 1976), it is important to protect any and all nature reserves in Madagascar as a crucial and important stepping stone for the conservation of this biodiversity hotspot. In recent years we had the opportunity to visit the largest protected area in northeastern Madagascar: The Masoala National Park (2100 km² of land plus three marine reserves; ANGAP 2000). Without roads leading into it, this peninsula has been isolated from much of the human activities on the island and is essentially free from tourism. Masoala includes the largest portion of primary, lowland tropical forest remaining in the country (Kremen et al. 1999) and has an annual rainfall exceeding 3500 mm (Nicoll & Langrand 1989). Our recent surveys provided the opportunity to study a diverse herpetofauna, including previously unknown species (Andreone et al. 2003). The Canopy Raft Program (Radeau des Cimes) enabled us (DBF, MEW, JFS, JER) to survey the herpetofauna of this forest from the ground up through the emergent canopy layer. Moreover, FA conducted a series of field surveys at particularly biologically rich areas, at Masoala and other northeastern localities, which yielded many new discoveries (Andreone 2004).

Frogs in the Masoala's wet forests exploit a variety of microhabitats ranging from the ground to the upper canopy (DBF & MEW pers. obs.), including plant-held waters,

referred to collectively as phytotelmata (Maguire 1971). While surveying the herpetofauna at various canopy levels and studying phytotelm community structure, we found a species of *Anodonthyla* that could not be classified as a currently recognized species. Subsequently we learned that it was genetically differentiated from *A. boulengeri* and *A. moramora* (11--12% uncorrected pairwise sequence divergence for a fragment of the mitochondrial 12S ribosomal gene, Glaw & Vences 2005), taxa that are close relatives of the species described here.

Materials and Methods

We collected all specimens described here on the Masoala Peninsula of northeastern Madagascar (Figure 2.1). The first specimens were collected in the years 1998 and 1999 by FA while conducting a diversity survey in some unprotected forest plots of Masoala. Subsequent specimens were collected on an expedition with the French-run canopy RAFT program (Radeau des Cimes) in 2001 along the southwestern coast of the peninsula.

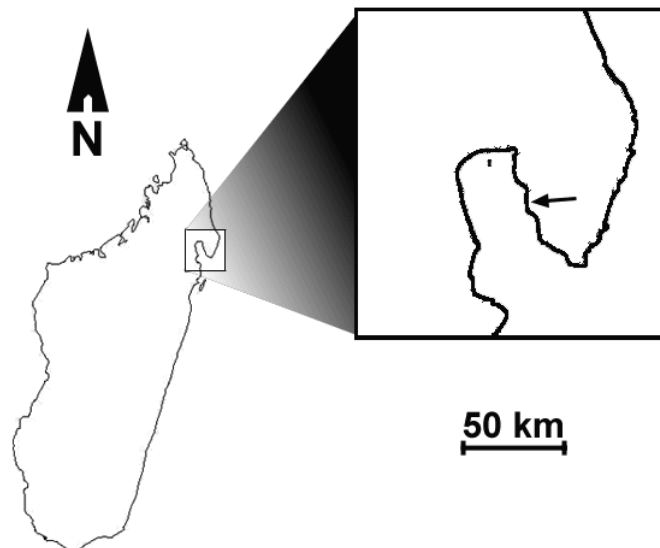


Figure 2.1 Map of Madagascar, inset of the Masoala Peninsula.

We searched for frogs opportunistically during the day and night along and just off of forest trails (from ground level to emergent canopy, 0--30 m) looking in phytotelmata, leaf litter, logs, and pools of water. We also employed bioacoustic searching methods. We used canopy ascension techniques to determine the presence of frogs higher in the forest canopy. Where possible for phytotelmata, we recorded dissolved oxygen, conductivity, and temperature of water using a digital DO/conductivity/temperature probe, and we measured pH with a separate digital pH probe (Cumberlidge et al. 2005).

Anuran vocalizations were recorded in 2001 with a Sony Walkman Professional WM-D6C stereo cassette recorder. We recorded a calling individual (OMNH 39030) located 40 cm from our microphone on the evening of 31 Oct 2001 and analyzed the calls using Raven (version 2.1), producing a sonogram with Canary (version 1.2.1). Air temperature near this individual was approximately 18°C, and the frog called from 3 m above the ground from a large tree trunk. Complications with the recording quality limit us to the preliminary call description below.

Specimens were anaesthetized by immersion in chlorobutanol. We fixed captured frogs in 10% formalin after writing a description of color in life and photographing most individuals. Subsequently, we transferred specimens to 70% ethanol for permanent storage at the Oklahoma Museum of Natural History or at the Museo Regionale di Scienze Naturali di Torino. Comparative materials were examined from the Naturhistorisches Museum Basel.

With digital calipers or a dissecting microscope micrometer, we took the following measurements of specimens: snout-vent length from the tip of the snout to the

venter (SVL); head width at the point of the widest dorsal view of the cranium (HW); head length from angle of jaw to tip of snout (HL); horizontal eye diameter from anterior to posterior edge of eye (ED); interocular distance from the edge of the ocular swelling of one eye to the edge of the opposing eye (IOD); eye to nostril distance measuring from the anterior edge of the eye to the center of the nostril (END); distance from the center of the nostril to the tip of the snout (NSD); internarial distance from the center of one nostril opening to the center of the other (NND); horizontal tympanum diameter from anterior to posterior edge of tympanic ring (TD); hand length from proximal edge of palmar tubercle to tip of the longest digit (HAL); forelimb length from axilla to tip of longest digit (FORL); foot length from proximal articulation of metatarsal tubercle to tip of the longest digit (FOL); tibia length measuring from tip of knee to base of tarsus on bent leg (TIBL); and maximum toe pad width on each forelimb digit (TPI--TPIV). All specimens were surgically sexed. We used analysis of variance, implemented in R, for all statistical comparisons of morphometrics (R Core Development Team 2005). We considered differences statistically significant at $p \leq 0.05$.

Abbreviations for museum specimens are as follows: OMNH = Sam Noble Oklahoma Museum of Natural History; MRSN = Museo Regionale di Scienze Naturali di Torino; NHMB = Naturhistorisches Museum Basel; NMBE = Naturhistorisches Museum der Burgergemeinde, Bern; ZFMK = Zoologisches Forschungsmuseum Alexander Koenig, Bonn; ZMA = Universiteit van Amsterdam, Zoologisch Museum; ZSM = Zoologisches Staatssammlung, München.

In their phylogeny of *Anodonthyla* and several other cophylina frogs, Glaw & Vences (2005) reported *A. boulengeri* from “Ilampy,” Madagascar as genetically distinct

from *A. bouleengeri* from Foulpointe, Madagascar. These specimens were reported as having a 12% uncorrected pairwise sequence divergence for a fragment of the mitochondrial 12S ribosomal gene. Glaw & Vences (2005) comment that, “The strong genetic differentiation among the two individuals of *A. bouleengeri* further demonstrates that this species may be composed of several cryptic, yet unrecognized species.” One of these specimens is mistakenly reported as, “Ilampy; Field number of F. Andreone, No. 10243; AY684182,” but is actually from field site Menamalona, Masoala Peninsula (Masoala camp 5) described in this paper and collected by FA. This specimen received the final museum number MRSN A4435 and has been included in our paper as a paratype of the new species of *Anodonthyla* described herein. Therefore, genetic differences referred to in Glaw & Vences (2005) between the Ilampy and Foulpointe *A. bouleengeri* specimens actually refer to differences between the new species of *Anodonthyla* described herein (MRSN A4435 from Masoala 5) and their *A. bouleengeri* specimen from Foulpointe.

Systematic Account

Anodonthyla hutchisoni, new species (Figures 2.1 and 2.2).

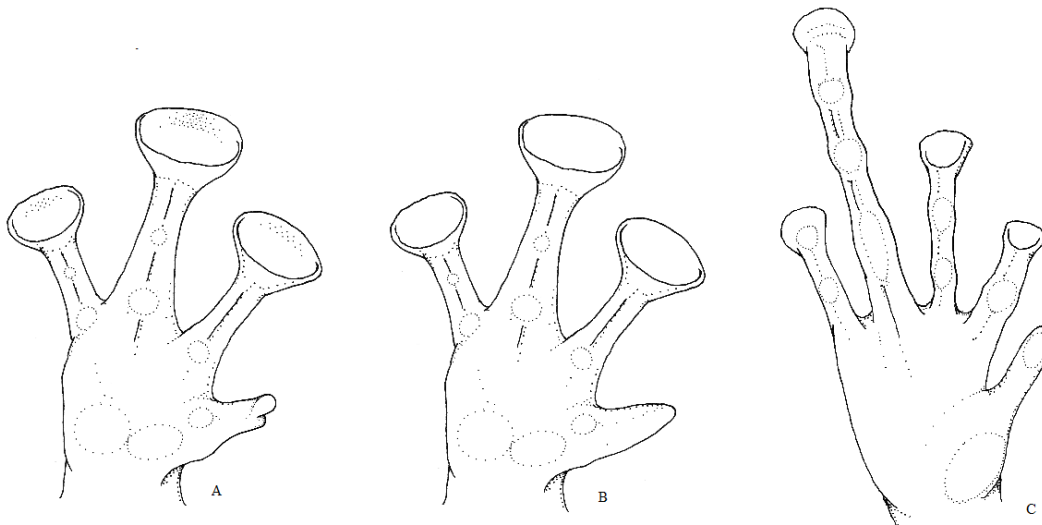


Figure 2.2 *Anodonthyla hutchisoni* A. Illustration of hand of male holotype, B. Illustration of hand of female paratype, C. Illustration of foot holotype.

Type material.---Holotype: OMNH 39029, adult male from the southern versant of the Masoala Peninsula, Andranobe campsite, Maroantsetra fivondronana (district), Toamasina faritany (province), Madagascar (Figure 2.1, arrow), 15°40.820'S, 49°57.750'E, 200 m, leg. DBF, MEW, JER, 31 Oct 2001.

Paratypes: OMNH 39026, adult male, same province, locality, and collectors as for the holotype, 28 Oct 2001; OMNH 39027, 39030, adult males, same province, locality, and collectors as for the holotype, collected on 31 Oct 2001; OMNH 39028, adult female collected with holotype; OMNH 39031, 39032 adult female and male respectively, same province, locality, and collectors as for the holotype, 1 Nov 2001; OMNH 39033, adult female, same province, collected south of Andranobe, 10 m above sea level, same collectors as for the holotype, 4 Nov 2001; MRSN A4435 [genbank accession number AY684182 for the 12S fragment], A4439, adult males, MRSN A4438, adult female, Masoala Peninsula, Menamalona (Masoala camp 5), Antalaha fivondronana (district), Antsiranana faritany (province), 15°22.87'S, 49°59.27'E, 780 m, leg. FA and JER, 17 Dec 1999; MRSN A4441, adult male, Masoala Peninsula, Antsarahana'Ambararato (Masoala camp 4), Antalaha fivondronana (district), Antsiranana faritany (province), 15°23.52'S, 50°02.82'E, 550 m, leg. FA and JER, 1 Dec 1999; MRSN A4442, adult female, Masoala Peninsula, Andasin'i Governera (Masoala camp 3), Antalaha fivondronana (district), Antsiranana faritany (province), 15°18'S, 50°01'E, 650 m, leg. FA and JER, 7 Dec 1998.

Additional material.---We tentatively leave the specimen MRSN A201, from Nosy Mangabe Island, Maroantsetra fivondronana (district), Toamasina faritany (province), leg. FA 23 Apr 1990 as unassigned to either *A. hutchisoni* or *A. boulengeri* until further genetic analysis can be done.

Diagnosis.---Assigned to the genus *Anodonthyla* based on the presence of a distinct prepollex visible in male specimens (Figures 2.2 and 2.3), and on molecular phylogenetic relationships (Glaw & Vences 2005). *Anodonthyla hutchisoni* is characterized by: 1) head as wide as long; 2) snout rounded and blunt in profile; 3) canthus rostralis barely distinct; 4) tympanum roughly one-half diameter of eye; 5) eyelid diameter usually greater than or equal to interocular distance; 6) light copper or gold iris; 7) fingers without webbing; 8) first finger shorter than second, third finger longer than others; 9) toe pads on hands truncate and large; 10) pads on feet more ovoid than circular in shape and smaller than pads on hands; 11) prepollex on digit I in males, absent in females; (12) feet without webbing; 13) first digit on hand shorter than second, third longer than all others; 14) first digit on foot shorter than second, third longer than fifth, fourth longer than all others; 15) inner metatarsal tubercle vague if present; 16) tarso-metatarsal articulation reaches eye or beyond; 17) dorsal surface smooth with small, dorsal, semi-prominent tubercles, brown in color, sometimes white tipped; 18) dorsal color ranges from brown, tan, or gray with vague darker pattern; 19) lower flank marked by irregular cream and brown markings, upper flank with fewer brown markings; 20) dorsal surface of thighs and legs with tan or brown bands sometimes outlined by thin cream lines; 21) ventral surface cream with numerous and minute brown dots, frequently aggregated to form large brown spots; 22) adult males 19.6--23.1 mm; females 19.0--24.4 mm SVL.

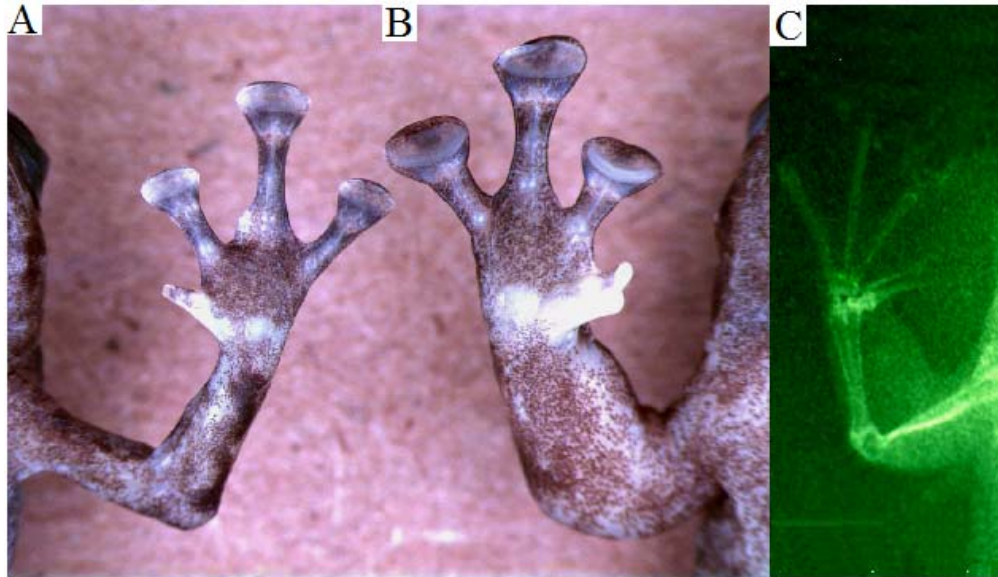


Figure 2.3 *Anodonthyla hutchisoni*. A. Digital image of hand of female paratype, B. Digital image of hand of male holotype, C. Enhanced x-ray image of hand of holotype.

Description of Holotype.---Body robust; head slightly wider than long, HW 35% of SVL, HL 31% of SVL; snout short, not protruding beyond margin of lip; rounded and blunt in dorsal view and in profile; END 67% of ED; END 25% of HL; eye large in size relative to head but comparable to other *Anodonthyla* (i.e., *A. boulengeri*); ED 38% of HL; upper eyelid with moderate tubercles, IOD 89% of ED. Pupil horizontal. Vocal sac moderate in size, single, and gular. Supratympanic fold absent, tympanum round and moderately distinct, TD 68% of ED. Top of head flat; cranial crests absent; canthus rostralis barely distinct; internarial area not depressed; nostril ovoid, protruding laterally at point above margin of lower jaw; choanae small, round, separated, partially obscured by palatal shelf of maxillary arch. Vomerine teeth absent. Tongue pear-shaped, widest at the free margin, no groove or notch, free behind for about four-fifths of its length.

Skin on dorsum of head, body, and limbs smooth but with numerous distinct tubercles. Ventral surfaces smooth. Cloacal opening elliptical, unmodified. Forearm

broad in males, fingers moderate in length bearing truncate, broad, large discs; disc on digit I smaller than others: III > II > IV > I; subarticular tubercles semi-distinct, circular, slightly elevated; supernumerary tubercles absent; palmar tubercle semi-distinct; prepollex attached to digit I in males (Figures 2.2 and 2.3). No webbing between digits on hands. Digits on hands and feet have slight flanges. Longest hand digit to shortest: III > IV > II > I. Feet without distinct tubercles. Small pads on digits of feet, ovoid in shape and smaller than those on hands (Figure 2.2). No webbing between digits of feet. Longest foot digit to shortest: IV > III > V > II > I.

Color of holotype in preservative.---(ethanol) Adult male (OMNH 39029), dorsum of head, body, and limbs brown or tan with vague darker markings, legs with tan bands outlined in thin cream lines. Two of four (in life) dorsal white spots apparent. Flanks gray, lower flanks with brown and cream irregular markings. Ventral surface gray to cream with numerous brown flecks sometimes conglomerating to form dark spots.

Color of holotype in life.---Adult male (OMNH 39029) (Figure 2.4), dorsum a combination of several shades of tan and brown, creating a vague background pattern of irregular dorsolateral stripes. Scapular region with two distinct white spots, two spots mid-dorsum, ovular or circular in shape. Dorsal spots may fade or intensify through time (in as little as five minutes). Inguinal region lighter in color with dark chevrons. Posterior parts of thighs cream with dark vermiculations. Gular region gray with heavy brown spotting. Small, brown, dorsal tubercles, sometimes white tipped. Ventral surface cream with brown flecks sometimes conglomerating to form dark spots. Venter cream. Iris gold with fine black striations.



Figure 2.4 Photograph of live *Anodonthyla hutchisoni* at the type locality, male Holotype.

Variation.---Thirteen adults from mainland Masoala. All standard measurements reported in Table 2.1. Males with a SVL ranging from 19.6--23.1 mm; females ranging from 19.0--24.4 mm. Head roughly as wide as long; HW 32--38% of SVL (ratio range of 1:2.7--3.1); HL 30--38% of SVL (ratio range of 1:2.6--3.3); snout short, not protruding beyond margin of lip; rounded and blunt in dorsal view and in profile; END 54--79% of ED (ratio range of 1:1.1--1.9); END 20--34% of HL (ratio range of 1:3.0--5.0); eye large in size relative to head but comparable to *A. boulengeri*; ED 32--48% of HL (ratio range of 1:2.1--3.1); upper eyelid with moderate tubercles, IOD 76--104% of ED (ratio range of 1:1--1.3). Tympanum round and moderately distinct with TD 41--68% of ED (ratio range of 1:1.5--2.4). Forearm broad in males and thinner in females (Figures 2.2, 2.3A, and 2.3B).

Table 2.1 Morphometric values (mm) for the type series of *Anodonthyla hutchisoni* and for a published series of morphometrics for *A. boulengeri* (Glaw & Vences 2005).

Species/locality	Museum/#	Status	Sex	SVL	HW	HL	EW	ED	IOD	END	NSD	NND	TD	HAL	FORL	FOL	TIBL
<i>A. hutchisoni</i> n. sp.																	
	OMNH	HT															
Andranobe	39029		M	22.9	8.1	7.2	1.9	2.7	2.35	1.8	1.4	2.3	1.3	5.7	11.4	8.9	11.1
	OMNH	PT															
Andranobe	39026		M	21.9	7.1	7.0	1.6	2.6	2.45	1.4	1.1	2.1	1.1	5.2	10.7	8.5	10.3
	OMNH																
Andranobe	39027	PT	M	19.6	7.2	6.8	1.8	2.6	2.2	1.7	1.1	2.1	1.1	4.4	9.9	7.6	10.2
	OMNH	PT															
Andranobe	39028		F	24.4	8.1	7.9	1.9	2.8	2.81	1.6	1.6	2.4	1.9	5.8	11.2	9.6	11.3
	OMNH	PT															
Andranobe	39030		M	21.5	7.3	7.2	1.6	2.3	2.38	1.6	1.2	2.2	1.1	5.1	10.7	8.7	10.5
	OMNH	PT															
Andranobe	39031		F	22.2	7.9	7.6	1.8	2.6	2.73	1.6	1.2	2.1	1.4	5.0	10.7	8.4	11.2
	OMNH	PT															
Andranobe	39032		M	23.1	7.7	7.6	1.6	2.5	2.3	1.9	1.2	2.2	1.2	5.0	11.0	8.4	10.7
	OMNH	PT															
Andranobe	39033		F	21.6	7.3	7.1	2.0	2.5	2.23	1.9	1.4	2.1	1.4	5.3	11.3	8.6	10.4
	MRSN	PT															
Masoala 5	A4435		M	21.8	7.6	6.6	2.1	2.9	2.20	1.9	1.7	2.0	1.5	6.5	9.6	10.0	10.6
	MRSN	PT															
Masoala 5	A4438		F	19.0	7.2	7.3	1.6	3.0	2.63	2.3	1.5	2.5	1.7	6.4	9.6	9.8	10.5
	MRSN	PT															
Masoala 5	A4439		M	20.5	6.6	6.3	1.6	2.9	2.52	1.5	1.6	2.7	1.7	6.4	8.9	9.3	10.5
	MRSN	PT															
Masoala 4	A4441		M	21.7	7.9	6.5	1.9	3.2	2.50	2.2	1.8	2.2	1.7	6.8	8.9	10.7	10.7
	MRSN	PT															
Masoala 3	A4442		F	21.0	7.4	7.4	1.6	2.8	2.09	2.2	2.0	1.8	1.9	6.5	9.7	9.7	11.8
<i>A. boulengeri</i>																	
	NHMB																
Madagascar	1448	HT	F	19.2	6.3	6.4	1.7	2.4	2.1	1.9	1.9	2.1	1.1	5.0	6.8	9.1	9.2

		NMBE														
Andasibe	1046031	M	16.2	6.4	5.3	2.1	2.2	2.1	2.2	2.1	1.7	1.4	5.1	8.6	7.7	7.2
		NMBE														
Andasibe	1046032	F	18.6	5.6	6.1	1.8	1.7	1.9	2.4	1.6	1.8	1.6	5.2	7.4	7.9	7.6
		MRSN/RJS														
Antara	0804	M	17.6	5.7	5.6	1.5	2.1	1.8	1.5	1.6	2.4	1.2	5.1	7.8	7.5	8.4
<i>A. sp.</i>																
		MRSN														
Nosy Mangabe	A201	M	21.6	7.2	6.6	1.7	3.2	2.4	1.6	1.8	2.5	1.8	5.9	7.2	10.4	10.5

Variation of color in preservative.---(ethanol) Two to four dorsal white spots often apparent, with posterior two spots faded to one light patch in one individual. Ventral surface gray to cream with numerous brown flecks sometimes conglomerating to form dark spots.

Variation of color in life.---Dorsum a combination of several shades of tan and brown, creating a vague background pattern of irregular dorsolateral stripes. Dorsal spots may fade or intensify through time (in as little as five minutes). In one individual at different times, we observed four spots, then only two spots, and infrequently, none of the spots were obvious. Gular region gray or cream with heavy brown spotting. Ventral surface cream with brown flecks sometimes conglomerating to form dark spots. Iris light copper to gold with fine black striations.

Sexual dimorphism.---Females with a more robust body than males. Males with a prepollex fused to thumb (Figures 2.2 and 2.3), absent in females. Males with broad forearms, females with relatively thin forearms (Figures 2.3A and 2.3B).

Vocalization.---We provide only a preliminary call description here due to our low recording quality. Males call from both exposed positions and from tree holes. The call is a repetitive single note produced for durations of 10--45 sec. The note repetition rate is approximately 1.0--1.3 notes/sec recorded at 18°C. From a spectrographic point of view, each single note shows two peaks at 3100--3200 Hz and 4600 Hz with a fundamental frequency of 1600--1700 Hz (Figure 2.5).

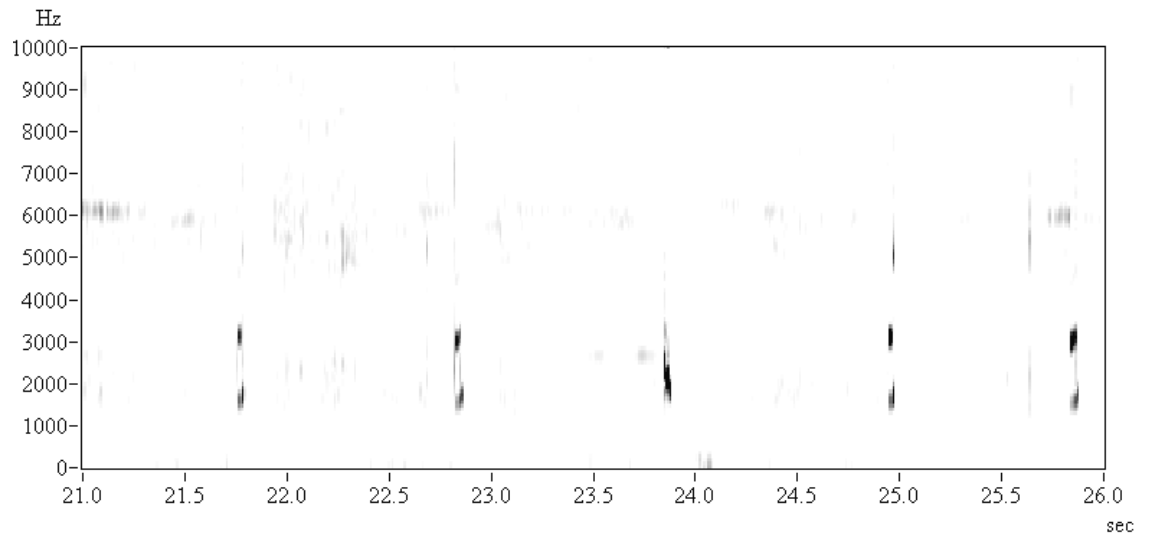


Figure 2.5 A. Advertisement call of *Anodonthyla hutchisoni*

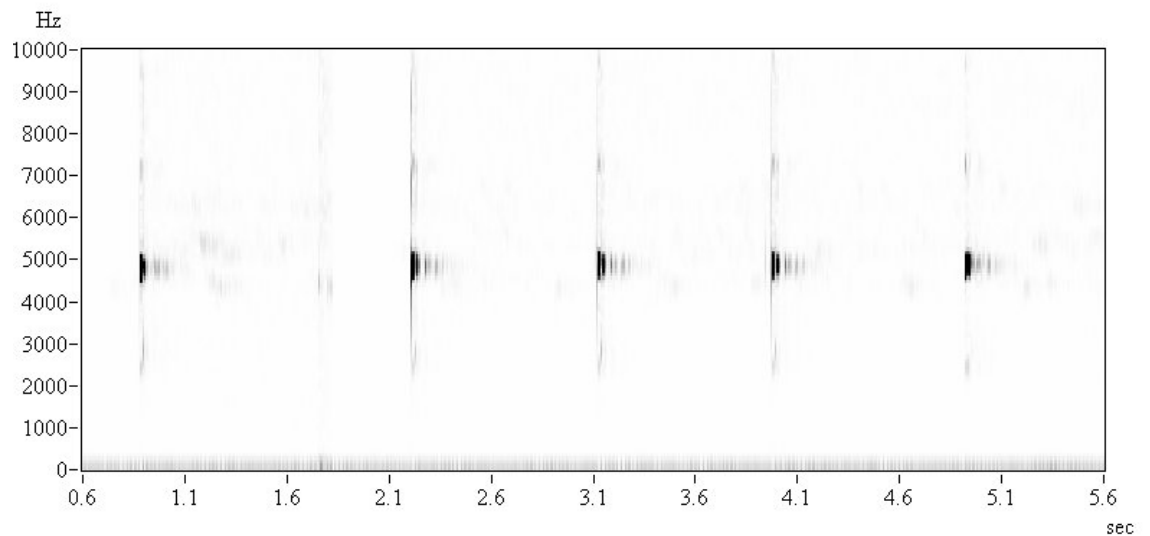


Figure 2.5 B. Advertisement call of *Anodonthyla hutchisoni*

Justification of the new species.---The phylogenetic tree provided by Glaw & Vences (2005) indicates that *A. hutchisoni* is closely related to *A. boulengeri* but genetically distinct. A paratype of *A. hutchisoni* (MRSN A4435 - see note in the Materials and Methods regarding this specimen) shows a strong genetic differentiation of 12% uncorrected pairwise sequence divergence for a fragment of mitochondrial 12S ribosomal gene, compared to a specimen assigned to *A. boulengeri* from Foulpointe. This indicates that at least two *Anodonthyla* species inhabit the north-central and northeastern portions of Madagascar. Smaller individuals from this range have been attributed to *A. boulengeri*. The female holotype of *A. boulengeri* (NHMB 1448) is only recorded as coming from “Madagascar.” Even with the ambiguous range designation for the holotype of *A. boulengeri*, we assume it was collected from central-eastern Madagascar based on the current recognized range of *A. boulengeri* and the specimen’s small size. The color of this specimen is faded and cannot provide any useful chromatic data.

The larger, differently patterned, and morphometrically distinct frogs from northeastern Madagascar’s Masoala Peninsula are here attributed to *A. hutchisoni*. Important morphological differences between *A. hutchisoni* and *A. boulengeri* exist: Male *A. hutchisoni* were significantly larger in the characters SVL, HW, HL, END, ED, and TIBL compared to male *A. boulengeri*, while male *A. hutchisoni* were significantly smaller than *A. boulengeri* in FORL ($F = 7.01, p = 0.01$; Table 2.2). Female *A. hutchisoni* were significantly larger than female *A. boulengeri* in the comparisons of HW, HL, END, ED, TD, and TIBL, with SVL showing a marginally significant difference ($F = 4.97, p = 0.056$; Table 2.2).

Table 2.2 Morphometric comparisons between *Anodonthyla hutchisoni* and *A. boulengeri*.

	Males				Females			
	<i>A. hutchisoni</i>	<i>A. boulengeri</i>	F value	P value	<i>A. hutchisoni</i>	<i>A. boulengeri</i>	F value	P value
SVL	21.6	18.6	13.19	= 0.002	21.6	19.4	4.97	= 0.056
HW	7.4	5.8	23.27	< 0.001	7.6	5.8	40.94	< 0.001
HL	6.9	5.6	34.22	< 0.001	7.5	5.9	52.01	< 0.001
END	1.8	1.3	19.9	< 0.001	1.9	1.4	6.23	= 0.037
ED	2.7	2.0	28.54	< 0.001	2.7	2.1	19.78	= 0.002
TD	1.3	1.2	2.75	= 0.115	1.7	1.3	7.6	= 0.024
TIBL	10.6	8.6	35.06	< 0.001	11.0	8.8	21.14	= 0.001
FORL	10.1	11.6	7.01	= 0.010	10.5	11.0	0.150	= 0.708

In addition to size differences, the dorsal pattern of *A. hutchisoni* typically has large, distinct cream spots not described in *A. boulengeri*. Finally, the vocalizations of *A. hutchisoni* appear to be distinct from *A. boulengeri*. *Anodonthyla hutchisoni* vocalizes at 1.0--1.3 calls/sec with frequency peaks of 3100--3200 Hz and 4600 Hz. In contrast, *A. boulengeri* has a call repetition rate of 1.8--3.1 calls/sec and a fundamental frequency of 3500--4500 Hz, while *A. moramora* has a call repetition rate of 0.6--0.9 calls/sec and a fundamental frequency of 2700--3150 Hz (Glaw & Vences 2005).

Anodonthyla hutchisoni differs from a population of *A. boulengeri* formerly recognized as “*Mantella pollicaris*.” These frogs are from a locality, Anevoka, approximately 400 km SSW of the range of *A. hutchisoni*. The type specimen of what was *M. pollicaris* (SMF 4299) has relatively smooth skin on its dorsal surface and little

indication of a pattern. *Anodonthyla hutchisoni* has tuberculate skin on its dorsal surface and preserved animals retain clear indications of their dorsal pattern.

A. hutchisoni is distinguished from *A. nigrigularis*, which has a blackish throat and is found in the southeast region of the island. The recently described species, *A. moramora*, is smaller than *A. hutchisoni*, and has a variegated dorsal pattern including a green color in its pattern (Glaw & Vences 2005).

Anodonthyla hutchisoni is also morphologically similar to some small microhylids of the closely related cophyline genus *Platypelis*. While males can be quickly differentiated in the presence (*Anodonthyla*) or absence (*Platypelis*) of a prepollex, females may not be as easily differentiated. *Platypelis tetra* is morphologically similar to *A. hutchisoni* (Andreone et al. 2003). However, *A. hutchisoni* is larger in size, (19.0--24.4 mm SVL vs. 15.7--19.4 mm SVL), with larger toe pads, bifurcate toe pads, second greatest toe pad diameter on digit four, presence of dorsal tubercles, increased brown spotting on the ventral surface, absence of a dorsolateral brown stripe bordered by cream bars on the flanks, and distinct white dorsal spots that, when present, do not interconnect.

Of the other *Platypelis* species that may be sympatric and similarly sized, *A. hutchisoni* differs from *P. barbouri* in lacking webbing between the toes and fingers, in lacking the red or green markings on the dorsal surface of the groin and thighs, in the possession of white dorsal spots, usually, and in a tympanum diameter half the diameter of the eye. *Anodonthyla hutchisoni* differs from *P. cowani* in lacking webbing between the toes, in lacking a uniformly white ventral surface, in the absence of a beige triangular marking on the dorsal surface, in the possession of dorsal tubercles, and in the possession

of white dorsal spots, usually. *Anodonthyla hutchisoni* differs from *P. occultans* in lacking vestiges of webbing between the toes, in lacking a triangular marking on the dorsal surface, in a tibio-tarsal articulation that extends at least to the eye, in the possession of dorsal tubercles, and in the possession of white dorsal spots, usually. In addition to morphological differentiation of the new species from frogs in the genus *Platypelis*, Glaw & Vences (2005) demonstrate that *A. hutchisoni* is phylogenetically nested amongst other species of *Anodonthyla*; a monophyletic group that has diverged from *Platypelis* (Andreone et al. 2005).

Distribution.---*A. hutchisoni* is currently known from the following localities of northeastern Madagascar: Ambanizana, Andasin'I Governora, Andranobe, Antsarahan'Ambararato, Menamalona. The localities fall within the mainland Masoala Peninsula, western coast and slope. The localities of Ambanizana, and Andranobe are on/near the coast, while the others are farther inland.

According to Glaw & Vences (2005) the similar species, *A. boulengeri*, is known from the following localities: Andasibe (ZFMK 53742, 52780, 62215, 62222), Nosy Boraha (ZFMK 52784--52786), Ranomafana (ZFMK 62313, ZMA 19430; ZSM 642/2003, 643/2003), and Foulpointe (ZSM 264/2002). Furthermore, we also attribute to *A. boulengeri* a specimen recently collected at Antara (MRSN-RJS 0804; Toamasina faritany, Mananara fivondronana; leg. JER, 15 Jan 2005). We suspect that the distribution of *A. boulengeri* extends from central-eastern Madagascar up to the Antongil Bay (as is supported by the finding of a specimen at Antara, which is south of Maroantsetra, and identified by FA as *A. boulengeri*), while *A. hutchisoni* is a potential endemic of the

mainland Masoala Peninsula. As far as is known, *A. Boulengeri* and *A. hutchisoni* do not live in syntopy.

A population of *Anodonthyla* from Nosy Mangabe is difficult to unequivocally ascribe to one species. A single analyzed female housed in Turin (MRSN A201) has a light dorsal coloration and has whitish spots at the level of the groin. The SVL is 21.6 mm, similar in size to *A. hutchisoni*. So, we wait for further information before we assign the Nosy Mangabe population to a species.

Ecology.---*A. hutchisoni* was found during the day and night from 0--3 m above the ground but was only found calling or out of refuges at night. Six of eight specimens found in 2001 were found in phytotelmata (tree holes), while the other two (OMNH 39030, 39027) were found moving freely on a tree trunk and vine respectively. A male-female pair (OMNH 39011, 39012) providing useful ecological data was only recently identified as *A. hutchisoni* and are therefore not included in the type series. These were collected 4 Nov 2001 near the locality of the holotype. They were located, by following the male's call to a tree hole 1.0 m above the forest floor in a 10 cm deep hole, and they were found with 23 eggs, which appeared viable (clear gel capsule and white, unpigmented embryos). The eggs were completely submersed in water (approx. 13 ml). A different male-female pair of adults (OMNH 39031, 39032) was also found in a tree hole 0.8 m above the forest floor with 29 eggs. The eggs were colored as above, contained embryos approximately 3.3 mm long, and appeared to be in Gosner stage 15 or 16 (Gosner 1960). They were completely submersed in the water (approx. 26 ml, pH = 5.4).

One specimen (OMNH 39026) was found syntopically with *Plethodontohyla notosticta* and an unidentified microhylid (juv.). Microhylids found in sympatry during this study were: *Anodonthyla* sp. (a juvenile specimen which we were unable to assign to a species), *Platypelis grandis*, *P. tetra*, *P. barbouri*, *P. tuberifera*, *P. sp.*, *Plethodontohyla notosticta*, *P. ocellata*, *Stumpffia tetradactyla*, *S. grandis*, and *S. roseifemoralis*.

Etymology.---The epithet “*hutchisoni*” is designated as a patronym in honor of Victor H. Hutchison to recognize his distinguished career and significant contribution to our understanding of herpetological biology, ecology, and physiology.

Chapter Summary

The new species of *Anodonthyla* is an arboreal member of the genus likely allied with *A. boulengeri*, with which it shares several morphological and chromatic characteristics. It is distinct from that species through differing genetic, morphological, and likely vocalization characteristics. Further field and genetic studies are required to understand the relationships of species in this genus, but the recent phylogenetic analysis carried out by Andreone et al. (2005) clarifies what is known to date.

The new species appears to be distributed only in northeastern Madagascar, although further data are needed to confirm this hypothesis. However, our intensive work in much of the pertinent Malagasy forests allows us to be rather confident of our reported distribution. In this context, *A. hutchisoni* is the only currently described species of this genus present in Masoala proper, where it seems to be restricted only to low altitude rainforests. This description adds to the already important data for Malagasy herpetodiversity (Andreone 2004). Further inventories should be conducted to determine

whether the new species is also present in the low altitude rainforests of the eastern slope of the Masoala Peninsula.

Serious threats challenge the anuran fauna of this region of Madagascar (Andreone & Luiselli 2003, Andreone 2004, Andreone et al. 2005). In terms of conservation, it is difficult to categorize the new *Anodonthyla* herein described. The few localities where it is present do not lend well to the determination of specific threats. For this reason, this species should be included in the “data deficient” species that already comprises a great number of Malagasy species (Andreone et al. 2005). Undoubtedly, deforestation represents a key threat for the Masoala Peninsula. Andranobe is currently protected, while the other known localities for *A. hutchisoni* are outside the Masoala National Park.

CHAPTER 3:

Redescription of *Rhinella ceratophrys* (Boulenger) (Amphibia:Bufonidae), with Notes on its Ecology and Distribution

In 1882, Boulenger described *Bufo ceratophrys*, now *Rhinella ceratophrys* (Frost et al., 2006), from a juvenile specimen (B.M. 80.12.5.151.) obtained in Ecuador. Early collections of specimens only involved juveniles, with sizes ranging from 12–35mm snout–vent length (SVL) (Boulenger, 1882; Cochran and Goin, 1970; Hoogmoed, 1977; Rivero, 1972). These collections led early workers to characterize the species as far smaller than in reality. Complicating our understanding of the species is the fact that few specimens of *R. ceratophrys* have been found, and scant information has been published on its ecology. Based on observations in the field and evaluation of museum specimens, we present a redescription of this distinctive toad, based primarily on a well preserved series from Vaupés, Colombia, housed in the herpetological collections of The University of Texas at Arlington.

Gallardo (1962) included *Rhinella ceratophrys* in the *Chaunus* (= *Bufo*) *marinus* subgroup. Blair (1972) and Hoogmoed (1977) included the taxon in the “*Bufo typhoni*” group. Hoogmoed (1977) considered *Rhaebo nasicus* the sister taxon to *Rhinella ceratophrys*, but at the time both were in the genus *Bufo*. Both *Rhinella ceratophrys* and *Rhaebo nasicus* have a projecting flap of skin above each eye and similar dorsal patterns and colors. However, as Hoogmoed (1977) noted, the eyelid process is more developed in *Rhinella ceratophrys* than in *Rhaebo nasicus*. Hoogmoed (1977) argued that *Rhinella ceratophrys* is the more derived of the two, with *Rhaebo nasicus* representing the “primitive condition.” He indicated important differences

including: (1) *Rhinella ceratophrys* not exceeding a snout–vent length (SVL) of 35mm, (2) *Rhaebo nasicus* not having pointed flaps of skin at the corners of its mouth, (3) the parotoid glands in *Rhaebo nasicus* being less elongate and less pointed, and (4) the presence of paired swellings of skin present above the five anteriormost vertebrae in *Rhinella ceratophrys*, which are absent in *Rhaebo nasicus*. While Hoogmoed (1977) suggested that these two species were likely closely related, he emphasized that he did believe that they represented distinct species. He based this supposition primarily on differences in size (*Rhinella ceratophrys* reaching approximately 35 mm SVL; *Rhaebo nasicus* reaching nearly 67 mm SVL) and differential degree of development of the fleshy projection over the eyes (large in *Rhinella ceratophrys*; small in *Rhaebo nasicus*). However, Hoogmoed (1977) had available to him only a small number of juvenile specimens of *Rhinella ceratophrys*. The discovery of additional specimens in the field and in museums indicates that Hoogmoed’s (1977) characterization of *Rhinella ceratophrys* must be modified to include variation among adult specimens. Furthermore, we note that the original description by Boulenger (1882) was based on a juvenile specimen, as are all other accounts prior to that presented by Rodríguez and Duellman (1994). Based on this history of inadequate accounts, we herein present a redescription of *Rhinella ceratophrys* (Boulenger, 1882).

Materials and Methods

In order to determine the distribution of *R. ceratophrys*, we compiled a list of all museum records of which we were aware. The diagnosis and redescription presented below are based on examination of well-preserved adult specimens from throughout their

known range. Museum acronyms used herein follow Leviton *et al.* (1985). The sex of individuals was determined by direct observation of the gonads and/or secondary sexual characters (e.g., vocal slits in males); very small specimens, lacking any secondary sexual characters were assumed to represent juveniles.

With digital calipers, we took the following measurements of specimens: snout–vent length from the tip of the snout to the vent (abbreviated as SVL throughout); head width at the point of the widest dorsal view of the cranium; head length from angle of jaw to tip of snout; eye-to-nostril distance measuring from the anterior edge of the eye to the center of the nostril; horizontal tympanum diameter from anterior to posterior edge of tympanic ring; hand length from proximal edge of palmar tubercle to tip of the longest digit; tibia length measuring from tip of knee to base of tarsus on bent leg; foot length from proximal articulation of metatarsal tubercle to tip of the longest digit. Comments on coloration are based on observation of living specimens and photographs of same. Ecological notes are derived from field experience and examination of museum specimens.

Systematic Account

Rhinella ceratophrys (Boulenger, 1882)

Bufo ceratophrys (Boulenger 1882)—Holotype: BMNH 80.12.5.151; Type locality: "Ecuador".

Bufo ceratophrys Boulenger, 1882; Gadow, 1901; Nieden, 1923; Gallardo, 1962; Gorham, 1963; Cochran and Goin, 1970; Blair, 1972; Hoogmoed, 1977; Frost, 1985; La Marca, 1992; Rodríguez and Duellman, 1994; Ruíz-Carranza *et al.*, 1996; Lamar, (Under Review).

Rhinella ceratophrys Frost *et al.*, 2006.

Diagnosis.---*Rhinella ceratophrys* is a distinctive species that differs from all other species of bufonid anurans in South America by having triangular dermal flaps projecting over the eyelids. While *Rhinella ceratophrys* historically has been taxonomically confused with *Rhaebo nasicus*, it may be easily distinguished from the latter by having: 1) triangular projecting dermal flaps on the eyelids; 2) projecting dermal flaps at the corners of its mouth; 3) a larger adult size (105 mm SVL in *Rhinella ceratophrys* vs. 67 mm SVL in *Rhaebo nasicus*). The utility of other putative diagnostic characters, such as shape of parotoid glands and other dermal textures and structures should await a more complete survey of variation in both species.

Description.---Body robust; head wider than long, approximately 33 % of SVL in females, 35 % of SVL in adult males. Head length in adult females is approximately 28 % of SVL, and approximately 33 % SVL in adult males. Snout pointed in dorsal view, moderately rounded in profile, rostral keel absent but *canthus rostralis* distinct, canthal, supraorbital, postorbital, supratympanic, and parietal crests absent; preorbital crest present, weakly developed, about 1/3 length of eye–nostril distance; skin on medial surfaces of canthal, supraorbital, postorbital, and lateral surfaces of head generally smooth; ocular skin fold present, approximately equal in length to eye diameter, extending from top of head to the beginning of the supratympanic fold, occasionally forming an elevated knob posteromedial to the eye; nostril protuberant, directed laterally; loreal region concave; upper lip barely distinct, rounded, lower lip distinct; one large, triangular tubercle present slightly posterior to the confluence of the upper and lower jaw, surrounded by several smaller tubercles; eye–nostril distance approximately 88 % of eye diameter in females, 70 % of orbit diameter in males; tympanum distinct, slightly ovoid,

approximately 60 % of eye diameter in females, 55 % of eye diameter in males; supratympanic fold distinct, terminating at the posterior edge of the parotoid gland. Forelimbs, short, robust, outer edge of forearm bearing a row of ulnar tubercles; prominent, triangular tubercles present at insertion of arm, and on anterior surfaces of arm and chest; hand broad, with long fingers; relative length of fingers $II < IV < III < I$; webbing absent between Fingers I and II, webbing vestigial between Fingers II and III, and between Fingers III and IV, all fingers bearing distinct lateral fringe; tips of fingers with slightly rounded tips, smooth dorsally; palmar tubercles prominent; subarticular tubercles distinct. Hind limbs robust, moderate in length, tibia approximately 48 % SVL in females, 49 % SVL in males; foot length approximately 39 % SVL in females, 41 % SVL in males, tarsal fold absent; inner metatarsal tubercle prominent, ovoid, toes long, slender, relative lengths of toes $I < II < V < III < IV$; lateral fringe on all toes, distinct; webbing thin, vestigial; tips of digits not enlarged, slightly rounded, smooth dorsally; subarticular tubercles prominent, raised and oblong in profile; supernumerary tubercles distinct; inner metatarsal tubercles prominent and ovoid; outer metatarsal tubercles prominent and rounded.

Skin on dorsum of body with irregularly spaced conical tubercles, many bearing keratinous apices; distinct dorsolateral ridge of large triangular tubercles extending from the posterior edge of parotoid gland to anterior edge of hindlimbs; parotoid glands larger than eyelids, distinctly raised, elongate and oblong in shape, color pattern of dorsolateral ridge splits the parotoid gland evenly, texture of gland tuberculate above, smooth below; dorsal surface of arms tuberculate over tibia and fibia, less tuberculate over humerus, tuberculate over the legs; skin on chest with small, distinct tubercles, ventral surface with

smaller conical tubercles. Nuptial excrescences in males small, poorly developed, appearing as simple fleshy thickenings on base of Finger I, no evidence of keratinous spicules (among specimens examined).

Choanae large, ovoid, widely spaced, clearly visible; teeth absent; tongue pear shaped, free posteriorly. Vocal slits in males are bilateral, approximately 1/3 length of tongue.

Coloration in Life.---This toad is medium to dark brown dorsally, occasionally with gray to reddish tan or golden brown highlights. A pale, thin, middorsal line, extending from the tip of the snout to the vent is often evident. A pattern of diffuse darker brown bands beginning posteriad to the parotoids and often continuing across the thighs and shanks is usually evident. The dorsal pattern may include chocolate brown bars radiating from the middorsal stripe to the tips of the supraocular processes. The snout, face, and flanks are primarily chocolate brown, with or without paler invasions beneath the eyes, tympanum, and in the paraventral region. The tympanum is usually medium brown to gray. The edge of the lower lip is usually cream colored. The forelimbs are pale brown with irregular darker markings. The overall effect is of a dead leaf and paler specimens also are counter shaded by the dark lateral pigment. Ventral color ranges from uniformly pale to dark brown, with or without darker mottling. The chin in adult males is uniformly blackish. Some specimens are so dark dorsally that a pattern is not evident. The iris is brownish bronze with gold highlights dorsally.

Distribution.---This species apparently has a patchy distribution including southern Venezuela, southeastern Colombia, undoubtedly northwestern Brazil, northeastern Peru and eastern Ecuador. It would not be surprising if *Rhinella ceratophrys*

occurs further north in Colombia, perhaps passing the Río Inirida and extending into eastern Vichada. The western and southern limits of the range in Ecuador and Peru have not been established. Notes on the distribution of this species in Venezuela have been provided by Rivero (1972), La Marca (1992), and E. La Marca (pers. comm.).

Distributional information from Colombia has been presented by Ruíz-Carranza et al. (1996) and Cochran and Goin (1970); similar information from Ecuador was presented by Coloma (1991). In Peru this species appears to be confined to the west and north banks of the Marañón and Amazon Rivers, respectively.

Ecological Notes.---*Rhinella ceratophrys* inhabits leaf litter on the floor of humid primary forests. In northeastern Peru it seems restricted to non-flooded forest although we have found specimens along riverbanks. Secondary areas close to undisturbed forest are also used. It is both diurnal and nocturnal (L. Coloma, pers. comm.; W. W. Lamar, pers. observ.). We have also found sleeping specimens at night so nocturnal activity may be associated with breeding or some other seasonal cue. When disturbed or approached, this toad presses its body down into the leaf litter and remains motionless. During ecdysis the eyelid processes are alternately rotated forward and backward to pull loose skin toward the mouth where it is consumed. Stomach contents of specimens collected in Vaupés, Colombia, (see Appendix 1) yielded termites of the genus *Nasutitermes*. A specimen from Orán, Loreto, Peru, was observed feeding on ants (*Dolichoderus bispinosus*) (A. Giardinelli, pers. comm.). Captive specimens in our care fed readily on termites.

Reproductive Notes.--- Three females from the series at UTA contained 1000–1500 small (< 1 mm diameter), pigmented eggs. Other aspects of the reproduction,

including behavior and advertisement call are unknown; the tadpole has never been observed.

Table 3.1 Morphometric variation in adults of *Rhinella ceratoprhys*.

Variable	Males n = 6	Females n = 9
Snout–vent length	56.5 ± 12.5 (39.9–69.8)	93.9 ± 15.5 (60.2–106.6)
Head width	20.0 ± 3.8 (14.4–24.4)	31.1 ± 4.6 (20.2–34.7)
Head length	18.4 ± 3.6 (13.1–22.0)	26.8 ± 3.1 (19.7–29.5)
Eye–nostril distance	4.3 ± 0.9 (3.3–5.7)	7.0 ± 1.3 (4.7–8.2)
Tympanum diameter	3.4 ± 0.6 (5.7–6.6)	4.8 ± 1.0 (2.8–5.8)
Hand length	14.7 ± 3.6 (10.2–18.6)	23.0 ± 3.2 (16.0–26.0)
Tibia length	27.8 ± 6.0 (19.5–33.27)	44.8 ± 7.5 (28.8–50.9)
Foot length	22.6 ± 5.7 (16.1–28.4)	37.4 ± 5.3 (26.0–41.9)

CHAPTER 4:

***Leptodactylus pustulatus* Peters, 1870 (Amphibia: Leptodactylidae): Notes on Habitat, Ecology, and Color in Life**

The Brazilian Cerrado remains one of the most poorly understood habitats in that country. Among the little known anurans from the Cerrado is a reclusive member of the genus *Leptodactylus* which Peters (1870) described as *Entomoglossus pustulatus*, collected from Ceará, Brazil (Bokermann 1966). Boulenger (1882) reassigned the species to the genus *Leptodactylus*. A number of subsequent publications briefly mention the species by either including it in faunal, distributional, or nomenclatural listings, or by providing short physical descriptions (Miranda Ribeiro 1926, 1927, Lutz 1926, 1930, Bokermann 1962, 1966, Heyer and Pyburn 1983, Frost 1985, Vanzolini and Heyer 1988, Liner 1992, Frank and Ramus 1995, Duellman 1999), but little else has been reported. The holotype was long believed lost, leading Heyer (1970) to designate a neotype (MCZ 373). Subsequently, the original type has been relocated in Berlin, Germany (ZMB 6951) (Bauer et al. 1995).

Habitat associations.---Little is known of the biology or ecology of the species. These frogs have been primarily associated with Amazon, Cerrado, and Caatinga habitats south of the Amazon River; the three drainage systems for which reliable locality records exist are the Paranaíba, the Araguaia, and the Tocantins in Central Brazil (Heyer 1970). The majority of the locality records associate the species with Cerrado (Heyer 1994) but frogs have also been found in a palm strewn habitat known in Brazil as “vereda.” The type specimen was collected in Ceará, Brazil (Peters 1870), an area dominated by Caatinga habitat. Bokermann (1962) reported finding individuals beneath an old boat

along the Xingu River. The locality, Posto Jacaré, is located in vereda between Cerrado and Amazonian forest (as was the locality for the author's specimens). No other habitat data for *L. pustulatus* has been published (Heyer 1994, Heyer pers. comm.), except for male calling sites (Brandão and Heyer in press).

In August 2000, the authors collected two specimens and observed a third along the upper Tocantins River in the state of Tocantins, Brazil. Vereda in the vicinity of the city of Tocantinópolis ranges from mildly disturbed to completely cleared for cattle grazing. We found our specimens in a heavily disturbed clearing with a natural spring that maintains a small wetland area, even in the dry season. During the wet season, the entire cleared area, approximately 50 ha², is inundated with water ranging from .2-1 m in depth. During the dry season, the wetland is reduced to small patches roughly one tenth the size of the wet season habitat. Tall grass covers the clearing at roughly .6 m in height. The ground is uneven and is arranged in a random series of slightly elevated mounds. These mounds become small, soggy "islands" in the wet season and are separated by shallow water. All *L. pustulatus* were found on these mounds at night. When approached, individuals crawl in a bufonid-like fashion rather than hopping away in a typical leptodactylid fashion. The following anurans were found sympatrically: *Leptodactylus ocellatus*, *L. podicipinus*, *Hyla raniceps*, *H. minuta*, *Scinax* sp., and *Pseudis tocantins*.

Color in life.--- The dorsal coloration of one individual was a dark, forest green, the other individual was dark brown. In contrast to other *L. pustulatus* without dorsolateral ridges (Heyer and Pyburn 1983), these specimens have six small, elevated dorsolateral ridges extending along the dorsum to the vent with an uneven surface. A maroon V-shaped pattern extends from behind each eye to the nasal openings. A faint

black line also extends from the base of each eye to the nasal openings. The pupils are horizontal and black. The iris is a faded copper color marked by irregularly spaced black reticulations that extend to the edge of the eye. The ventral pattern and coloration of *L. pustulatus* is a distinguishing character. The ventral ground color ranges from charcoal gray to black and can change within 20 minutes from gray to jet black or vice versa. Circular and ovoid spots cover the ventral surface extending from the throat covering to the groin and undersides of the legs, as well as onto the flanks. The spots wrap around the throat and onto the lower lip. Syntopic *L. ocellatus* and “*L. podicipinus*” also have white spots of similar proportion on their lower lip which confuses visual identification from a distance. The ventral spots on *L. pustulatus* do not connect and are white in the throat and chin region. All of the spots near the groin and lower abdomen range from a brilliant orange to canary yellow. Individual variation exists in the relative number of colored versus white spots, and in the progression of the orange spots up the ventral surface toward the animal’s head (Figure 4.1). In a resting position, the spots are visible on the chest and lower lip when approaching the animal from the front.



Figure 4.1 The ventral surface of *Leptodactylus pustulatus* demonstrating ventral color in life and variation in coloration of the markings

Table 4.1 Morphometric measurements for two specimens of *Leptodactylus Pustulatus*.

Specimen #	SVL	HW	HL	IOD	END	TD	FeL	TL
HLR20	56.3	19.2	22.4	14.3	4.8	3.8	21.5	16.9
HLR21	49.0	17.7	20.2	11.9	5.0	2.8	14.1	21.6

CHAPTER 5:

Exposure to Common Heavy Metals in Embryonic and Larval Development Between Related Species of Xenopus

Although metals occur naturally and almost ubiquitously throughout the environment (Paquin et al, 2003), they tend to be concentrated by and enter the environment through various anthropogenic activities. Common mechanisms are direct application of chemicals to the environment, atmospheric deposition, and non-point source run off (e.g., Mailman, 1980; Gunn, 1984; Fowler, 1989; Depledge, 1995; Thornton, 1996; Linder and Grillitsch, 2000; Haywood et al., 2004). Additional activities, such as watershed acidification through fossil fuel burning (acid rain), can increase metal bioavailability in aquatic systems indirectly, by changing attributes like ionization and bonding in metals (Linder and Grillitsch, 2000). While at trace concentrations, some metals are essential nutrients and are indispensable to life processes, for example: selenium, zinc, copper, and chromium (Linder and Grillitsch, 2000). At higher concentrations, these metals may reach threshold concentrations whereby they can exert varied negative biological effects in exposed organisms such as, oxidative stress, carcinogenesis, teratogenesis, and lethality (Gunn, 1984; Thornton, 1996; Calevro et al., 1999; Linder and Grillitsch, 2000; Buzzard and Kasprzak, 2000; Ercal et al., 2001; Haywood et al., 2004; Henson and Chedrese, 2004).

Particularly susceptible to aquatic contamination are the amphibians. Life cycle ties to aquatic ecosystems render amphibian larvae vulnerable to metal bioaccumulation. Metals are absorbed through gills and the integument as well as digested from

contaminated food (Linder and Grillitsch, 2000). Once in the amphibian system, metals cause diverse and profound problems (Fort and Stover, 1997; Linder and Grillitsch, 2000; Chen et al., 2006, 2007; Gross et al., 2007). For example, drastic morphological alterations before and during metamorphosis create significant periods of susceptibility for teratogenesis through metal contamination (Cooke, 1981; Haywood et al., 2004). In addition, sensitivity to contaminants likely varies within and among anuran amphibians lineages (Linder and Grillitsch, 2000); however, it remains unclear if closely related species have common responses, such as shared toxicological endpoints, much less if widely divergent species share common responses. Because previous studies have employed various and incomparable methods (e.g., water conditions, developmental starting points, phenotypic measurements, toxicological endpoints, and exposure concentrations), differences in sensitivity cannot be adequately drawn (Linder and Grillitsch, 2000).

The African aquatic frogs in the genus *Xenopus* offer opportunities for comparative work on environmental sensitivities. One species, the “African clawed frog,” *Xenopus laevis*, has been used in research for over 100 years (Touart, 2002). Studies with *X. laevis* include various environmental contaminants, heavy metals among them (e.g., Bantle et al., 1989; Fort et al., 2004a; Haywood et al., 2004). A close relative, *Xenopus tropicalis*, is also an aquatic frog that has recently garnered research attention due to three significant advantages: (1) *X. tropicalis* has a diploid genome, (2) *X. tropicalis* represents opportunities for inbred and transgenic lines, and (3) *X. tropicalis* has a much shorter life cycle and less than half the time span to sexual maturity (Touart, 2002; Fort et al., 2004a, 200b). Although *X. tropicalis* is being used in the laboratory on

an increasing basis (Fort et al., 2004a, 2004b), in contrast to *X. laevis* scant work has been published evaluating *X. tropicalis* as a tool in ecotoxicology study (Fort et al., 2004a, 2004b). Comparisons between the species are inevitable (Song et al., 2003; Fort et al., 2004a). Comparing the toxicological response to metals between these related species is warranted in assessing *X. tropicalis* as a good replacement for *X. laevis* in toxicology studies. I performed 72h embryotoxicity studies directly comparing *X. laevis* and *X. tropicalis* for sublethal exposure effects to cadmium, chromium, lead, aluminum, and zinc. As well I calculated teratogenic index values for each species to each metal in order to better compare relative sensitivities to each metal between the species pair.

Materials and Methods

Approaching Xenopus husbandry.--- I maintained each species at its recommended culture and maintenance temperature: *X. tropicalis* and *X. laevis*, 26°C vs. 23°C respectively. In a comparative study, Fort et al. (2004a) found that the difference in recommended culture and maintenance temperature between *X. tropicalis* and *X. laevis* (25–27°C vs. 23°C respectively) did not have a significant impact on the reactions by either species to contaminants. The recommended culture and maintenance temperature takes into account mean water temperatures from the regions where each species is found naturally. Fort et al. (2004a) suggested including recommended temperature requirements into an experimental design. They argued that by maintaining *X. tropicalis* at cooler temperatures (e.g., those preferred by *X. laevis*), frogs might demonstrate responses to contaminants that are in part a reflection of being maintained at less than optimal temperatures.

Husbandry and reproduction, Xenopus laevis.--- Adult *Xenopus laevis* (Xenopus Express, Plant City, FL) were maintained in groups of four within 106L plastic tubs (holding 90L of water). Frogs were maintained in laboratory prepared water (pH 7.2–7.5, Total Hardness 136 mg/L CaCO₃, 23°C) with established sponge filtration. These tubs were maintained in a climate controlled animal facility on a 12h :12h light/dark photoperiod throughout the experiment. Frogs were fed every 48 h with floating frog food (Xenopus Express, Plant City, FL) and uneaten food was removed from the tubs within 20 minutes after feeding behavior ended. Water changes (30L) were performed 24h after feedings.

Adult female *X. laevis* were injected with 1000 units of human chorionic gonadotrophin (HCG) (Sigma-Aldrich) to induce ovulation. Males were injected with 250 units of HCG to increase sexual drive and induce amplexus. All frogs were injected in the dorsal lymph sac and two or more pairs of frogs were injected for each trial. Egg deposition followed from 12h to 18h after injection.

Husbandry and reproduction, Xenopus tropicalis.--- Each breeding pair (n = 48 pairs) of adult *Xenopus tropicalis* (Nasco, Fort Atkinson, WI) were maintained in 8L of UV sterilized laboratory prepared water (pH 7.5–7.8, Total Hardness 170 mg/L CaCO₃, 26°C) and at a photoperiod 14h light: 10h dark in a climate controlled animal facility throughout the experiment. Frogs were fed Juvenile Frog Brittle (Nasco, Fort Atkinson, WI) every 48h and allowed to eat ad libitum overnight with 90% water changes occurring with 12h of feeding. For each contaminant trial at least 8 pairs of *Xenopus tropicalis* adults were bred. All *X. tropicalis* adults used in this study were 3 years old, bred every

3–4 months, and breeding success (since animals were 7-8 months old) and embryo survival to 72h per clutch were monitored prior to initiating this study.

Adult male and female *X. tropicalis* were injected (primer dose) with 20 units of HCG (Chorulon). 48h after the priming dose, female *X. tropicalis* were injected (booster dose) with 200 units of HCG to induce ovulation. Likewise, males were injected with 100 units of HCG and pairs were allowed to undergo amplexus in 22°C water. All frogs were injected subcutaneously with a 26 gauge needle in the tissue folds immediately dorsal and anterior to the cloaca. Fertilization usually proceeded within 4h of the booster dose.

Heavy metals and doses.--- Six heavy metals were chosen because they are relatively common heavy metal contaminants, and some published EC₅₀ and LC₅₀ toxicity data existed to bracket our selected doses (DeYoung et al., 1991; Rayburn, 2000; Haywood et al., 2004; Fort et al., 2004a; Gross et al., 2007). The focal heavy metals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) as: chromium anhydride (CrO₃), copper sulphate (CuSO₄), lead nitrate (PbNO₃), sodium selenate (Na₂SeO₄), cadmium chloride (CdCl₂), and zinc sulphate (ZnSO₄). Stock solution was created by dissolving each metal in 1L of millipure water. Each dilution was prepared by diluting the initial stock solution with FETAX solution (Frog Embryo Teratogenesis Assay — *Xenopus* (Dumont et al, 1983)) to achieve 1L of the appropriate exposure concentrations.

Exposures.--- Starting points for our toxicology studies were pulled from studies that used the FETAX model with focal contaminants (Haywood et al., 2004; Fort et al., 2004a). Using common embryotoxicity methodologies, we created a data set where

species sensitivities are directly comparable. The duration of the exposures in this study, 72h and 96h intervals, were chosen based on (1) different optimal culture temperature and likely differences in developmental rates, and (2) limitations to experimental run times as determined in published studies. For example, findings from Fort and Stover (1996) identified the importance of an experimental run exceeding 24h. This work in an aquatic system demonstrated that a heavy metal, copper, was deleterious at the highest treatment dose at 24h. In lower doses, periods longer than 24h were crucial to detect decreased survival, delayed development, and increased developmental malformations. Thus, the 72h and 96h studies provide more information on developmental changes than shorter treatment regimes. Metals can inhibit development in amphibians indirectly by slowing metabolic rates (Dawson et al., 1985). Further inhibition can be different between unhatched and hatched embryos (Dawson et al., 1985). For these reasons, 72h and 96h toxicity studies, that encompass both unhatched embryos and hatched larvae, were used.

Experimental approach.--- Standardization of the experiment environment is critical because water chemistry can affect metal toxicity in aquatic systems (e.g., Linder and Grillitsch, 2000; Paquin et al., 2003). FETAX (Dumont et al, 1983) is a standardized toxicity screening assay that examines the effects of contaminants on developing amphibian embryos and larvae. FETAX protocol standardizes water conditions whereby experimenters use a cookbook recipe to make “FETAX solution.” I used FETAX solution with a neutral Ph value (7.0–7.3) to standardize my experimental environment.

Embryotoxicity models often use FETAX solution to standardize aquatic conditions but deviate from the FETAX model in isolating experimental subjects rather than collectively pooling them. From the use of FETAX solution, I departed from FETAX assay methodology and employed an embryotoxicity model. With experimental subjects all inhabiting a common container, group effects might have impacted my study. For example, should an embryo/larvae die close enough to a water change so that the corpse begins to decompose before the next water change, byproducts of the decomposition process could affect the results and outcomes, misrepresenting the real effects of the contaminant under study. With these potential problems in mind, I used an embryotoxicity model employing cell culture petri dishes (Falcon). The individual wells isolate each embryo/tadpole within an experimental group and eliminate the potential FETAX “shared-pool” problem.

In a comparative ecotoxicology study, it is critically important to understand lethal concentrations and effective concentrations, LC_{50} and EC_{50} s respectively, to produce a teratogenic index. LC_{50} and EC_{50} are defined here as follows: LC_{50} = the lethal concentration at which 50% or more of the experimental subjects expire; EC_{50} = the effective concentration at which 50% or more of the experimental subjects demonstrate teratogenic effects. “Expire” is defined as no movement in a test subject combined with no visible evidence of a heart beat. A teratogenic index, the division of the LC_{50} by the EC_{50} , produces a value that demonstrates separation between lethal and non-lethal concentrations of a given contaminant. For example, Fort et al. (2004a) suggested that a teratogenic index value equal to or smaller than 1.5 was of no consequence. An inconsequential value is produced when little or no separation exists

between non-lethal and lethal concentrations, leaving little room for teratogenic effects to manifest. I used teratogenic index values as a comparative character to help determine the potential in both species of *Xenopus* for teratogenic effects to manifest.

Experimental protocol. --- Temperature controlled rooms were employed with experimental runs for both *X. tropicalis* and *X. laevis*, strictly maintained at 26°C vrs. 23°C respectively. To accommodate differences in developmental rates between the two species of frogs, experiments were conducted for 72h with *X. tropicalis* and for 96h with *Xenopus laevis*.

With both species of *Xenopus*, equal numbers of embryos were used from 3 clutches; embryos chosen were staged using the Neiwenkoop and Faber (1975) method chosen at stages 4–8 for *X. tropicalis* and stage 5 for *X. laevis*. Eggs were randomly distributed between 6 well plastic culture Petri dishes (Falcon). Falcon Petri dishes were pre-washed in a 3% nitric acid solution and rinsed in distilled water before use in these experiments. Egg jelly coats remained intact and embryo viability was confirmed by dissecting scope prior to initiating the experiment.

Each embryo was exposed to 5 ml of treatment solution in an individual well of a six well cell culture dish. Every 24h, prior to a complete water change and replenishment with equivalent experimental solution, all embryos were evaluated for survival, developmental stage, and malformations. Each trial used twice the number of controls, with regard to experimental group numbers. At the termination of each experimental run, larvae were euthanized by adding 3-aminobenzoic acid ethyl ester, MS222, (1g/1L) to their water. In each experimental run, if the mortality or malformations rose above 3%, the experiment was run again.

Statistics. --- Probit analyses were used to calculate LC₅₀ and EC₅₀ values (Hayes, 1994). Teratogenic indexes were calculated (EC₅₀/LC₅₀) (Fort et al., 2004a). Concentration data were log transformed to fit a normal distribution curve (Hayes, 1994). Proportion data for survival, teratogenesis, and percent metamorphosis, were adjusted with an arcsine square-root transformation for normalization (Chen et al., 2007). In early trials, when initially determining the rough concentrations equating to LC₅₀ and EC₅₀ values, or “range finding,” these values were not used in calculation of LC₅₀ and EC₅₀ values. Outliers, three or more standard deviations out, were also not used in calculating LC₅₀ and EC₅₀ values. All statistics were conducted using Systat 11 software.

Results

Table 5.1 Comparisons between *X. laevis* and *X. tropicalis* with regard to 6 heavy metals.

Test Metal	Species	LC ₅₀	EC ₅₀	TI (LC ₅₀ /EC ₅₀)
Cadmium chloride (CdCl ₂)	<i>X. laevis</i>	1.87	1.12	1.67
	<i>X. tropicalis</i>	1.28	0.72	1.78
Copper sulphate (CuSO ₄)	<i>X. laevis</i>	0.30	0.15	2.00
	<i>X. tropicalis</i>	0.20	0.04	5.00
Sodium selenate (Na ₂ SeO ₄)	<i>X. laevis</i>	46.30	9.64	4.80
	<i>X. tropicalis</i>	5.84	7.99	0.73
Chromium anhydride (CrO ₃)	<i>X. laevis</i>	39.94	>5.00*	N/A
	<i>X. tropicalis</i>	20.87	17.24	1.21
Lead nitrate (PbNO ₃)	<i>X. laevis</i>	N/A	N/A	N/A
	<i>X. tropicalis</i>	N/A	N/A	N/A
Zinc sulphate (ZnSO ₄)	<i>X. laevis</i>	94.67	6.96	13.60
	<i>X. tropicalis</i>	17.08	5.60	3.05

Table 5.2. Data collected for *X. laevis*

Test Metal	Concentration (ppm)	Numbers at the initiation of the experiment (n ₀)	Survival post 24 h (n)	Teratogenic effects at 96 h	Survivorship at 96 h	
Cadmium chloride (CdCl₂)	0.18	24	24	1	23 (96%)	
	0.72	24	24	5	23 (96%)	
	1.44	24	17	6	15 (63%)	
	1.88	24	23	20	18 (75%)	
	2.35	24	23	22	10 (42%)	
	2.82	24	23	16	2 (8%)	
Control	0.00	48	48	0	48 (100%)	
Copper sulphate (CuSO₄)	3.07x10 ⁻³	24	24	0	23 (96%)	
	0.02	24	24	4	23 (96%)	
	0.05	24	24	4	19 (79%)	
	0.13	24	24	8	17 (71%)	
	0.26	24	24	18	19 (79%)	
	0.52	24	24	11	2 (8%)	
	0.70	24	24	7	6 (25%)	
	Control	0.00	48	48	1	48 (100%)
Sodium selenate (Na₂SeO₄)	4.18	24	24	5	23 (96%)	
	41.80	24	24	20	17 (71%)	
	83.60	24	24	14	10 (42%)	
	104.50	24	24	24	0 (0%)	
	209.00	24	24	24	1 (4%)	
	418.00	24	24	24	0 (0%)	
	Control	0.00	48	48	1	48 (100%)
Chromium anhydride (CrO₃)	5.00	24	24	24	22 (92%)	
	10.00	24	24	24	24 (100%)	
	20.00	24	24	24	23 (96%)	
	25.00	24	24	24	23 (96%)	
	30.00	24	24	24	20 (83%)	
	35.00	24	24	24	18 (75%)	
	40.00	24	24	24	11 (46%)	
	Control	0.00	72	72	0	72 (100%)
	Lead nitrate (PbNO₃)	1.4 x 10 ⁻²	24	23	3	23 (96%)
0.14		24	22	3	22 (92%)	
1.40		24	23	0	23 (96%)	
14.4		24	23	0	23 (96%)	
Control	0.00	48	48	0	48 (100%)	
Zinc sulphate (ZnSO₄)	1.28	24	24	5	23 (96%)	
	12.80	24	24	10	22 (92%)	
	32.00	24	23	11	22 (92%)	
	64.00	24	23	23	20 (83%)	
	96.00	24	24	24	13 (54%)	
	112.00	24	21	19	9 (38%)	
	128.00	24	23	23	6 (25%)	
	Control	0.00	48	48	0	48 (100%)

Table 5.3 Data collected for *X. tropicalis*.

Test Metal	Concentration (ppm)	Numbers at the initiation of the experiment (n ₀)	Survival post 24 h (n)	Teratogenic effects at 96 h	Survivorship at 72 h
Cadmium (Cd)	0.40	18	18	3	17 (94%)
	0.80	18	17	11	16 (89%)
	1.08	18	18	11	15 (83%)
	1.28	18	18	15	15 (83%)
	1.70	18	9	4	1 (6%)
Control	0.00	36	36	3	35 (92%)
Copper sulphate (CUSO₄)	0.10	18	18	10	17 (94%)
	0.25	18	18	15	10 (56%)
	0.50	18	7	2	0 (0%)
	0.70	18	6	0	0 (0%)
	3.40	18	10	2	0 (0%)
Control	0.00	35	33	0	33 (94%)
Sodium selenate (Na₂SeO₄)	2.61	24	22	0	22 (92%)
	10.45	24	23	23	8 (33%)
	20.90	24	23	22	0 (0%)
	41.80	24	23	21	0 (0%)
	418.00	24	24	10	0 (0%)
Control	0.00	48	48	1	47 (98%)
Chromium anhydride (CrO₃)	3.13	21	21	2	18 (86%)
	12.50	23	23	4	20 (13%)
	25.00	24	24	20	15 (63%)
	50.00	24	23	23	0 (0%)
Control	0.00	40	40	6	35 (88%)
Lead nitrate (PbNO₃)	0.90	24	22	2	23 (96%)
	1.80	24	23	3	23 (96%)
	3.60	24	24	1	24 (100%)
	7.20	24	23	5	20 (13%)
	14.40	24	24	2	20 (13%)
	Control	0.00	48	48	6
Zinc sulphate (ZnSO₄)	1.00	18	16	2	15 (83%)
	4.00	18	16	3	16 (89%)
	16.00	18	17	15	6 (33%)
	32.00	18	18	14	8 (33%)
	64.00	18	15	15	0 (0%)
	128.00	18	0	0	0 (0%)
	Control	0.00	36	32	3

Cadmium. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to cadmium are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3.

Concentration-response curves are represented in Figures 5.1 and 5.2. While slightly higher, the mean LC₅₀ value for *X. laevis* at 23°C (1.87 ppm) was not substantially different (order of magnitude) to that found at 26°C for *X. tropicalis* (1.28 ppm).

Similarly, while slightly higher in *X. laevis*, the EC₅₀ values were not substantially

different between the species pair (1.12 ppm and 0.72 ppm respectively). The TI values between *X. laevis* and *X. tropicalis* are similar for cadmium (1.67 and 1.78 respectively).

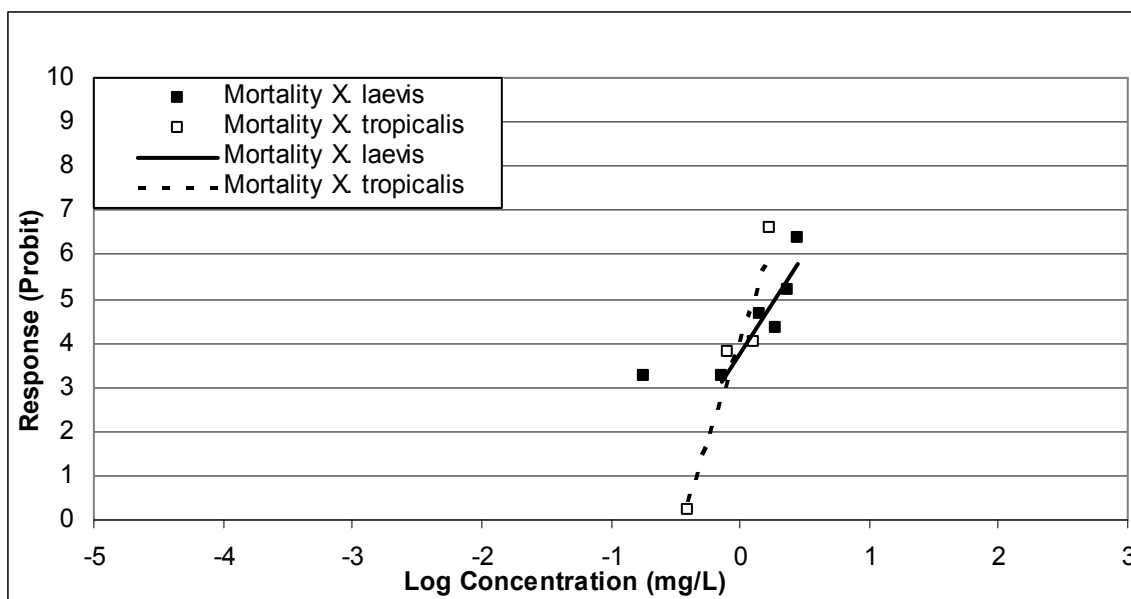


Figure 5.1 Graphic estimation of cadmium LC50 by Probit Analysis.

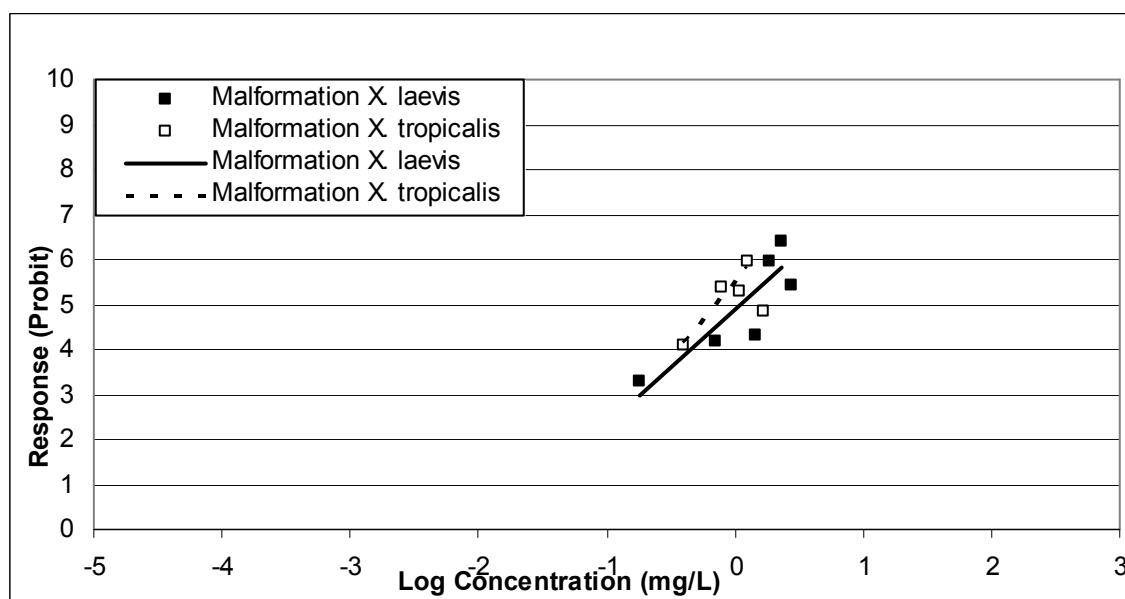


Figure 5.2 Graphic estimation of cadmium EC50 by Probit Analysis.

Copper. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to copper are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3.

Concentration-response curves are represented in Figures 5.3 and 5.4. While slightly higher, the mean LC₅₀ value for *X. laevis* at 23°C (0.30 ppm) was not substantially different (order of magnitude) to that found at 26°C for *X. tropicalis* (0.20 ppm). Higher in *X. laevis*, the EC₅₀ values were different between the species pair (0.15 ppm and 0.04 ppm respectively); *X. laevis* has an EC₅₀ value nearly 4 times higher than *X. tropicalis*. The TI values between *X. laevis* and *X. tropicalis* are different for copper (2.00 and 5.00 respectively); the difference in TI between the species pair is a 2.5 fold difference greater in *X. tropicalis* than in *X. laevis*.

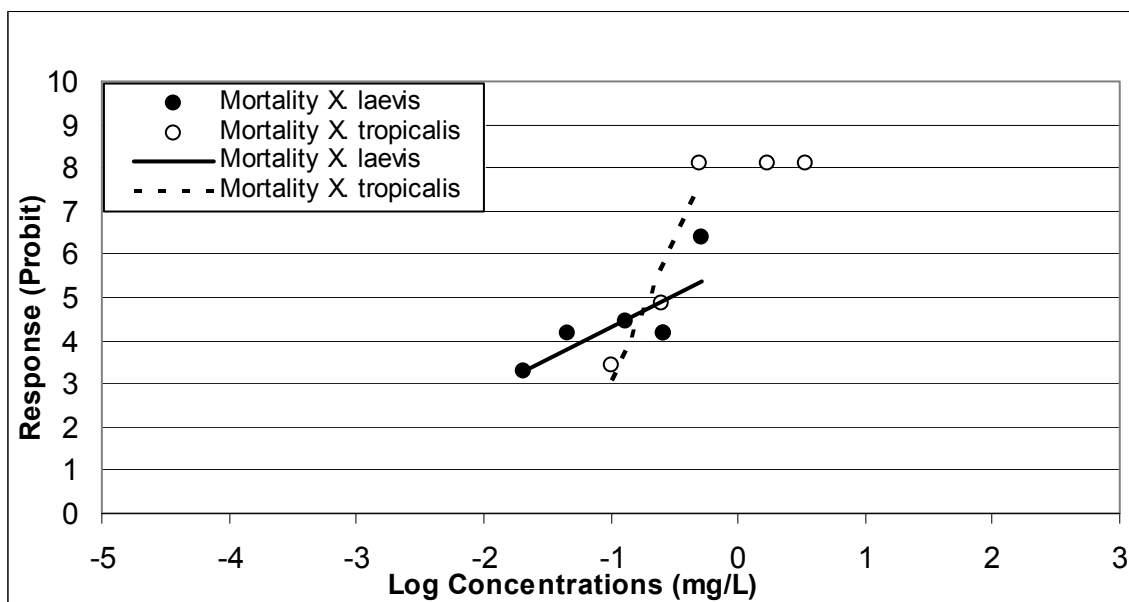


Figure 5.3 Graphic estimation of copper LC₅₀ by Probit Analysis.

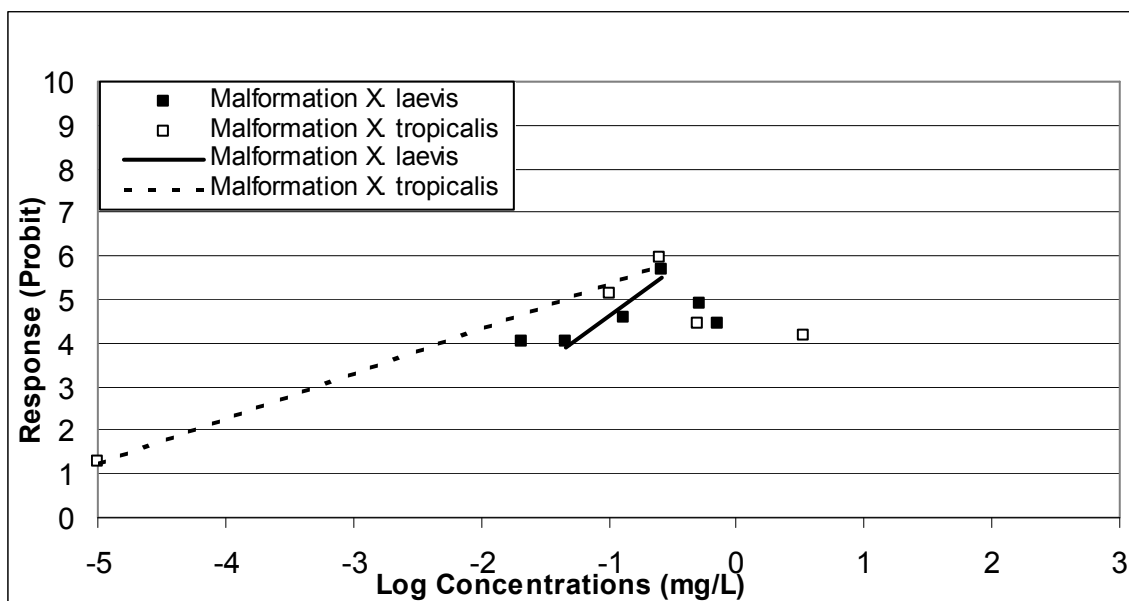


Figure 5.4 Graphic estimation of copper EC₅₀ by Probit Analysis.

Selenium. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to selenium are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3.

Concentration-response curves are represented in Figures 5 and 6. While higher, the mean LC₅₀ value for *X. laevis* at 23°C (46.30 ppm) is not substantially different (order of magnitude) to that found for *X. tropicalis* at 26°C (5.84 ppm); the LC₅₀ value for *X. laevis* is nearly 8 times greater than that found in *X. tropicalis*. Conversely, while slightly higher in *X. laevis*, the EC₅₀ values were much closer between the species pair (9.64 ppm and 7.99 ppm respectively). The TI values between *X. laevis* and *X. tropicalis* are different for cadmium (4.80 and 0.73 respectively); 6.5 times greater in *X. laevis* than in *X. tropicalis*.

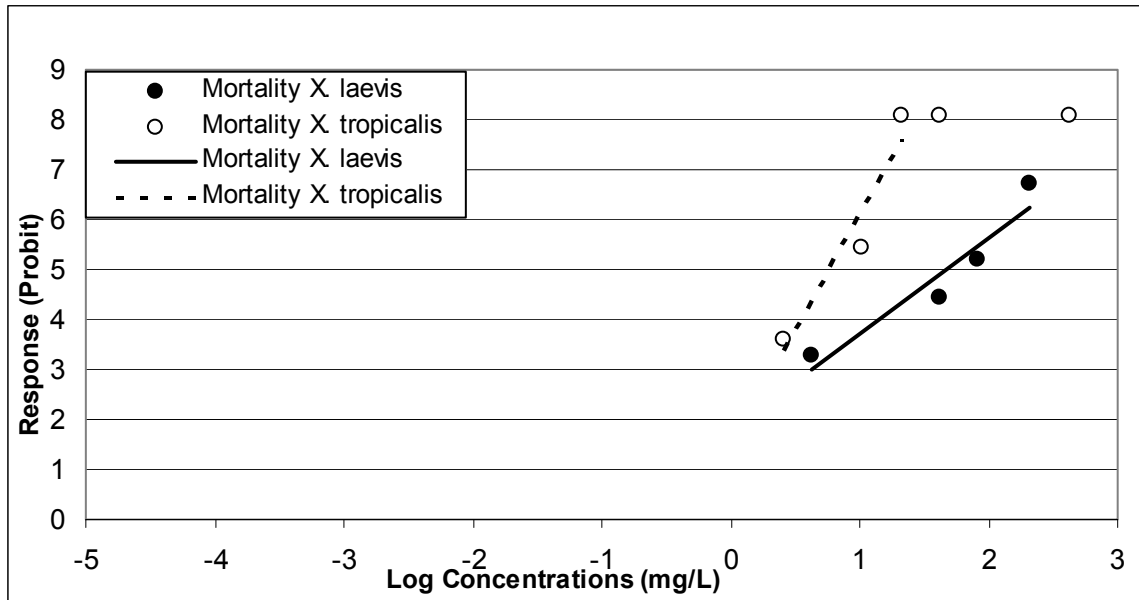


Figure 5.5 Graphic estimation of selenium LC50 by Probit Analysis.

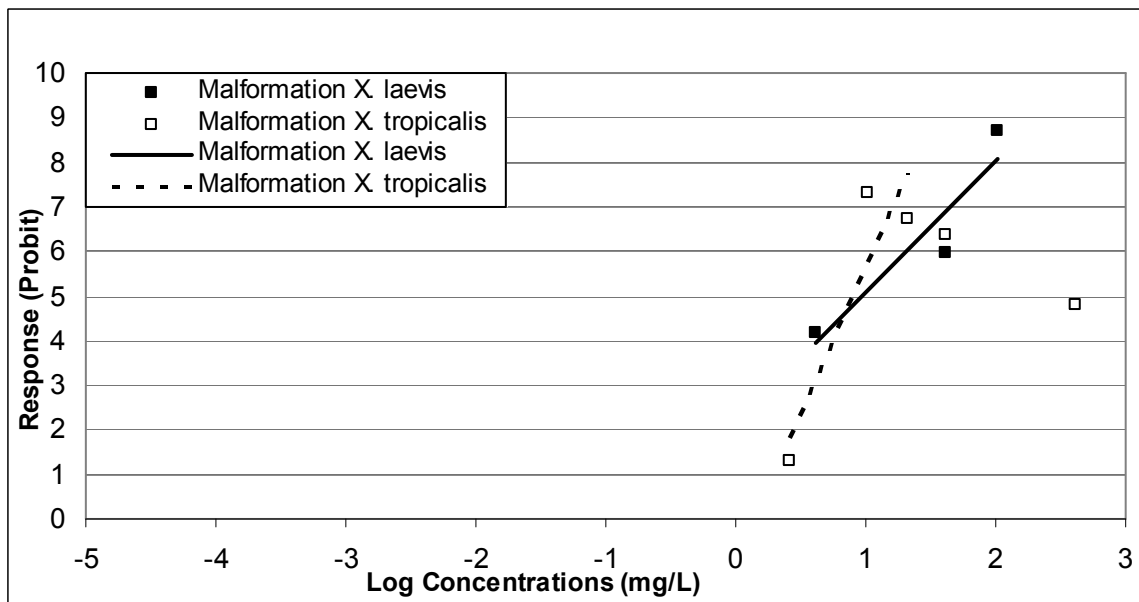


Figure 5.6 Graphic estimation of selenium EC50 by Probit Analysis.

Chromium. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to chromium are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3. Concentration-response curves are represented in Figures 5.7 and 5.8. The mean

LC₅₀ value for *X. laevis* at 23°C (39.94 ppm) was different to that found at 26°C for *X. tropicalis* (20.87 ppm) but not substantially different (order of magnitude); the LC₅₀ values between the species pair are two times greater in *X. laevis* than in *X. tropicalis*. EC₅₀ values were not found for *X. laevis* as our testing schedule was above the EC₅₀ value at our lowest dose (5 ppm). However, for *X. tropicalis*, the EC₅₀ value was found to be 17.24 ppm with a TI value of 1.21.

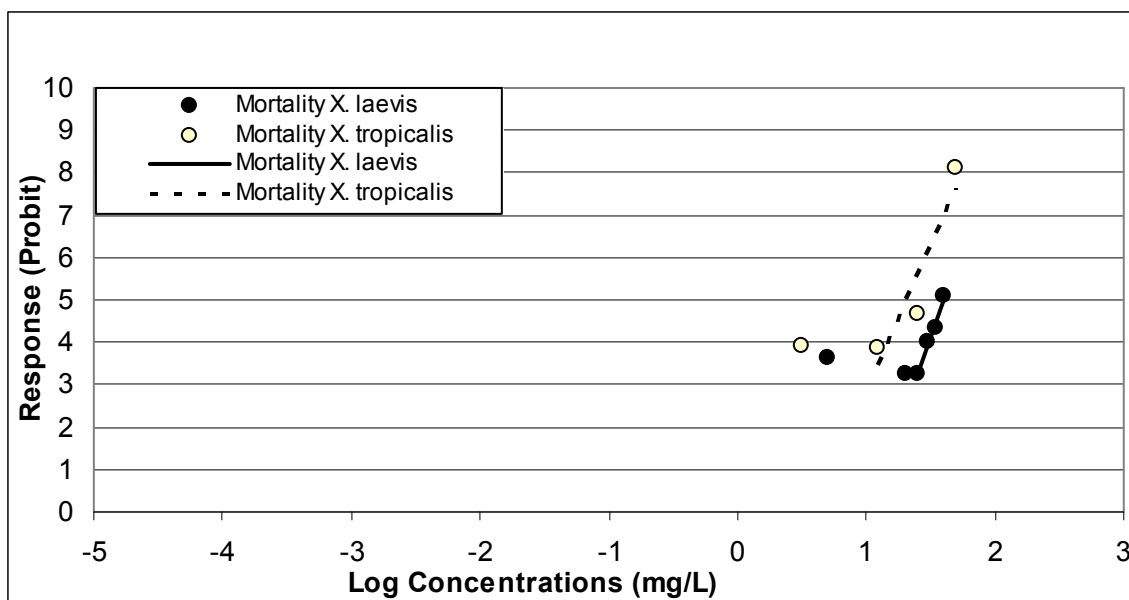


Figure 5.7 Graphic estimation of chromium LC₅₀ by Probit Analysis.

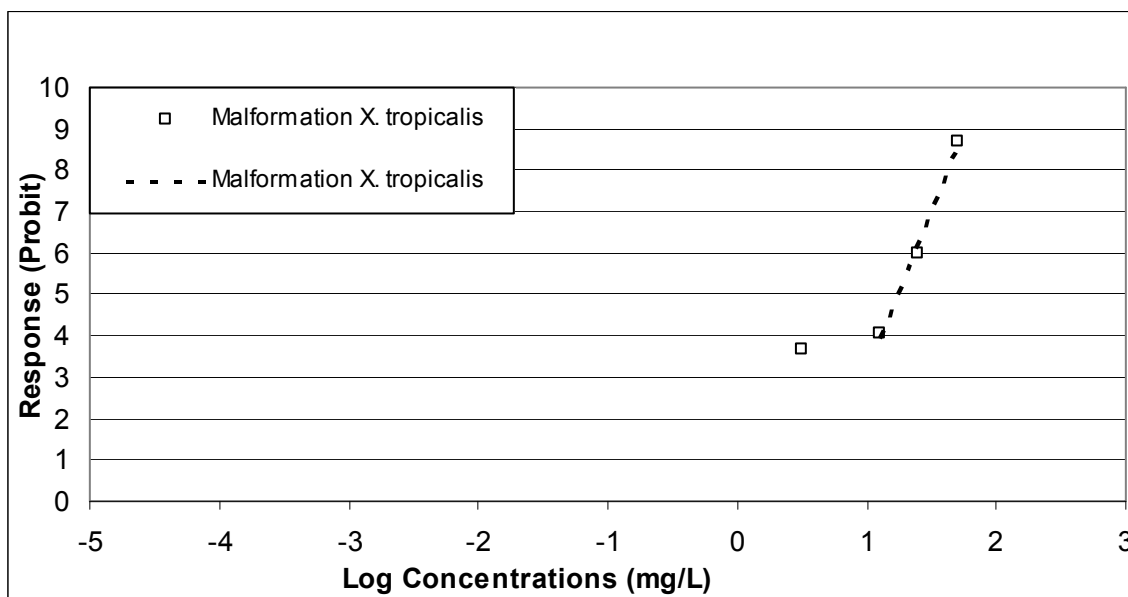


Figure 5.8 Graphic estimation of chromium EC₅₀ by Probit Analysis.

Lead. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to lead are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3. Lead was problematic in that regardless of the dosage we tested, we did not find any significant difference in mortality or teratogenic effects to our controls. For *X. laevis*, we tested as high as 14.4 ppm, a concentration at which metal was precipitating back out of solution, and had no significant mortality or teratogenic effects relative to the control groups.

Zinc. The stage 46 EC₅₀ and LC₅₀ values for *X. laevis* and *X. tropicalis* that had been exposed to zinc are represented in Table 5.1. Exposure concentrations for each experimental group and species are represented in Tables 5.2 and 5.3. Concentration-response curves are represented in Figures 5.9 and 5.10. The mean LC₅₀ value for *X. laevis* at 23°C (94.67 ppm) was different to that found at 26°C for *X. tropicalis* (17.08

ppm) but not substantially different (order of magnitude), the values were over five times greater in *X. laevis* than in *X. tropicalis*. Similarly, while slightly higher in *X. laevis*, the EC_{50} values were not substantially different between the species pair (6.96 ppm and 5.60 ppm respectively). The TI values between *X. laevis* and *X. tropicalis* are different for zinc (13.60 and 3.05 respectively); the difference in TI values between the species pair is over four times greater in *X. tropicalis* than in *X. laevis*.

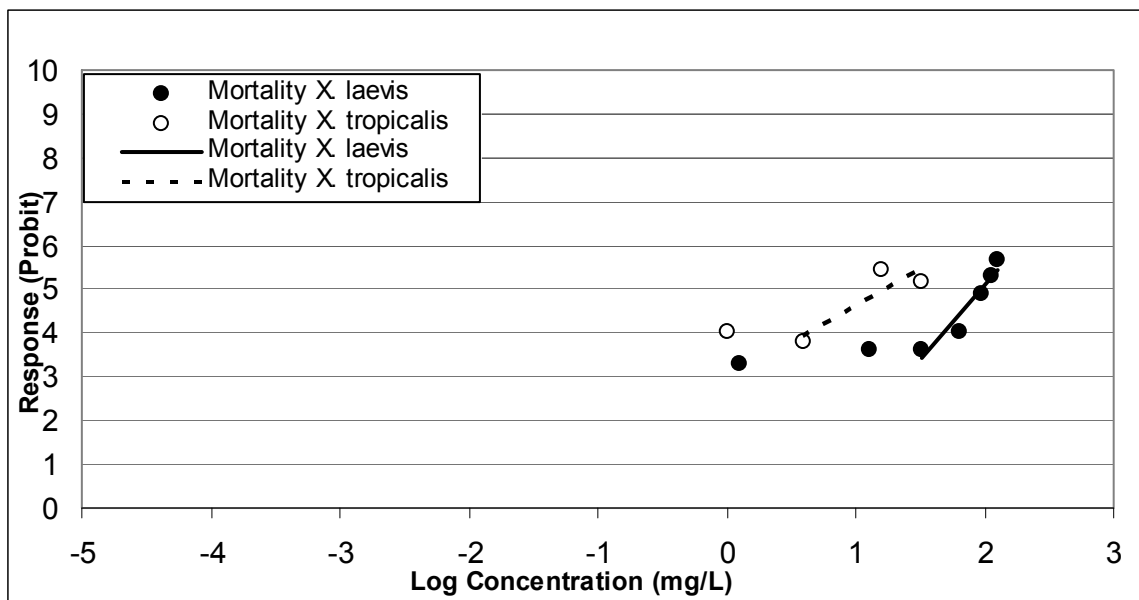


Figure 5.9 Graphic estimation of zinc LC50 by Probit Analysis.

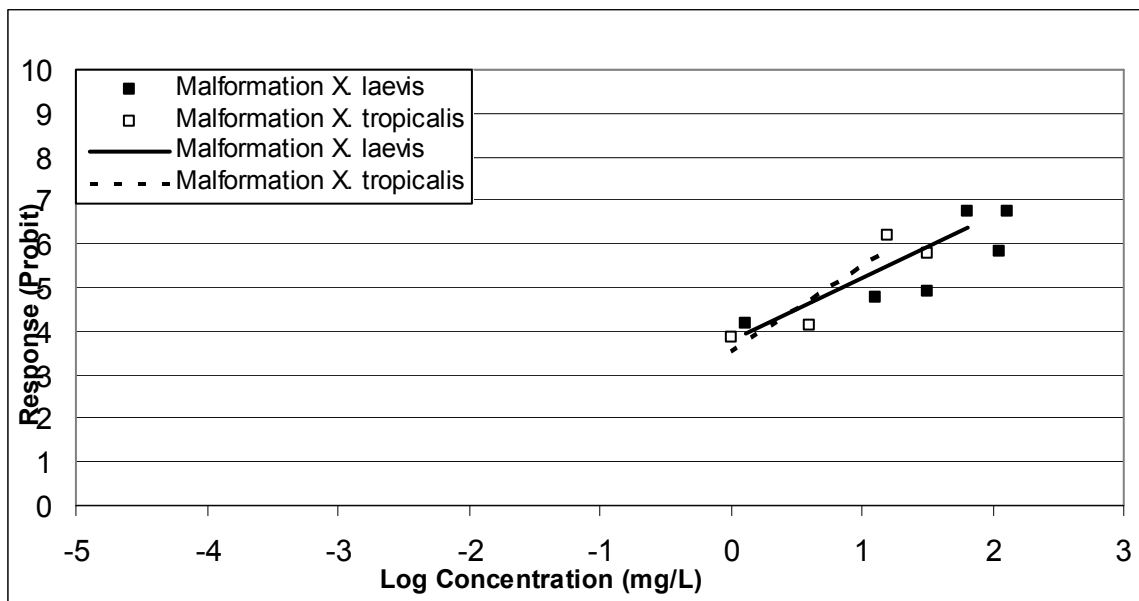


Figure 5.10 Graphic estimation of zinc EC50 by Probit Analysis.

Discussion

This experiment compares the relative sensitivities of two *Xenopus* species to heavy metals. While numerous studies examine effects of contaminants on *X. laevis*, far fewer have examined responses to contaminants in *X. tropicalis* (e.g., Fort et al., 2004a, 2004b). This study demonstrates that in laboratory conditions, *X. tropicalis* is equally or more sensitive to the metals examined relative to *X. laevis*. Our findings suggest further comparisons are warranted, which may identify *X. tropicalis* as an appropriate substitute for *X. laevis* in metal toxicology studies.

Cadmium. When tested in comparison trials, cadmium is a heavy metal that has proven generally more toxic to most amphibian species than to most fishes tested (Birge et al, 2000). For example, sixteen of eighteen amphibian species tested were more sensitive to cadmium than was the fathead minnow, *Pimephales promelas*; fifteen of these same amphibian species were more sensitive than rainbow trout, *Oncorhynchus*

mykiss (Birge et al., 2000). Sunderman et al. (1991) reported a FETAX LC₅₀ value (3.60 ppm) and an EC₅₀ value (0.42 ppm) for *X. laevis*. In contrast, I recorded an LC₅₀ value nearly two times lower (1.87 ppm) than this reported value. Conversely, we recorded an EC₅₀ value almost three times higher (1.12 ppm). One possible explanation for my different results is that, unlike prior studies cited here, I left the jelly coat on the embryos. The jelly coat may provide some additional protection to the embryo before it hatches, which would be expected to produce lower FETAX EC₅₀ and LC₅₀ values. However, although this probable explanation could explain differences in EC₅₀ values between the *X. laevis* methodologies, it does not support the differences in *X. laevis* LC₅₀ values reported in this study compared to the higher FETAX values from the literature.

***Xenopus tropicalis* in contrast to *X. laevis* for cadmium.** Although cadmium sensitivities were similar between the species pair, *X. tropicalis* had a 32% lower LC₅₀ value and a 36% lower EC₅₀ value than found in *X. laevis*, demonstrating a greater sensitivity. *X. tropicalis*, with a slightly higher teratogenic index value than *X. laevis*, demonstrates more potential for teratogenic effects.

Copper. Copper is a heavy metal for which different amphibian species have mixed sensitivities relative to one another and relative to salmonid fishes (Birge and Black, 1979). Nonetheless, copper is a heavy metal that is considered to be more toxic to amphibians relative to heavy metals like zinc (Birge et al., 2000). Spehar and Cardwell (2000) reported a FETAX LC₅₀ value (1.05 ppm) and an EC₅₀ value (0.53 ppm) for *X. laevis*. Our recorded values were lower in both cases (.30 and .15 respectively).

***Xenopus tropicalis* in contrast to *X. laevis* for copper.** Copper sensitivities were similar between the species pair. However, *X. tropicalis* again had a 33% lower LC₅₀

value and a 73% lower EC₅₀ value than found in *X. laevis*, demonstrating a greater sensitivity. *X. tropicalis*, with a higher teratogenic index value, demonstrates a TI value 2.5 times greater than *X. laevis*. This value suggests *X. tropicalis* is a much stronger candidate for detecting teratogenesis than *X. laevis* for copper.

Selenium. Published toxicology values for *Xenopus* and selenium are few. However, a published LC₅₀ value for selenium and the frog *Gastrophryne carolinensis* is 0.09 ppm (Birge et al. 2000). It is important to note that this value for *G. carolinensis* is likely at the high end of a sensitivity spectrum owing to *G. carolinensis* being generally more sensitive to contaminants compared to other species tested (Birge et al., 2000).

***Xenopus tropicalis* in contrast to *X. laevis* for selenium.** Selenium sensitivities were widely different between the species pair in LC₅₀ values, but not substantially different (less than an order of magnitude in difference). EC₅₀ values were similar but *X. tropicalis* was more sensitive. As with the metals discussed above, *X. tropicalis* had an 87% lower LC₅₀ value and a 17% lower EC₅₀ value than found in *X. laevis*, demonstrating a greater sensitivity. With a higher teratogenic index value in *X. laevis*, selenium represents one of only two circumstances whereby *X. tropicalis* demonstrated a lower potential for teratogenic effects than *X. laevis*.

Chromium. Little published information regarding amphibians and chromium toxicity exists. Spehar and Cardwell (2000) indicated that embryos of *Xenopus laevis* were more tolerant (a higher LC₅₀ value) than embryos of the freshwater shrimp, *Palaemonetes pugio*. Spehar and Cardwell (2000) reported a FETAX LC₅₀ value of 93.59 ppm and an EC₅₀ value of 0.43 ppm (for *X. laevis*). Our recorded LC₅₀ value was lower (39.94).

***Xenopus tropicalis* in contrast to *X. laevis* for chromium.** In comparing *X. laevis* and *X. tropicalis*, chromium sensitivities were similar between the species pair with LC₅₀ values. However, *X. tropicalis* had a 48% lower LC₅₀ value than found in *X. laevis*, demonstrating a greater sensitivity. Unexpectedly, I missed collecting an EC₅₀ value for *X. laevis* as teratogenic effects were observed in 100% of the experimental subjects in my lowest testing concentration (5 ppm). I did collect an EC₅₀ value for chromium in *X. tropicalis* of 17.24ppm. Therefore, with 100% of my *X. laevis* embryos demonstrating teratogenic effects at 5ppm, *X. laevis* appears far more sensitive to chromium with regard to an EC₅₀ than *X. tropicalis*. If future testing of *X. laevis* also produce lower EC₅₀ values for chromium in *X. laevis* than in *X. tropicalis*, chromium may be a metal whereby *X. laevis* is actually a better suited model species, and a more sensitive species for use in ecotoxicology studies, than *X. tropicalis*.

Lead. For both *X. laevis* and *X. tropicalis*, EC₅₀ and LC₅₀ values were not produced despite running tadpoles through progressively stronger concentrations of lead. The lack of an effect may be due to poor solubility of lead in FETAX solution (Bantle, 1995). Additionally, the Mg²⁺ in FETAX solution may interfere with the toxicity of lead (Bantle, 1995). Owing to the low levels of mortality and teratogenic effects observed in my experiments, even with high concentrations of lead at the precipitation point, I concur with Bantle (1995) in that special deviations from FETAX solution are necessary to determine lead toxicity. However, those deviations would diminish comparative value to the other metals examined here under this testing regime.

Zinc. Zinc is considered a heavy metal with reduced toxicity to amphibians relative to metals like copper and cadmium (Birge et al., 2000). Birge et al. (2000)

reported widely ranging LC₅₀ values in five amphibian species (from 0.01 to 87.00 ppm) but none of these frogs were in the genus *Xenopus*. We reported an LC₅₀ value of 94.67 which might be expected since *X. laevis* is regarded as more tolerant of metals than many other frogs.

***Xenopus tropicalis* in contrast to *X. laevis* for zinc.** Zinc sensitivities were widely different between the species pair in LC₅₀ values, but not substantially different (order of magnitude). EC₅₀ values were far more similar but *X. tropicalis* was more sensitive. As with earlier metals discussed above, *X. tropicalis* had an 82% lower LC₅₀ value and a 20% lower EC₅₀ value than found in *X. laevis*, demonstrating a greater sensitivity overall. With a higher teratogenic index value than *X. tropicalis*, this is the second of only two circumstances whereby *X. tropicalis* demonstrated a lower potential for teratogenic effects than *X. laevis*.

Conclusion

As a larger question, we performed these experiments to determine whether *X. tropicalis* is an appropriate alternate to *X. laevis* for use in toxicology experiments with metals. All other considerations aside, we wanted to investigate an alternate for *X. laevis* that is equally or more sensitive to metal contaminants. Our results suggest that with five of the six metals tested, *X. tropicalis* is more sensitive than is *X. laevis*. The sixth metal, lead, was inconclusive. Our teratogenic index values (TI values) show that with two metals, copper and chromium, *X. tropicalis* demonstrates a stronger potential for teratogenic effects than *X. laevis*. Particularly with copper, *X. tropicalis* demonstrated a sensitivity 2.5 times greater than *X. laevis*. With selenium and zinc, *X. tropicalis*

demonstrates a lower potential for teratogenic effects than *X. laevis*. However, with zinc, the teratogenic value recorded for *X. tropicalis* (3.05) is still considered significant as per the definition of an informative TI value provided by Fort et al. (2004a). This straight comparison between *X. tropicalis* and *X. laevis* demonstrates that with regard to metal toxicology, *X. tropicalis* may be an appropriate substitute for *X. laevis* owing to an increased degree of sensitivity to the metals examined.

In regard to teratogenic index values (TI value), the definition of an important TI value from Fort et al. (2004a) suggests that when equal to or smaller than 1.5, the value is of no consequence because there is so little separation between lethal and non-lethal contaminant concentrations. The only two instances in our study where inconsequential values were produced came with *X. tropicalis* exposed to selenium and chromium. The small selenium value is likely a product of the separation between our concentrations used to produce the EC₅₀ value rather than an actual value lower than 1.

Basic toxicology tests, such as those we performed here, are critical in moving toward the next step in contaminant research, life cycle assessment models. Life cycle assessment models examine contaminant exposure effects in one life stage and how that exposure affects subsequent stages (USEPA, 2003). For example, hormone-mediated changes affecting gene expression, especially early in life, can be altered by endocrine disrupting compounds and have profound impacts throughout the remaining course of the organism's life and subsequent life stages (USEPA, 2003). Further, some metals have been identified as having significant influence on the endocrine system, including a role as endocrine disrupting compounds (Henson and Chedrese, 2004; Beckett and Arthur, 2005). The United States Environmental Protection Agency has prioritized research into

endocrine disrupting compounds, these include metals like cadmium (USEPA, 2003).

Before we can apply life cycle assessment models, we need a model species that is sensitive to the contaminants in question and we need to know the concentrations of those contaminants at which lethality and teratogenic effects exist. Frogs of the genus *Xenopus* culture well in the laboratory and provide a biphasic life cycle, ideal for life cycle assessment models. As a potential new model species for contaminant research, *Xenopus tropicalis* offers shorter generation times, a diploid genome, and transgenic and hybrid lines. Our laboratory comparisons here suggest *X. tropicalis* compares favorably to *X. laevis* with regard to metal sensitivities. Improving our understanding of metal toxicity in *X. tropicalis*, and the doses at which effects are observed, meet a key step outlined in the USEPA research plan with regard to endocrine disrupting compound research (USEPA, 2003).

APPENDICES

APPENDIX I Specimens Examined

COLOMBIA: **Nariño:** La Guayacana, 400m (KU 145061); **Putumayo:** Puesto de Bombeo Guamez [= Guamués](KU 140332); Santa Rosa de los Kofanes, middle course of Río Guamés, tributary of upper Putumayo (CM 50609); **Vaupés:** Wacará (UTA 3852–55, 4060–62, 6137, 8526, 8365); Yapima (UTA 6138). **ECUADOR:** eastern Ecuador (AMNH 53339); Pastaza River, Canelos to Marañón (MCZ 19601); **Napo:** Cuyabeno, along the Río Cuyabeno (UIMNH 59422–24); Limón Cocha (UIMNH 93099); **Pastaza:** Andoas, Río Pastaza (AMNH 53335) [authors' comment: this is actually in Loreto, Peru]; Río Capahuary, at point of confluence with Río Pastaza (USNM 196962) [authors' comment: this should be the Río Copahuari, not to be confused with the Río Copahuari which joins the Río Pastaza at Andoas, a site in Loreto, Peru]; Río Rutuno, tributary of Río Bobonaza (USNM 196963–65, 266104); Río Bufeó, Bajo Bobnonaza (USNM 196695, 266105–06); Río Pindo (USNM 196966–67); **PERU:** **Loreto:** Santa Luisa, Río Nanay, 160m (FMNH 109824); Yagua Indian Village, headwaters of Río Loretoyacu [100+ km NW Leticia] (AMNH 96009–10); Quebrada Orán, ca 5 km N Río Amazonas, 85 km NE Iquitos, 110m (KU 206135).

APPENDIX II

Additional Material in Collections and field observations (WWL)

Material in Museums. — **COLOMBIA: Putumayo:** Puesto de Bombeo Guamez, 1000m [= Guamués] (KU 140330–31); **Vaupés:** Yapima (UTA 6138). **ECUADOR: Pastaza:** upper Río Pastaza drainage (KU 154655); Río Pindo (USNM 196966); andaos [= Andoas], Río Pastaza (AMNH 53337); Río Capaguaría (AMNH 53338); Amundai (Kapawi Lodge), 300m, 2 degrees 52' S, 72 degrees 20' W (QCAZ 11114–17); north of Jatún Molina (east of Sarayacu, between the rivers Bobonaza and Jatúnrutunoyacu, 1 degree 48' S, 77 degrees 18' W (QCAZ 10638). **PERU: Loreto:** Quebrada Orán, ca 5 km N Río Amazonas, 85 km NE of Iquitos, 110M, KU 206134; Estirón, Río Ampiyacu, MZUSP 27.368–27.369; Aldeia dos Indios Borra, 2 km da boca do Río Zumón, MZUSP 54.140; San Joaquín de Omaguas, Río Amazonas (WWL); Pebas (WWL); Sabalillo, Río Apayacu (WWL); Supay Cocha, Río Apayacu (WWL); Nauta (WWL); lower Río Itaya (WWL); Brillo Nuevo, Río Yaguasyacu (WWL); Orán, Río Amazonas (WWL), ExplorNapó Lodge, Río Sucusari (WWL), Explorama Lodge, Quebrada Yanamono (WWL); Nueva Colonia, Río Zumón (WWL); between the Río Tacshacuraray and Río Mazan, 3 days walking distance(WWL); middle Río Nahuapa, tributary of the lower Río Tigre (WWL); El Paraíso, left bank of Río Tigre (WWL); Gueppí (P. Venegas, pers. comm.). **VENEZUELA: Amazonas:** Marahuaca (U.P.R. 253, cited by Rivero, 1972).

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