ABSTRACT

Title of Document: EPIDEMIOLOGIC ANALYSIS OF RISK FACTORS FOR LOCAL DISAPPEARANCES OF NATIVE RANID FROGS IN ARIZONA

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This study used epidemiologic case-control methodology to examine habitat and environmental factors contributing to amphibian declines in Arizona. Risk factors were compared between sites where frogs disappeared (cases) and persisted (controls) using univariate and multivariable logistic regression analyses. Thirty-six percent (117/324) of all sites became cases during the study period. Elevation, non-native predators, hydrologic characteristics, aspect, and effects of nearby sites were significantly associated with frog persistence or disappearance. In the final multivariable model, risk for disappearance increased with increasing elevation (OR=2.7 for every 500 meters, $P<0.01$). Sites where disappearances occurred were 4.3 times more likely to have other nearby sites that also experienced disappearances ($P<0.01$), while having an extant population nearby decreased risk of disappearance by 85% (OR=0.15, $P<0.01$). Sites experiencing disappearances were 2.6 times more likely to have crayfish than control sites ($P=0.04$). Identification of risk factors associated with frog disappearances will guide future research and conservation efforts.

EPIDEMIOLOGIC ANALYSIS OF RISK FACTORS FOR LOCAL DISAPPEARANCES OF NATIVE RANID FROGS IN ARIZONA

By

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

The decline and extinction of amphibian populations has gained global recognition, as massive population losses have now been reported on all inhabited continents (Stuart et al. 2004). Leading hypotheses to explain the recent declines include emerging infectious diseases, global climate change, alterations in land use introduction of non-native species, and commercial over-exploitation (Collins and Storfer 2003). Although many declines are known to be direct results of human habitat destruction and overexploitation, some species have disappeared without any identifiable causes. Species experiencing declines often share the same characteristics of living in high elevations and breeding in streams. (Berger et al. 1998; Lips et al. 2003a, 2004; Stuart et al. 2004).

Patterns of declines in the Western United States are consistent with those in other parts of the world; while species in some areas decline, other populations appear unaffected (Carey 1993; Sredl 1997). Range reductions and population declines have been recognized in all seven species of ranid frogs native to the Sonoran Desert of Arizona, including *Rana blairi, Rana chiricahuensis, Rana onca, Rana pipiens, Rana subaquavocalis, Rana tarahumarae, and Rana yavapaiensis* (Clarkson and Rorabaugh 1989; Sredl 1997). Factors implicated in Arizona declines include introduction of predator and competitor species, habitat alteration and pollution, drought, and infectious disease. Many of these hypotheses draw from field observations of a few, intensely studied populations (Hale and Jarchow 1987; Rosen et al. 1996; Sredl et al. 2000). However, the desert climate of Arizona produces extreme variations in local conditions making it difficult to measure variables related to population declines in one locality and generalize them to a larger, statewide population. For example, sporadic flash flooding, seasonal forest fires, and drought may severely diminish a local population, but may not be important factors at the metapopulation or regional level. Investigating trends in risk across many sites statewide may help discern between site-specific factors that are important regionally versus those that are only important locally.

Identification of environmental and spatial risk factors through multivariable modeling is useful in understanding the degree and magnitude to which different variables act independently and/or synergistically to contribute to frog declines. Until recently, most studies have examined only the effects of a single factor on amphibian mortality (Storfer 2003). It is now recognized that the dramatic declines observed in amphibian populations are more likely the result of many factors working in complex, synergistic ways (Kiesecker et al. 2001a; Blaustein et al. 2002) and recent research further substantiates this concept by demonstrating that more than one factor can modulate morbidity and mortality of amphibians in laboratory and mesocosm experiments (Berger et al. 2004; Parris and Beaudoin 2004). However, few studies have examined multiple factors associated with the declines in naturally occurring populations due to complex analyses and need for large data sets.

The Arizona Game and Fish Department (AZGFD) has collected and maintains a large, statewide frog database with more than 2000 localities with time periods that span over a century. Researchers began surveying native ranid frog habitat to study the present and historical distribution of the species and inadvertently captured potential decline events. Although dead and/or moribund frogs were rarely observed in the field, many of the surveys captured time points when frogs disappeared and never returned to a site. Observational epidemiologic methodology lends itself well to the analysis of such data; collected for alternative purposes and possessing a considerable degree of survey-to-survey variation through time. The purpose of this study was to use epidemiologic methodology and multivariable analytic techniques to investigate risk factors associated with disappearances of frogs throughout Arizona. This investigation highlighted important factors that may be implicated in the worldwide amphibian decline phenomenon. As awareness for the need of long-term amphibian monitoring programs increase, it is expected that more datasets will lend themselves to these types of dynamic analyses. This project provides an important foundation for future studies aiming to examine multiple risk factors associated with amphibian declines.

Chapter 2: Background

2.1 Global amphibian declines

In the late 1980's and early 1990's, controversy surrounded the reality of amphibian declines. Anecdotal accounts of disappearing frogs from locations around the globe surfaced at the First World Congress of Herpetology in 1989 and were further examined in 1990 at the National Research Council Workshop (Collins and Storfer 2003). Much of the controversy stemmed from the lack of long-term monitoring data sets distinguishing between natural population fluctuations and significant declines (Pechmann et al. 1991; Pechmann and Wilbur 1994). Many amphibian populations are characterized by dramatic yearly fluctuations in number of individuals due to variation in breeding success and recruitment of juveniles to adults. Factors such as precipitation, temperature or predation that vary yearly could influence the population by orders of magnitude (Collins and Storfer 2003). Pechmann et al. (1991) illustrated this problem by analyzing long-term census data for four amphibian species that regularly experience large fluctuations in population size. The authors concluded that the overall population was stable and observed fluctuations could be attributed to drought. However, if shorter segments of the same data set had been analyzed separately, one might conclude that populations were declining.

Although lack of long-term data and difficulty in monitoring highly fluctuating populations are still issues in amphibian research, there is consensus within the scientific community that the observed declines are real and have increased in the latter part of the 20th century (Houlahan et al. 2000; Alford et al. 2001).

Biologists became especially alarmed by declines, disappearances, or extinctions of amphibians in pristine and protected habitat in remote locations without any obvious indicators of environmental change (Berger et al. 1998). Green and golden bell frogs (*Litoria aurea*), among many other species, have declined in the Australian tropics without any identified anthropogenic disturbance (Mahoney 1996). Declines have been more severe in the protected forests of eastern Puerto Rico than in other areas where deforestation has occurred (Stallard 2001). Declines in upland Mexico have occurred at remote, undisturbed sites (Lips et al. 2004). Habitat seemed unchanged in the forests of Costa Rica where declines have affected over 40% of the anuran fauna at one protected upland site in the Monteverde Cloud Forest Preserve. Rebounds of affected populations in Costa Rica were never observed (Pounds et al. 1997).

Disappearing species often share the same features of living in high elevation, aquatic habitat (Berger et al. 1998, Lips et al. 2003, 2004). Declining species may also share low fecundity and a high degree of habitat specialization and restricted geographical ranges (Williams and Hero 1998; Lips et al. 2003a). While some species are declining and disappearing, others are thriving in healthy populations (Hedges 1993; Richards et al. 1993; Mahoney 1996; Carrier and Beebee 2003; Bell et al. 2004; Crochet et al. 2004). Shared commonalities between declining and non-declining populations may indicate that the widespread geographic declines are related. Leading hypotheses include emerging diseases, climate change, changes in land use, introduction of non-native species, and commercial overexploitation. These factors could work independently or synergistically in complex ways to induce widespread, catastrophic declines and disappearances of amphibians (Kiesecker et al. 2001a).

2.2 Amphibian declines in Arizona and North America

Patterns of declines in the Western United States are consistent with those in other parts of the world; while species in some areas decline, other populations appear unaffected (Carey 1993; Sredl 1997). Data from well-documented declines in the Cascade, Sierra Nevada and Rocky mountain ranges show populations of frogs have been mysteriously disappearing since the 1970's (Corn and Fogleman 1984; Clarkson and Rorabaugh 1989; Carey 1993; Fellers and Drost 1993; Fisher and Shaffer 1996; Drost and Fellers 1996; Houlahan et al. 2000). Most declines have occurred in sites at high elevations during the winter months and, although rare, some massive die-offs have been observed in the field (Carey 1993; Bradley et al. 2002). Spread of infectious disease coupled by immunosuppression is hypothesized to have contributed to many of these declines, (Carey 1993; Fellers et al. 2001; Bradley et al. 2002; Muths et al. 2003) but habitat destruction, environmental change, and introduced exotic predators are also implicated (Hayes and Jennings 1986; Fisher and Shaffer 1996).

Declines have been recognized in all seven native ranid species in Arizona, including *Rana blairi, Rana chiricahuensis, Rana onca, Rana pipiens, Rana subaquavocalis, Rana tarahumarae, and Rana yavapaiensis.* All of these species are currently protected in Arizona and one species, *Rana chiricahuensis*, is listed as threatened in the United States' Endangered Species Act. Declines and range reductions likely began in the 1970's and many species are now missing from historic localities (Clarkson and Rorabaugh 1989; Sredl 1997). Dead and dying frogs have been observed in the field, and in some cases, the proximate cause of death has been attributed to infectious disease (Sredl 2000, Bradley et al. 2002). Other studies have linked declines to introduced predatory species, habitat destruction, climate variability and environmental contamination with heavy metals (Hayes and Jennings 1986; Hale and Jarchow 1987; Schwalbe and Rosen 1988; Clarkson and Rorabaugh 1989; Rosen et al. 1995).

2.3 Overview of Anuran Biology

Fundamental to understanding the mechanisms through which massive declines may operate is the appreciation for ecological and physiological complexities of amphibians, such as their highly permeable skin and bi-phasic life cycle. These and other characteristics contribute to susceptibility to disease and vulnerability to environmental stressors. The following section will review important aspects of amphibian (Order: Anura) biology that may be implicated in amphibian declines.

2.3.1 The skin

One of the most distinctive and important features of amphibians is their skin. Equally adaptable to both aquatic and terrestrial environments, it serves as a permeable, yet protective, barrier functioning in osmoregulation and gas exchange, thermoregulation, and respiration. Amphibians regulate biochemical and physiological processes through their skin by exchanging dissolved ions (such as oxygen, carbon dioxide, sodium and chloride) with their aqueous environment via osmosis (Hofrichter 2000a; Lametschwandtner 2000). Continuous absorption and water loss through their skin causes many amphibian species to be highly, if not completely, dependent on water. Regulation of water flux occurs by moving in and out of water, or by increasing and decreasing the surface area of their body that is in contact with water (Pough et al. 2000). Preventing dehydration is a crucial issue for all frogs, especially for terrestrial and semi-aquatic species, or those living in arid climates and brackish waters (Hofrichter 2000a). Structural, physiological and behavioral adaptations have provided frogs with the ability to maintain their necessary internal homeostasis through water uptake and regulation. Structural adaptations the thin, permeable "pelvic patch" on the ventral surface that aids in water uptake, lipid glands that secrete mucous to help keep the skin moist, and retention of dead layers of skin to help prevent water loss (Stebbins and Cohen 1995). Physiologic adaptations include high toleration for water loss, the ability to produce concentrated urine for water retention, and production of water through fat metabolism (Stebbins and Cohen 1995). Finally, behavioral adaptations that aid in water uptake and regulation include reducing or prolonging exposure to moist surfaces to slow or increase the rate of water uptake, ground burrowing to prevent water loss, and nocturnal activity to avoid water loss during the hottest times of the day (Pough et al. 2000; Sinsch 2000)

Skin permeability and water flux aids in body temperature regulation, which turn serves to regulate the rate of several physiological processes (Carey and Alexander 2003). Thermoregulation occurs by moving in and out of water, through basking, heat exchange with substrates, and diurnal and annual avoidance behaviors such as moving to shelter during the day for cooling and hibernation in the winter to avoid lethal temperatures (Sinsch 2000).

Amphibians obtain oxygen from both water (through gills, the oral epithelium, and skin) and air (through pulmonary respiration). Both types of respiration are performed to varying degrees, but often occur simultaneously. The skin performs up to 60 percent of the oxygen intake in tadpoles, and may perform between 25 and 80 percent of adult respiration. The thinness of skin, its low degree of keratinization, its pronounced vascularization, and its moist surface makes amphibian skin an effective respiratory organ (Lametschwandtner and Tiedeman 2000). Adaptations to increase the blood oxygen levels are effected through increased surface area of the skin, including skin folds, wrinkles, ridges, and large numbers of subcutaneous capillaries (Lametschwandtner 2000).

The high degree of permeability of amphibian skin and its importance in the regulation of fundamental processes renders amphibians vulnerable to toxins. Toxic chemicals from industry, heavy metals, and pesticides have been known to act directly in killing frogs (further discussed in section 2.5.4.2, reviewed in Blaustein et al. 2003). Direct deaths may be related to high levels of toxicity from chemicals directly infiltrating the thin, permeable skin.

Diseases may also successfully attack amphibians through their skin. *Pseudocapillaroides xenopi,* a nematode parasite of African clawed frogs, causes skin thickening and excessive secretion of mucous. This organism may disturb the osmotic balance that is maintained by the skin (Faeh et al. 1998). A pathogenic chytrid, implicated in amphibian declines, may also attack amphibian hosts via the skin (Berger et al. 1998, Pessier et al. 1999). Although mechanisms by which this pathogen induces mortality are currently unknown, it has been hypothesized that the parasitic fungus attaches to the skin and interferes with water and ion flow. Obstruction or interference with any of the vital osmoregulatory, thermoregulatory, or respiratory processes of the skin may be fatal to an individual.

2.3.2 Development and metamorphosis

Anuran developmental processes usually involve three stages; immobile eggs hatch into free swimming tadpoles, which then transform into terrestrial or semi-terrestrial adults. These complex life forms expose amphibians to both aquatic and terrestrial habitat hazards. The life cycle begins with the depositing of gelatinous encapsulated eggs. A variety of egg laying processes have evolved, but the most common involves the female depositing large egg masses numbering in the thousands into the water for external fertilization by a male's sperm (Zug et al. 2001). Inability to freely move makes the embryonic stage vulnerable to stressors such as predation, desiccation, or ultra-violet (UV) radiation exposure. Increased UV exposure induces embryonic mortality in some species, supporting the hypothesis that differential exposure to UV and the ability to repair DNA damage caused by UV may play a role in hatching success (Blaustein 1994).

Surviving eggs hatch into free-swimming tadpoles that remain in their aquatic tadpole form for as little as a few days, or, in the case of the bullfrog (*Rana catesbieana*), over a year (Collins 1979). Tadpoles develop in response to environmental stimuli such as population density, food availability, temperature tolerance and susceptibility to predation (Shi 1999). As the tadpole develops, the levels of intracellular thyroid hormones (TH) and the numbers of TH receptors increase in tissues. The activation of TH and its receptors has largely been found to induce metamorphosis (Rollins-Smith 1998; Kalezic 2000), but corticosteroid hormones (CH) have also been implicated (Rollins-Smith 1998). Metamorphic change involves reorganization of several tissues and organ systems, including reabsorption of the tail, development of limbs, development of lungs and loss of gills, replacement of larval hemoglobin with adult hemoglobin, a shift from ammonia excretion to urine excretion, regression of the jaw, expansion of the mouth opening, shortening of the intestine, immune system reorganization, regression of the tadpole lateral line into the adult middle ear and a change from herbivorous to carnivorous diet (Rollins-Smith 1998; Kalezic 2000).

Exact environmental mechanisms inducing metamorphosis are currently unknown. Metamorphosis has been experimentally induced or retarded by food availability, oxygen, water temperature, population density of larvae, predation, light, and environmental iodine levels (cited in Shi 1999; Semlitsch 2001). Denver (1997) found that environmental stress induces the release of a stress response hormone, which, subsequently, activates the release of TH, supporting the hypothesis that stress may induce premature metamorphosis in stressed anurans. Certain stressors, such as presence of predators, can promote early metamorphosis at a small size (Lawler et al. 1999, Nyström et al. 2001). In general, studies have shown that metamorphosis occurring at a less than optimal size affects adult fitness (Semlistch 2001).

Dramatic changes in immune responses, anatomical structures, physiological processes and behaviors make frogs extremely vulnerable during metamorphosis. The immune system is not fully developed, making them more susceptible to disease and parasites (Rollins-Smith 1998). Additionally, energy may not be readily available for fighting infection; during metamorphic climax, feeding ceases and energy is only provided by reabsorption of the tail (Kalezic 2000). Increased vulnerability of an individual frog during metamorphosis may have far reaching impacts; success at a population level is determined by the number and quality of metamorphosing larvae that leave the pond and are recruited into adulthood (Semlistch 2001). Among species studied, fewer than 4.5% of the deposited eggs hatch and survive through metamorphosis to become adults (cited in Carey and Bryant 1995). Thus, factors leading to compromised metamorphosis may leave amphibian populations at greater risk of significant declines.

2.3.3 The immune system

The amphibian immune system, which contains both innate and adaptive responses, is fundamental in protecting against invading pathogens. The innate immune system provides rapid, non-specific protection from pathogens until the adaptive immune system can be activated. The amphibian's innate immune system has macrophages and neutrophils that can phagocytize a pathogen, a complement system for killing pathogens via a membrane attack complex, and natural killer cells which provide an immediate response to virus-infected or tumor cells (Rollins-Smith 1998). An important first defense of the anuran innate immune system is antimicrobial skin peptides that can protect against a variety of organisms including bacteria, yeast and fungi. Little is known about the effects of environmental changes (i.e. temperature, pH, skin hydration, exposure to toxins, ect.) on peptide production and efficacy in protecting against pathogens (Carey et al. 1999). Antimicrobial peptides may also play an important role in defense against *Batrachochytrium dendrobatidis*; a pathogen that attacks the non-vascularized stratum corneum of the skin which may prevent it from adaptive immune recognition (Rollins-Smith and Conlon 2005).

The adaptive immune system provides a more potent immune response, but requires time for activation. It is highly specific to a given pathogen, and results in memory cells that can rapidly respond to a repeated antigenic attack. Similar to other vertebrates, the anuran adaptive immune system is composed of T- and B-lympohcytes expressing immunoglobulin receptors, leukocyte-derived cytokines, and major histocompatibility complex (MHC) class I and class II genes (Carey et al. 1999). T-lymphocytes destroy intracellular pathogens and help coordinate other cells during an immune response. B-lymphocytes are responsible for the production of antibody which can bind soluble antigen, neutralize toxins, and opsonize microbial pathogens.

Altered immune response or change in the pathogenicity of the agent may provide favorable conditions for disease to cause population-level mortalities. Carey et al. (1999) list six hypotheses that have been proposed to explain recent amphibian declines in the context of emerging disease and immunological responses.

1. New, emerging pathogens are highly virulent and kill the host before the amphibian can mount an adaptive immune response.
2. The new pathogens are immunosuppressive.

3. Alterations in the environment (temperature, moisture, etc.) have altered the proportions of microorganisms in the soil and water in way that a rare pathogen has become prevalent.
4. Environmental change may stress the host, resulting in release of stress hormones that result in immunosuppression. Maintenance of immune systems is costly and stressed animals may need to divert energy from immune functions for other vital processes (Lafferty and Gerber 2002).
5. Exposure to environmental contaminants directly diminishes the components of the innate and/or adaptive immune response, (e.g. skin peptides).
6. Neuroendocrine changes are being caused by lethal or sublethal changes in the environment, releasing stress hormones and thereby causing immunosuppression.

Immunosuppression naturally occurs during critical events in the amphibian's life, including metamorphosis, seasonal temperature change, and breeding congregations (Rollins-Smith 1998; Carey et al. 1999). These important periods may be critical in evaluating the role immunosuppression plays in amphibian declines. During metamorphosis the immune system is reorganized, along with many other body tissues. Immunosuppression occurs at the peak of transformation and may have evolved to prevent an adaptive immune system attack on the newly formed tissue. Most amphibians probably survive metamorphic immunosuppression with no deleterious effects, but additional stressors that induce early metamorphosis may damage the adult immune system, posing greater risk to amphibian health (Rollins-Smith 1998).

Temperature can strongly influence the immune functions of amphibians as well. Prolonged exposure to cold temperatures during hibernation can diminish immune function (Carey et al. 1999). Risk of infection during cold-induced immunosuppression depends on the temperature-dependence and virulence of the pathogen (Carey et al. 1999). Pathogens that have evolved with optimal growth at temperatures that affect amphibian immune functions may pose the greatest threat to amphibian populations. *B. dendrobatidis* may be one such pathogen. With optimal pathogen growth at 23 degrees Celsius in laboratory experiments (Longecore et al. 1999), the amphibian immune response may become compromised allowing the pathogen to easily invade the host. Additional studies examining the impacts of temperature-induced immunosuppression are needed.

The role of social congregations on the amphibian immune response is not well understood. Carey et al. (1999) suggests overcrowding may induce immunosuppression and promote disease spread. Outbreaks of iridoviruses in Arizona have occurred frequently among high population densities of tiger salamanders (*Ambystoma tigrinum*) (Green et al. 2002).

2.4 Overview of Arizona native ranid frogs

2.4.1 Family *Ranidae*

Ranid frogs are classified into the following taxonomic groups: 1) Kingdom: Animalia 2) Phylum: Chordata 3) Subphylum: Vertebrata 4) Class: Amphibia 5) Order: Anura and 6) Family: Ranidae. Frogs belonging to the family *Ranidae* are distributed worldwide (with limited distributions in South America and Australia) and occupy a variety of habitat that range from dry to temperate to tropical (Duellman

1999). Physical characteristics often include smooth skin, a slim waist, long legs with pointed toes, extensive webbing on their hind feet, and dorsolateral folds. Ranids, also known as “true frogs”, are often loyal to their spawning sites and return every year to the same breeding ponds. They usually lay eggs in large clots in open water and have free-swimming larvae (Hofrichter 2000b). Breeding often occurs in the spring, with males calling in females to breeding sites (Duellman 1999).

The family *Ranidae* is among the top four families (fourth after *Bufonidae*, *Leptodactylidae*, and *Hylidae*) that contribute overwhelming numbers of rapidly declining species (Stuart et al. 2004). Overexploitation for human consumption and habitat loss are important issues for declines in this family, but unexplained massive population loses, called “enigmatic declines” by Stuart et al. (2004), are also contributing. Enigmatic declines are well recognized in the tropics (especially Central America and Australia), and are more likely to occur among stream-breeding frogs at high elevations (Laurance et al. 1996, Lips et al. 2003a, Williams and Hero 1998). Enigmatic declines among ranids are confounded by their wide distribution; it is unknown whether these groups are susceptible to declines due to habitat specialization or whether they occupy localities where enigmatic declines have occurred (Stuart et al. 2004).

2.4.2 Native ranid frogs

The six species of Arizona leopard frogs (*Rana blairi, Rana chiricahuensis, Rana onca, Rana pipiens, Rana subaquavocalis,* and *Rana yavapaiensis*) are similar in appearance and ecologic needs and vary in geographic distribution (Figure 1). All ranid frogs native to Arizona, except *Rana tarahumarae*, were classified as *Rana*

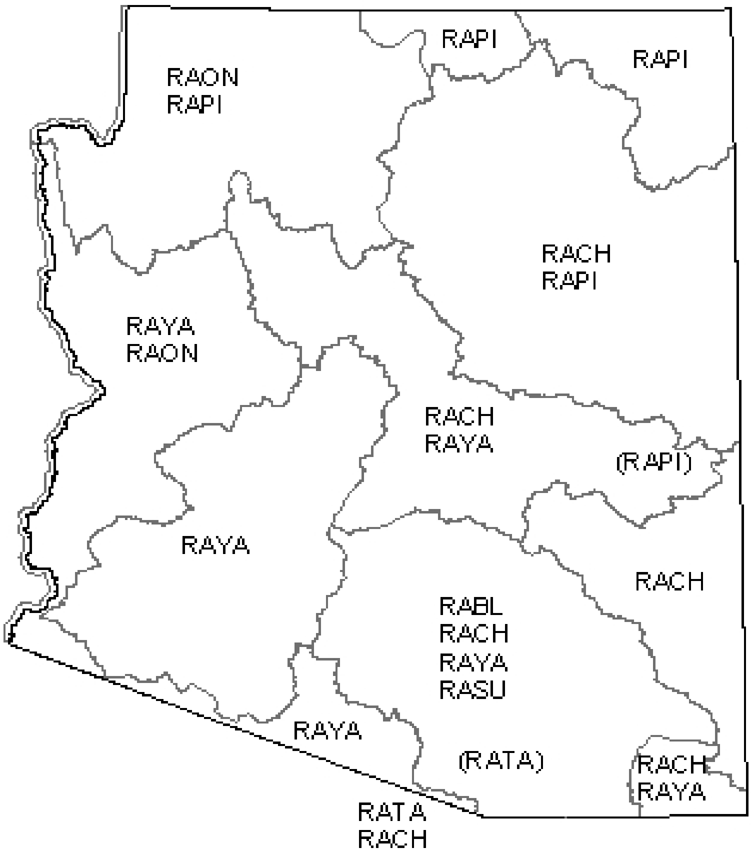

Figure 1. Distribution of Arizona native ranid frogs by major river basin. *Rana blairi* (RABI), *Rana chiricahuensis* (RACH), *Rana onca* (RAON), *Rana pipiens* (RAPI), *Rana subaquavocalis* (RASU), *Rana tarahumarae* (RATA), and *Rana yavapaiensis* (RAYA). Courtesy of Sredl, M.J., Arizona Game and Fish Department, Pheonix, Arizona.

pipiens until the late 1960's when scientists subdivided this group into multiple species based on slight variations in genetics, vocalizations, appearance and breeding characteristics (Hillis 1988). *Rana tarahumarae* lacks dorsolateral folds and can easily be distinguished from the leopard frogs. All species have experienced declines and range reductions since the 1970's. They are currently protected by the state of Arizona and one species, *R. chiricahuensis*, is federally listed as threatened under the Endangered Species Act.

Plains leopard frogs, *Rana blairi*, are restricted to the Sulphur Springs Valley in southeastern Arizona and are found in cattle tanks, irrigation sloughs, and settling ponds of a power plant. They typically breed from March through early June, and again from August through October. Due to its restricted range, this species is one of the most endangered leopard frogs in Arizona (Sredl 1997).

Chiricahua leopard frogs, *Rana chiricahuensis,* occupy habitat in central Arizona and mountains and valleys in southeastern Arizona. These are one of the most aquatic species of ranid frogs in Arizona. They are habitat generalists and occupy a variety of natural and human-made aquatic systems. Egg masses have been observed in all months except from November through January, but are uncommonly observed in June. The larval period is from 3 to 9 months and tadpoles may overwinter (Sredl and Jennings 2005). Populations of the Chiricahua leopard frog have declined dramatically; the Chiricahua Leopard Frog Recovery Team is currently drafting a plan to recover this species.

The Relict leopard frog, *Rana onca*, is a relatively small leopard frog that was declared extinct in 1984, but rediscovered in Nevada in the early 1990's. The species is currently found in a fewer than 10 natural and re-established populations near the Arizona/Nevada border (Jaeger et al. 2001).

Northern leopard frogs, *Rana pipiens,* have distributions ranging from the central to the northern portion of the state. Breeding occurs from April through July and tadpoles likely develop within 3 to 6 months, but may overwinter. The status of

Northern leopard frogs in Arizona is poor. Populations have disappeared from the White Mountains and the Mogollon Rim (Sredl 1997).

Ramsey Canyon leopard frogs, *Rana subaquavocalis,* get their scientific name from the male's characteristic underwater vocalizations. This species currently occupies the two canyons on the east side of the Huachuca Mountains in southeastern Arizona. Adult and juvenile habitat consists of springs, cienegas, cattle tanks, creeks, and slack water. Breeding occurs from late March through October and tadpoles may overwinter (Sredl 2005a). The small number of extant breeding populations makes this species highly vulnerable to extinction from disease, anthropogenic pressures, and stochastic events (Sredl 1997).

The Tarahumara frog, *Rana tarahumarae*, originally occupied plunge pools in higher gradient systems in southeastern Arizona and typically bred in April and May. The Tarahumarae frog was extirpated from Arizona in 1983 (Sredl 1997). Declines of the Tarahumara frog have been linked to the leaching of heavy metals from acid rain pollution caused by copper smelters in Mexico, but findings have not been verified (Hale and Jarchow 1987).

The Lowland leopard frog, *Rana yavapaiensis,* has a known range extending through central and southern Arizona. Lowland leopard frogs are habitat generalists; they occupy and breed in a variety of aquatic systems, including rivers, streams, pools, ponds, cienegas, cattle tanks, livestock drinkers, canals and other natural and human made aquatic habitat. They breed from early spring (April) through early fall (October), and may cease breeding activity during warmer summer months (Sredl 2005b). Tadpoles may metamorphose in the same year of oviposition or may overwinter, depending on conditions. Lowland leopard frogs are currently considered the most stable ranid frog populations in Arizona (Sredl 1997).

2.5 Amphibian decline hypotheses

A number of hypotheses have been proposed to explain loss of amphibian fauna, including emerging diseases, climate change, alterations in land use, introduced species, and commercial overexploitation. While each of these factors will be discussed independently, it is likely that one or more of these factors act synergistically, through direct or indirect means, to contribute to massive, widespread population declines (Kiesecker et al. 2001a; Blaustein et al. 2002).

2.5.1 Emerging disease

Although amphibians are susceptible to a range of pathogens, two diseases have received major attention as being directly associated with widespread die-offs and declines: *B. dendrobatidis* and iridoviruses (Carey et al. 2003b; Collins and Storfer 2003).

2.5.1.1 *Batrachochytrium dendrobatidis*

The newly recognized pathogenic fungus, *B. dendrobatidis,* was discovered in the United States (Pessier et al. 1999) and Australia (Berger et al. 1998). This fungus has the characteristics of an emerging disease spreading through frog and toad populations and causing worldwide morbidity and mortality (Berger et al. 1998; Bosch et al. 2001; Fellers et al. 2001; Bradley et al. 2002; Lips et al. 2003b; Bell et al. 2004), but not all individuals are affected (Collins and Storfer 2003; Mazzoni et al. 2003; Bell et al. 2004). *B. dendrobatidis* belongs to a group of parasitic chytrid fungi that are common in lakes, streams, ponds, and moist soil where they degrade cellulose, chitin, and keratin (Powell 1993). Other chytrid species are parasites of plants and invertebrates, but this is the first known to parasitize vertebrates (Daszak 1998).

B. dendrobatidis is aquatic with a two stage life cycle; an infective zoospore and a growing zoosporangium. The motile, uniflagellated zoospore attaches to keratinized amphibian epithelial cells and develops into a stationary, proliferating zoosporangium. When mature, the zoosporangium discharges more zoospores that infect nearby epithelial cells or swim through an aqueous environment to infect a new host (Longecore et al. 1999). For this reason, it is believed that transmission must occur in the water and may be more likely with close contact between individuals (Daszak 1998; Pessier et al. 1999; Bradley et al. 2002). Solely infecting keratinized epithelial frog tissue and keratinized tadpole mouthparts suggest that *B. dendrobatidis* uses keratin as a nutrient (Berger et al. 1998; Daszak 1999). Clinical manifestations include epidermal sloughing of roughened and disorganized skin, small ulcers, hemorrhaging or skin reddening (Pessier et al. 1999). Few internal lesions suggest the cause of death originates in the skin. Epidermal hyperplasia may impair cutaneous respiration or nutrient uptake, or the fungus may produce an immunosuppressant toxin (Pessier et al. 1999). Antimicrobial peptides in the skin do illicit a response to *B. dendrobatitidis* (Rollins-Smith et al. 2002). Additional studies are needed to understand mechanisms for *B. dendrobatidis* related mortality.

Rapid, large scale (50-100%) mortality is often observed with *B. dendrobatidis* infection (Laurance et al. 1996; Bosch et al. 2001; Bradley et al. 2002;

Lips et al. 2003b; Bell et al. 2004). Mortality only occurs in metamorphosed adults, but infected tadpoles can have deformed mouthparts (Green et al. 2002). Declines are often detected at higher altitudes or during cooler seasons and among species associated with riparian habitat (Carey 1993; Williams and Hero 1998; Young et al. 2001; Bradley et al. 2002; Lips et al. 2003a; Bell et al. 2004; Lips et al. 2004). In many cases, infectious disease appears to be the direct cause of death (Berger et al. 1998; Lips 1999).

Frog chytrid has now been isolated from amphibians in widespread geographic regions. Documented cases have been identified in North America, Central America, South America, Europe, Africa, Australia, and New Zealand (Speare and Berger 2000). In the state of Arizona, numerous die-offs have been observed in the field and many are attributed to *B. dendrobatidis*. It has been isolated from native and non-native species of ranids, hylids and caudates (Sredl 2000; Bradley et al. 2002; Davidson et al. 2003).

2.5.1.2 Iridoviruses

Iridoviruses are commonly found in bony fish have been identified as a newly emerging disease of amphibians, causing systemic infection in anuran and caudate hosts (Chinchar 2002). These viruses, also called *ranaviruses*, invade the liver, kidneys, and digestive tract and cause hemorrhaging lesions in the skeletal tissue (Daszak et al. 1999). External clinical signs include skin sloughing, erosion and ulceration (Chinchar 2002), but many infected tadpoles and adults die without any evident signs (Daszak et al. 1999). Histology and bacterial culture are used to diagnose disease (Daszak et al. 1999).

Iridoviruses can cause >90% mortality in its host and generally infects tadpoles, metamorphosing, and post-metamorphic individuals (Daszak et al. 1999; Green et al. 2002). Outbreaks are recurring and often associated with altered habitat or high population densities (Carey et al. 1999; Daszak et al. 1999; Green et al. 2002). None of the affected species are known to be species in decline, threatened or endangered, therefore some believe this disease may just be a hazard of overcrowding (Green et al. 2002).

Disease has been reported in *Rana temporaria* in the United Kingdom. It has also been found occurring commonly in tiger salamanders in Arizona and the Western United States as well as Canada (Jancovich et al. 1997; Bollinger et al. 1999). However, iridoviruses have never been found in native ranid species in Arizona and therefore, is unlikely to be implicated in their declines.

2.5.2 Climate change

Dramatic changes in climate in the past thirty years may contribute to amphibian declines. The frequency, intensity, and duration of El Nino/Southern Oscillation (ENSO) events have increased since 1976 (Carey and Alexander 2003). In the past thirty years, the most rapid global warming has occurred, raising the zero degree isotherms about 100 meters per decade in tropical mountains (Diaz and Graham 1996; Carey 2000; Carey and Alexander 2003). Climate change that directly affects one region may promote a multitude of events that could alter habitat far from the source affecting large groups of amphibians (Collins and Storfer 2003).

Ectothermic characteristics of amphibians may cause them to be sensitive to climate change. Amphibians are vulnerable to the effects of rapid climate change compared to other terrestrial vertebrates, because of their limited mobility and their dependence on moisture and temperature (Donnelly 1998). However, it is uncertain whether variation in climatic patterns would cause massive mortalities. Carey and Alexander (2003) point out that the survival of amphibians over time has depended on their ability to track climate variation. For climatic factors to be determined as a direct cause of decline, they must be shown to exceed lethal limits for a given population. Current studies demonstrate only a tenuous association between climatic factors and amphibian declines (Carey and Alexander 2003; Collins and Storfer 2003). Discussions of climatic change involving temperature and moisture, and ultraviolet-B (UV-B) radiation will follow.

2.5.2.1 Temperature and Moisture

Temperature and moisture directly impact amphibian biology. Amphibian body temperatures are regulated by exchange of heat with the air, water or soil. Body temperature, in turn determine rates of biochemical, cellular and physiological processes, including larval growth, metabolism, respiration, excretion, circulation, and digestion (Carey and Alexander 2003). Physiological and biochemical processes can increase by two to three folds for every 10 degree Celsius increase in body temperature (cited in Carey and Alexander 2003). Seasonal fluctuations in air and water temperature may be responsible for initiating certain behaviors such as emergence from hibernation and reproductive activity (Carey and Alexander 2003). Cool temperatures may also induce immunosuppression, leaving amphibians vulnerable to infection (Carey 1993).

Water availability is a key determinant in global amphibian distributions (Duellman 1999; Carey and Alexander 2003). Annual variation in rainfall, including the amount and timing of precipitation, can dramatically affect the yearly reproductive output. Carey et al. (2003a) found too much precipitation at critical times during egg/larval development can cause egg/larval mortality. Lack of snowmelt in early spring that supplies water to temporary breeding ponds may cause reproductive failure in some amphibians (Carey et al. 2003a). Non-permanent waters pose challenges for amphibians as eggs and larvae are at risk of desiccation and adult survivorship may be affected by high rates of water loss through their skin. Reduction in pond size also affects food supply, density of tadpoles, size at metamorphosis, and the number and efficiency of predators (Carey and Alexander 2003).

A number of studies have found correlations between changes in temperature and moisture patterns and amphibian declines. Abnormally low rainfall and record high temperatures associated with a 1986-1987 El Nino/Southern Oscillation event was correlated with population collapses of Harlequin frogs (*Atelopus varius*) in Costa Rica (Pounds and Crump 1994). Drought in Arizona and Colorado has been linked to the decline of ranid populations (Corn and Fogleman 1984, Sredl 1997). Some studies have documented changes in breeding phenology induced by large-scale changes in climatic patterns. Breeding phenology was significantly correlated with climatic warming in Great Britain and New York (Beebee 1995; Gibbs and Breisch 2001; Beebee 2002). Amphibians in North America may be showing trends toward earlier breeding (Blaustein et al. 2001), which may result from changes in the amount and timing of snow melt in the mountains (Corn 2003). Climate-induced interruptions in evolved breeding phenologies through global changes in temperature and moisture patterns may have large, population-level impacts on amphibians.

Other studies have found no associations between temperature and moisture and declines. Analysis of temperature and moisture patterns from areas around the world experiencing declines (Australia, Puerto Rico, Central America and Western United States) found no extreme climatic events occurring prior to or coincident with massive amphibian mortalities (Alexander and Eischeid 2001). The authors concluded that climate change was unlikely a direct cause of the observed declines. Declines of the red-legged frog (*Rana aurora draytonii*) were not associated with climate change (Davidson et al. 2001).

Pounds and Crump (1994) hypothesized that extreme climatic events may encourage outbreaks of pathogens. Pathogens may differentially respond to temperature and moisture; or temperature and moisture may alter host susceptibility or vectors that transmit disease (Pounds 2001). Reductions in water depth due to altered precipitation patterns exposed embryos to damaging ultra-violet radiation, thereby permitting an outbreak of lethal *Saprolegnia* (Kiesecker et al. 2001b). Cool temperatures may have contributed to immunosuppression (Carey 1993) and subsequent chytrid infection in *Bufo boreas* of the Rocky Mountains (Muths et al. 2003). Additional studies are needed to determine the effects of temperature and moisture on the outbreak of pathogens implicated in amphibian declines.

2.5.2.2 Ultraviolet Radiation

Recent increases in ambient UV-B radiation have stimulated interest in the role of UV-B in amphibian declines. Evidence of chemically induced reductions in the thickness of the ozone layer over the entire earth has led to increased UV-B levels (Middleton et al. 2001). Data demonstrate seasonal increases in UV-B at Polar regions and some evidence demonstrates increases in temperate latitudes. In general, it is assumed UV-B levels are greater at higher altitudes (Middleton et al. 2001). Remote sensing data has demonstrated UV-B levels have significantly increased in tropical and temperate regions, especially since 1979 (Blumthaler and Ambach 1990; Middleton et al. 2001).

UV-B radiation can directly kill embryos or cause sublethal effects, such as cell mutations, cell death, or slow growth rates (Jablonski 1998; Blaustein and Belden 2003). Damage to the immune system by UV-B exposure may limit the body's ability to detect allergens and antigens. Skin cells could also be damaged, leading to secretion of immunosuppressant chemicals, which may then facilitate pathogenic infection (Carey et al. 2001). Field studies have shown that *Rana cascadae* and *Bufo boreas* are more susceptible to *Saprolegnia ferax*, a common fish pathogen that causes amphibian embryo mortality, when exposed to ambient UV-B radiation (Kiesecker and Blaustein 1995). Defense mechanisms to repair UV induced damage may differ interspecifically, making some species less able to cope with recent increases in UV-B (Blaustein and Belden 2003).

It is debatable whether UV-B is directly contributing to amphibian declines. Research in this area has solely focused on differential hatching success of embryos exposed to sunlight and the ability of eggs to repair UV-induced DNA damage.

Some studies show a significant relationship (Blaustein 1994; Anzalone et al. 1998; Lizana and Pedraza 1998; Starnes et al. 2000) while others do not (Nagl and Hofer 1997; Corn 1998; Crump et al. 1999). Significant increases in UV-B radiation have been described in parts of Central and South America in areas where declines have occurred (Middleton et al. 2001) however, increases have not been demonstrated in all areas affected by major declines (Daszak et al. 1999). The UV-B induced declines hypothesis does not account for the decline of canopy-protected tropical forest species, nocturnal species, those who lay eggs in shaded areas, and massive declines occurring among adult frogs. Some investigators argue that the role of UV-B most likely acts through a set of complex interactions (Long et al. 1995; Kiesecker et al. 2001a; Blaustein et al. 2003). Additional research is needed to determine how UV-B is implicated in amphibian declines. To date, there have been no studies in Arizona examining the role of UV-B in ranid frog declines.

2.5.3 Alterations in land use

Changes in land use have drastically affected amphibian populations worldwide. This section will discuss impacts of habitat destruction and alteration, acidification and pollution of amphibian habitat.

2.5.3.1 Habitat destruction and alteration

It is not surprising that habitat destruction and alteration has resulted in the loss of total biodiversity, including amphibian species. The availability of aquatic and terrestrial habitat conducive to the needs of a species for mating, reproduction, and embryonic and larval development are vital for species survival. Both terrestrial and freshwater habitats have been drastically altered in the United States for land consolidation, wetland draining, regulation of flowing waters, agriculture, and forestry. Further damage to amphibian habitat is caused by erosion, lowering of ground water levels, and fragmentation (Grillitsch 2000). One of the primary effects of fragmentation is the immediate loss of individuals, species and biodiversity in an area. Secondarily, the landscape is fragmented into increasingly smaller patches, altering natural linkages and introducing barriers to animal movement between patches (Dodd and Smith 2000).

Alteration of habitat can affect amphibians directly or indirectly by killing them, removing their habitat, or preventing access to breeding sites (Collins and Storfer 2003). Over time, loss of habitat can lead to the loss of genetic diversity by creating small, isolated populations that are vulnerable to the accumulation of deleterious mutations and loss of adaptive potential and are in danger of extinction from stochastic events (Lynch 1996). Disruption at the landscape level can disturb metapopulations that regularly migrate between patches and alter population density, which may then impact disease emergence (Dobson and May 1986; Daszak et al. 2001). In extreme cases of fragmentation, the possibility of recolonization of empty patches decreases and a species may eventually disappear from a region (Dodd and Smith 2000).

Habitat modification has been implicated in Arizona amphibian declines. Waterway alterations have produced deep, still, perennial waters (Jennings and Hayes 1994) that may not be suitable for native species or that may favor persistence of non-native species. The Colorado river has been highly modified from a warm water, fluctuating, and meandering turbid river into a cold water habitat constrained by channels for transportation of flood waters for urban and agriculture use (Jennings and Hayes 1994). *Rana yavapaiensis* has disappeared along the Colorado and Gila rivers in Arizona, probably due to altered habitat, but invading *Rana berlandieri* might also be implicated (Clarkson and Rorabaugh 1989; Jennings and Hayes 1994). Overgrazing of riparian vegetation by cattle negatively affects amphibian habitat by removing cover, increasing ambient ground and water temperatures, destroying bank structure, trampling egg masses, and adding organic waste to the system. Effects of overgrazing are conspicuous in ranid frog habitat (Clarkson and Rorabaugh 1989; Sredl and Wallace 2000), but in most cases, do not appear to be a factor contributing to amphibian population losses (Clarkson and Rorabaugh, 1989).

In a climate where flash flooding is common, sedimentation may occur in amphibian habitat due to loosened soil from the building of roads, trails, firebreaks and recreational use. These disturbances alter hydrology by increasing runoff and erosion (Sredl and Wallace 2000). The degree to which flash flooding has affected Arizona native ranids has not been studied.

Lastly, ground water depletion from urban and agricultural demands may also impact amphibian habitat. Depression of Arizona groundwater levels in the upper San Pedro River Valley may negatively impact amphibian communities by eliminating or depleting flows in springs, creeks, and rivers and alter plant communities (see citations in Sredl and Wallace 2000). Breeding populations can not persist at sites where surface water has been lost (Sredl and Wallace 2000).

Although habitat destruction is a probable direct cause of many amphibian declines, it does not appear to explain the disappearances of species and massive die-offs of pristine and protected habitat in Arizona as well as in forests of Australia and Central America.

2.5.3.2 Habitat acidification

Episodic habitat acidification can occur naturally during snow melt or when air pollutants deposit sulfate and nitrate into water systems (Gillis 1993; Vertucci and Corn 1996). Due to their dependence on water, the developmental embryonic and larval stages are most affected by alterations in pH. Acidic conditions induce embryonic mortality by altering the consistency and elasticity of the egg membrane and inhibiting the hatching enzyme. Sublethal damage includes embryonic curling, which results in larval spinal deformities (Faber 2000). Reduced larval growth rates, which have a variety of consequences resulting in or including reduced fitness, have also been observed under acidic conditions (Pierce and Wooten 1992). Net loss of body sodium and deformities in adults are also indicators of acidic conditions (Dunson et al. 1992; Faber 2000). Finally, alterations in food supply and stress as a result of altered pH may compromise the immune system (Vertucci and Corn 1996).

It is unclear whether habitat acidification has contributed to widespread population declines. Persistent, long-term acidification of breeding habitat has been investigated as a possible cause of the decline of Natterjack toads (*Bufo calamita*) in Great Britain (Beebee et al. 1990). In the United States, literature on habitat acidification focuses on the decline of Rocky Mountains amphibians in the Western United States. Vertucci and Corn (1996) point out that snowmelt is the dominant hydrologic event that influences the chemistry of high-elevation amphibian breeding habitat and causes temporary acidic conditions by lowering the buffering capacity of surface waters. This usually occurs before there is open water in the ponds and amphibians breed. They examined published data and concluded that there was no indication that egg deposition coincided with snow melt (acidic conditions) for the species studied. Corn and Vertucci (1992) and Vertucci and Corn (1996) conclude that there is little evidence implicating episodic acidification in amphibian declines in the western United States. Habitat acidification and its role in ranid declines has not been examined in Arizona, however some studies indicate that ranid frogs may be exceptionally intolerant of highly acidic conditions compared to other frog species (Pierce 1985).

2.5.3.3 Xenobiotics and pollution

A wide array of contaminants affect amphibians, including heavy metals, pesticides, herbicides, fungicides, fertilizers and other pollutants (reviewed in Blaustein et al. 2003). According to Cowman and Mazanti (2000), over 100 types of carbamates and organophosphates alone are applied at a yearly rate of approximately 200 million acre treatments in the United States. The potential impact becomes tremendous if one considers pesticide, fungicide, herbicide and fertilizer use by the rest of the world. Many of these chemicals have high levels of toxicity and may pose a risk to amphibians through lethal or sublethal effects (Cowman and Manzati 2000).

Few studies have examined the role of xenobiotics in the natural environment. Spraying of dichloro-diphenyl-trichloroethan, commonly known as DDT, may have caused mortality among western spotted frogs (Rana pretiosa) in Oregon (Kirk 1988). Reproductive success of *Rana temporaria* was reduced after spraying atrazine nearby, which resulted in 100% mortality of eggs (Hazelwood 1970). Davidson et al. (2001)

concluded that patterns of decline among red-legged frogs in California were likely caused by pesticides carried upwind from the Central Valley.

Most of the amphibian xenobiotic literature focuses on the lethal toxicities and subsequent developmental effects on eggs or larvae in laboratory tests. Embryos, originally hypothesized to be the most vulnerable to xenobiotics, may actually be resistant to toxins due to their protective jelly coating (Cowman and Mazanti 2000). Tadpoles are often highly susceptible to poisoning, but effects vary with species, age, and experimental conditions (Bridges and Semlitsch 2000; Cowman and Mazanti 2000). Many authors conclude that sublethal effects, such as paralysis, decreased swimming speed, and reduced activity levels would have detrimental effects on larval ability to forage and escape predation (Cowman and Mazanti 2000). Carrie and Bryant (1995) add to this discussion, suggesting toxins can suppress the immune system and increase potential for infection by pathogens. Chemical compounds have also been found to disrupt the endocrine system by mimicking estrogen and androgens which may lead to reproductive consequences (Carey and Bryant 1995, Blaustein et al. 2003). More on amphibian responses to toxic chemicals and lethal doses can be found in Sparling et al. (2000).

Hale and Jarchow (1987) did speculate that the leaching of heavy metals from soil was caused by acid rain deposition from copper smelters in Arizona and Mexico, and was responsible for the decline of the Tarahumara frog (*Rana tarahumarae*). However, evidence to support this hypothesis is lacking. Point source pollution was not evident in other ranid surveys (Clarkson and Rorabaugh 1989). Additional studies examining the role of toxic substances in the declines of Arizona ranids have not been done.

2.5.4 Invasive Species

Introduced species of frogs (especially bullfrogs), fish and crayfish may negatively affect native amphibians. Invasive species can be very successful in a new habitat since they are often released from natural predators and parasites (Lafferty and Gerber 2000). They can directly prey on native species or compete for food and habitat (Kats and Ferrer 2003).

In addition to direct predation, Rosen et al. (1995) speculate that non-native species exclude native frogs from optimal habitat, thereby increasing their vulnerability to environmental stochasticity, such as drought. In the southwestern United States, native species are often observed in temporary ponds that frequently dry, increasing their vulnerability to desiccation, whereas non-native species are generally found in permanent bodies of water.

Non-native species may also introduce pathogens. The introduction of exotic pathogens into naive populations with no innate or adaptive resistance to the disease can be devastating (Daszak and Cunningham 1999). Often non-native species act as agents of disease dissemination. *B. dendrobatidis* has been identified in both bullfrogs (*Rana catesbeiana*) and cane toads (*Bufo marinus*), two widely introduced species through the aquaculture and pest control industries, respectively. This disease, which is lethal for many frogs, has been shown to induce low level infection in some individuals without any apparent clinical manifestations (Mazzoni et al. 2003; Daszak 2004). Bullfrogs and cane toads may serve as environmental reservoirs for the maintenance and/or spread of *B. dendrobatidis*. Another pathogen that has caused amphibian die-offs, *Saprolegnia ferax*, has been linked to introduced species of fish in the Pacific Northwest (Kiesecker et al. 2001a).

2.5.4.1Bullfrogs

Introductions and subsequent expansions of the American bullfrog (*Rana catesbieana*) to Western North America has raised concern about their role in amphibian declines. Since its introduction, investigators have repeatedly reported declines and range reductions in native ranid species (Hayes and Jennings 1986). The often-invoked bullfrog hypothesis suggests bullfrogs are implicated in the decline amphibian through direct predation or competition for food and habitat. Adult bullfrogs are opportunistic predators and feed on a variety of prey, including other frogs. Tadpoles also prey on the tadpoles of other amphibian species (Lawler et al. 1999; Kiesecker 2003). They breed between June and August and larvae can overwinter, taking one to three years to metamorphose. Consequently, competition may occur between native larvae and larger, older, bullfrog larvae (Kiesecker 2003). Bullfrogs may also be less susceptible to pathogens that cause mortality in other species (Mazzoni et al. 2003; Daszak et al. 2004).

Continued invasion by bullfrogs has been implicated in the decline of native ranid species in the desert Southwest. Surveys in Central and Southern Arizona have shown bullfrogs to successfully expand their distribution through translocation and within drainage dispersal (Hayes and Jennings 1986; Schwalbe and Rosen 1988; Rosen et al. 1995). Removal of riparian vegetation or reductions in stream flow may affect competitive interactions between native species and bullfrogs. Bullfrogs may be favored by conversion of wetlands into small, permanent ponds with increased water temperature (Hayes and Jennings 1986; Kiesecker 2003). However, data demonstrating that bullfrogs directly affect native Arizona ranids are lacking. Hayes and Jennings (1986) question the importance of the bullfrog hypothesis and point out that its origin was based mostly on observed inverse correlations between distribution of ranids and bullfrogs. The authors stress the need to test and consider alternative hypotheses as well as interactions with other variables.

2.5.4.2 Fish

The introduction of non-native fish to new habitat where they previously did not occur has become a global problem (Kiesecker 2003). As many as 120 predatory fish have been introduced into 160 countries around the world for recreational sport fishing and control of mosquito populations (cited in Kiesecker 2003). Access to early, vulnerable life stages provides a compelling argument that fish could have dramatic, negative effects on amphibian populations (Hayes and Jennings 1986). Non-native species of fish may compete for food and habitat with amphibians, directly prey on the vulnerable aquatic life stages, or introduce deadly pathogens into naïve amphibian populations (Hayes and Jennings 1986; Blaustein et al. 2002; Kiesecker 2003).

Correlational studies have found negative associations between the occurrence of fish and amphibians (reviewed in Kats and Ferrer 2003). Paedomorphic newts were extirpated from all sites in Southern Europe where fish had been introduced (Denoel et al. 2005). Introduced trout may have contributed to the decline of the mountain yellow-legged frog (*Rana muscosa*) (Bradford 1989; Knapp and Matthews 2000) and populations of these species showed rapid recovery when fish were removed from sites in California's Sierra Nevada mountains (Vredenburg 2004). Introductions of mosquitofish (*Gambusia affinis*) are thought to have contributed to declines of California red-legged frogs (*Rana aurora draytonii*). Fischer and Shaffer (1996) found that these native amphibians do not often co-occur with non-native predatory fish. Historically, California red-legged frog populations occurred at significantly lower elevations than presently known extant populations. Many of these lower elevation sites are now occupied by mosquitofish. However, abundant numbers of mosquitofish did not significantly affect the survival of the California red-legged frogs in laboratory experiments, but presence of fish was correlated with increased injury and decreased size at metamorphosis, possibly implicating competition between native frogs and fish (Lawler et al. 1999). Reduced metamorphic size and rate in the presence of fish have been found in other experimental studies as well (Nyström et al. 2001; Tyler et al. 1998)

Experimental studies have also demonstrated that presence of fish decrease the survivorship of amphibian embryos through direct predation (Gamradt and Kats 1996; Goodsell and Kats 1999, Gillispie 2001) and can transfer deadly pathogens to amphibians (Kiesecker et al. 2001b). Differential hatching success was found in western toad (*Bufo boreas*) embryos exposed to trout experimentally infected with *Saprolegnia ferax* compared to embryos exposed to non-experimentally infected trout (Kiesecker et al. 2001b).

Introduced species of fish in Arizona that may negatively affect native amphibians include centrarchid fish (family *Centrarchida*e) trout (*Onchorhynchus mykiss, Salmo trutta*), fathead minnows (*Pimephales promelas*), and mosquitofish (*Gambusia affinis*) (Rosen et al. 1995). Centrarchid fish have been widely introduced into Western North America (Moyle and Light 1996) and are generally considered to be the most offensive introduced fish to amphibian assemblages in Arizona aquatic systems. Centrarchid fish, namely bluegill (*Lepomis macrochirus*) have been shown to decrease the survival of *Rana aurora* and *Hyla regillia* in field studies (Adams 2000) and have been shown to affect amphibian assemblages and abundances (Smith et al. 1999). Brown trout (*S. trutta*) and Rainbow trout (*O. mykiss*) were found to reduce survivorship of *Litoria spenceri* and *Litoria phyllochroa* in Australia (Gillispie 2001), reduce metamorphic size and rate among Rana temporaria, (Nystrom et al. 2001), and were shown to directly prey on *R. muscosa* (Needham and Vestal 1938, Mullally and Cunningham 1956). Fathead minnows may alter ecological characteristics of aquatic systems (Zimmer et al. 2001). Mosquitofish have been linked to decreased larval survivorship in amphibians (Gamradt and Kats 1996, Goodsell and Kats 1996; Lawler et al. 1999). Decreased survivorship may be due to reduced metamorphic rate or size, alterations in normal activity, or increased tail injuries that occur when mosquitofish cohabitate with frogs (Gamradt and Kats 1996; Goodsell and Kats 1999; Lawler et al. 1999). Additionally, *G. affinis* readily eat native Arizona ranid hatchlings in laboratory experiments (Rosen, unpublished data).

Negative correlations between distributions of introduced fish and native ranid frogs have been documented in Arizona (Rosen et al. 1995).Rosen et al. (1995,

1996) found that Chiricahua leopard frogs were replaced by American bullfrogs and centrarchid fish. Most localities where Chiricahua leopard frogs occurred lacked non-native vertebrates, and historical localities without Chiricahua leopard frogs supported non-native vertebrate populations.

2.5.4.3 Crayfish

Crayfish may be harmful predators and competitors of native herpetofauna. Microcosm experiments have demonstrated that crayfish directly prey on amphibian eggs, tadpoles, and adults (Fernandez and Rosen 1996). Laboratory and field experiments have shown that crayfish consume and decrease survivorship of eggs and larvae of the California newt (*Taricha torosa*) (Gamradt and Kats 1996). Sharing habitat with aggressive crayfish may deter amphibian breeding (Gamradt et al. 1997). Crayfish may also negatively affect frog populations by reducing aquatic vegetation and decreasing the amount of habitat heterogeneity and cover available for native amphibians. Pristine, clear aquatic habitat with abundant plant life became murky, lacked aquatic vegetation, and appeared to decrease in animal diversity when dense populations of crayfish became established (Fernandez and Rosen 1996). Some native organisms have been completely eliminated from a habitat once crayfish were introduced, including native ranid frogs in Arizona (Fernandez and Rosen 1996).

The spread of crayfish is newly recognized threat facing Arizona’s aquatic systems. Crayfish are not native to Arizona, but two species, the northern crayfish (*Orconectes virilis*) and the red swamp crayfish (*Procambarus clarkii*), have been successfully introduced since the 1970’s to aquatic systems through stocking with game fish, use as fish food, or dumping of bait buckets (Fernandez and Rosen 1996, Gamradt and Kats 1996, Taylor et al. 1996, Kats and Ferrer 2003). Field studies have also documented negative correlations between the distributions of native ranid frogs and crayfish (Fernandez and Rosen 1996). Additional research is needed, however, to evaluate the impact that crayfish have had on native ranid frog populations. The role of crayfish in worldwide declines is unknown.

2.5.6 Commercial Exploitation

Researchers are just beginning to examine the effects of commercial exploitation on amphibian populations. Amphibians have been historically traded for food, pets, research, education, medicine, and bait (Jensen and Camp 2003). Over-harvesting can directly impact numbers of breeding amphibians, while indirect effects include intentional and unintentional non-native introductions. Establishment of amphibians outside their natural ranges is a problem that accompanies exploitation. Intentional establishment of individuals occurs for commercial or industrial purposes. Unintentional introduction occurs through the release of unwanted pets, experimental animals, or bait as well as commercial escapees (Jensen and Camp 2003). Introduced non-native species, discussed in section 2.5.5, can have serious impacts on native fauna, including direct predation, competition or pathogen introduction.

Over-harvesting and trade in frog legs for consumption has contributed to the endangered status of several species of ranids, primarily native to Eastern Asia (Schmuck 2000; Stuart et al. 2004). According to Jensen and Camp (2003), primary markets exist in the United States, Canada and Europe. Countries in the European Union imported over 6,000 metric tons of frog legs in the 1990's. Secondary markets exist in Asia, with over 6 million frogs shipped from Thailand to Hong Kong in one year. Poaching continues to be a problem where collection and trade are banned.

Collecting frogs as pets has led to the decline of several rare salamanders and newts (i.e. *Ranodon sibiricus*, *Salamandra salamandra*, *Triturus vittatus, Andrias davidianus*). Poison dart frogs from Central and South America (*Dendrobatides* and *Phyllobates*) and Malagasy poison frogs (*Mantella*) from Madagascar are brightly colored and highly sought after specimens in pet trade. These pricey frogs are sold in pet stores, but 80% of the 16,000 reported in international trade between 1987 and 1993 came from the wild (Jensen and Camp 2003).

The use of amphibians for research and education has led to a commercial market. Many of these frogs are still caught in the wild (Jensen and Camp 2003) while others, such as *Xenopus laevis*, has been shipped globally for research purposes. To date, the earliest diagnosed case of chytridiomycosis has been found in a museum specimen of *Xenopus laevis* from 1938 (Weldon et al. 2004). Some investigators hypothesize that that *B. dendrobatidis* may have originated from *Xenopus sp.* in South Africa (Weldon et al. 2004).

2.6 Population monitoring and declines

Validated techniques have been developed to monitor amphibian populations by capturing, counting, and repeatedly registering as many individuals as possible to make quantitative population estimates (Jehle 2000). Each method has advantages and disadvantages with associated biases and limitations; goals of the study should be considered in order to select an appropriate survey method. The visual encounter survey (VES) monitoring method used by AZGFD to monitor target amphibian species and their respective habitats in this study is a widely used method for amphibian inventory and monitoring. In VES surveys, field personnel walk through an area of habitat for a period of time, systematically searching for target species. The VES can be used to determine species richness, compile a species list and to estimate relative abundance of species within an assemblage. This survey technique offers the advantages of being simple to conduct, with the ability to rapidly evaluate a large area, but is limited by inability to sample all habitat and microhabitat types with equal success (Crump and Scott 1994). Crump and Scott list four main assumptions of the VES methodology that should be considered in data interpretation: 1) every individual has an equal chance of being observed during the survey (no differential effects of coloration, size, behavior, activity, etc.) 2) Each individual/species is equally likely to be observed during sampling sessions (no seasonal effects of activity, weather, precipitation, etc.). 3) An individual is recorded only once during a survey (observers are able to keep track of all movement so as to not record multiple encounters for the same individual.) 4) Results from two or more observers surveying the same area will be identical (there is no inter-observer variation in species and numbers observed).

Despite the use of appropriate monitoring techniques, assessing declines in naturally occurring amphibian populations poses many challenges. Populations often fluctuate with substantial magnitude in the size of the breeding population and the recruitment of juveniles to adults (Pechmann et al. 1991; Blaustein et al. 1994; Pechmann and Wilbur 1994). Many factors may influence population fluctuations, including rainfall, predation, competition, population density, and environmental disturbance (Pechmann et al. 1991; Meyer et al. 1998). Additionally, amphibians are usually considered to exist in metapopulations, or groups of subpopulations that exchange migrants and are subject to recolonization and local extinction events. Metapopulation models predict that subpopulations will blink in and out of existence and will be colonized at a rate related to the spatial arrangement of habitat patches (Marsh and Trenham 2001).

Field observations of dead and dying frogs may further substantiate significant decline events. Some declines have been preceded by observations of dead and dying frogs in the field (Berger et al. 1998; Lips 1999; Sredl 2000), but amphibian population crashes may go unnoticed due to insufficient monitoring, removal of carcasses by scavengers, efficient predation of sick frogs, or death in cryptic refuge (Green et al. 2002). Often frogs mysteriously disappear with no or few field casualties detected (Green et al. 2002). Some authors have emphasized a need for caution in interpreting local extirpations as actual population declines without sufficient long-term, quality datasets (Pechmann et al. 1991; Travis 1994; Green 2003).

Several monitoring recommendations have resulted from difficulties in distinguishing between processes that produce turnover versus those that have lead to true declines and/or extinctions. Marsh and Trenham (2001) stress that presence/absence data should be calibrated with known distributions, mark-recapture methods should be employed to identify turnover due to skipped breeding or movement between ponds, and that individuals should be absent from breeding ponds for longer than the time it takes to reach maturity before calling a population extinct.

Travis (1994) used a model to demonstrate that it would take at least three generations before an extinct site may be recolonized at a 0.25 colonization success rate (the highest colonization rate examined). Thus, a site may need to be visited for several generations to determine if a population is truly extinct. Skelly et al. (2002) reported that decline and distributional change were sensitive to duration of resurvey effort and the type of historical data used. Multiple-year resurveys were more conservative in estimating declines than single-year resurveys. Restricting analysis to estimating loss of amphibian presence tended to increase estimates of decline, compared to analysis of data with both known historical presence and absence.

Although many monitoring recommendations have been made that aid in data interpretation with respect to declines, these recommendations may not always be realized when studying dynamics of naturally occurring populations. As conservation goals change through time, resurvey efforts in long-term monitoring studies often must reflect such changes (Howland et al. 1996). Additionally, many of the recommendations appropriate for smaller subpopulations (such as mark-recapture studies) are not feasible for monitoring geographically widespread areas with large numbers of sites. Thus, the need continues for developing innovative study designs and efficient analytical methods to draw out meaning from dynamically captured data.

2.7 Epidemiologic observational studies

Powerful methods borrowed from the field of observational epidemiology may facilitate analysis of declines in naturally occurring amphibian populations. Observational studies, the study of disease or another outcome of interest in natural populations, are used to identify risk factors, their quantitative effects and the various components that contribute to the occurrence of disease (or another outcome) (Thrusfield 1995). Observational studies differ from experimental studies in that the investigator is not free to randomly allocate factors. Study designs and methods have been developed that utilize datasets which collected for alternative purposes and possess numerous biases.

Study designs and analytical methods have been developed in epidemiology to facilitate the control of extraneous variables and elucidate meaning out of difficult-to-analyze datasets (see Martin et al. 1987; Thrusfield 1995; Rothman and Greenland 1998). The two main types of observational study designs are the cohort study design, and the case-control study design (Rothman and Greenland 1998). Cohort studies follow groups of individuals exposed and unexposed to a specified risk factor/s forward through time to determine whether the outcome of interest develops (Thursfield 1995). They are particularly useful for evaluating incidence and the prospective study design allows for flexibility in choosing variables to be recorded. Cohort studies are often expensive, require large numbers of subjects and have considerable loss to follow-up (Thursfield 1995; Rothman and Greenland 1998). Cohort studies examining factors relating to amphibian declines may become useful as large groups of amphibians are prospectively monitored.

Case-control studies identify subjects with and without disease (or another event of interest) and retrospectively follow them to determine prior exposure to single or multiple risk factors (Thursfield 1995). These study designs are often used to study rare diseases. Case-control studies are advantageous in that relatively few subjects are needed because analytical methods are statistically efficient, they are inexpensive and quick to conduct, and existing records can be used. Drawbacks of case-control studies include, relying on recall and records for past exposures, difficulty in selecting appropriate control groups, and difficulties in controlling numerous inherent biases (Thursfield 1995; Rothman and Greenland 1998). Case-control study designs may lend themselves well to the analysis of retrospective amphibian decline datasets where the outcome of interest (whether a decline event, disease, or another outcome) is rare, few subjects are available, monitored populations were not randomly selected, and data from existing records were used. Because many amphibian monitoring datasets contain these features, the case-control study design offers an exceptionally useful alternative for examining risk factors related to amphibian declines.

2.8 Hypothesis testing

Based on published information documenting amphibian declines, sites experiencing disappearances should be characterized by different traits than sites with persisting populations. Variation between declining and persisting populations is hypothesized to be caused by a combination of factors: 1) negative effects of introduced predator/competitor species on native species, 2) disease caused by *B. dendrobatidis*, 3) environmental factors affecting hosts or pathogens, and 4) the spatial distribution of sites and metapopulation movement.

Specific hypotheses examined that correspond to the preceding numbers include: 1) Crayfish, fish and bullfrogs may directly compete with, prey upon, or introduce pathogens into amphibian populations, thereby increasing the risk of having a disappearance. 2) *B. dendrobatidis*-related population declines can not be directly assessed due to lack of information on presence of disease in these Arizona populations. However, hypotheses related to spread of this infection will be indirectly examined by evaluating variables from the other hypotheses. 3) Temperature (measured by elevation), pH, water type (still versus moving waters), soil characteristics (soil organic matter and available water capacity), UV exposure (measured by southern aspect), and wind exposure (measured by northwestern aspect) may interact with the amphibian immune system or modify pathogen virulence to influence disappearances. 4) Nearby disappearances may promote local extinctions due to disease transmission, while nearby extant populations may decrease this risk due to recolonization after a decline event.

Chapter 3: Manuscript

3.1 Introduction

The decline and extinction of amphibians has gained global recognition with large population losses reported on all inhabited continents (Stuart et al. 2004). Leading hypotheses for the recent declines include introduction of non-native species, commercial over-exploitation, alterations in land use, global climate change, increased chemical usage and habitat pollution, and emerging infectious diseases (Collins and Storfer 2003). Although many declines are known to be direct results of human habitat destruction and overexploitation, other species have disappeared without any identifiable causes. These more mysterious declines have shared some characteristics, mainly affecting species which live at higher elevations, breed in streams and have restricted geographical ranges (Berger et al. 1998; Lips et al. 2003, 2004; Stuart et al. 2004).

Patterns of declines in the Western United States have been consistent with those in other parts of the world; while species in some areas decline, other populations appear unaffected (Sredl 1997). Range reductions and population declines have been recognized in all seven species of ranid frogs native to the Sonoran Desert of Arizona, including *Rana blairi, Rana chiricahuensis, Rana onca, Rana pipiens, Rana subaquavocalis, Rana tarahumarae, and Rana yavapaiensis* (Clarkson and Rorabaugh 1989; Sredl 1997). Causal processes hypothesized in Arizona are analogs to the global concerns and include introduction of predator and competitor species, drought, habitat alteration, pollution, and infectious disease (Hayes and Jennings 1986; Hale and Jarchow 1987; Schwalbe and Rosen 1988;

Clarkson and Rorabaugh 1989; Jennings and Hayes 1994; Rosen et al. 1995; Sredl 1997; Sredl and Wallace 2000; Bradley et al. 2002).

In other naturally occurring wildlife populations, quantitative epidemiologic methods have been used to identify factors underlying health and disease (Brown et al. 2003; Gordon et al. 2004; Krueder et al. 2005). Epidemiologic study designs and analyses, developed to minimize bias in observational data, may prove useful for understanding the degree and magnitude to which different variables contribute independently and/or synergistically to frog declines. Most previous studies have examined only the effects of a single factor on amphibian mortality (Storfer 2003). Dramatic declines are more likely the result of many factors working in complex, synergistic ways (Kiesecker et al. 2001a; Blaustein et al. 2002) and recent research further substantiates this concept by demonstrating that more than one factor can modulate morbidity and mortality of amphibians in laboratory and mesocosm experiments (Berger et al. 2004; Parris and Beaudoin 2004). However, few studies have examined multiple factors associated with the declines in naturally occurring populations due to complex analyses and the need for large data sets.

The Arizona Game and Fish Department (AZGFD) has created and maintains a large, statewide database with data from over 2000 localities on the present and historical distribution of native ranid frogs. Researchers began active field surveys of sites throughout the state in the early 1990s. Many of the surveys captured time points when frogs disappeared and never returned to a site. This data resource provided a unique opportunity to investigate patterns in frog declines using detailed, field data from natural frog populations. The objective of this study was to use epidemiologic methodology and multivariable analytic techniques to identify risk factors associated with disappearances of frogs throughout Arizona. This information was needed for management of Arizona frogs as well as to provide a foundation for future studies of risk factors for other amphibian declines.

3.2 Methods

3.2.1 Source data and study design

The AZGFD database describes statewide native ranid frog localities from 1891 to present. Historical distributions were determined from published literature and AZGFD Technical Reports (for a complete list of citations, see Sredl 1997). Present distributions of frogs were established by AZGFD visual encounter surveys (VES) through multiple visits to sites with suitable riparian habitat. The survey methodology has been previously described in detail (Sredl, 1997). Briefly, numbers of target species (*Rana blairi, Rana chiricahuensis, Rana pipiens, Rana subaquavocalis, Rana tarahumarae*, and *Rana yavapaiensis*), local site characteristics, environmental data, and survey conditions were recorded during each visit. The location of the site was georeferenced by either manually calculating Universal Transverse Mercator (UTM) units from 7.5 minute topographical maps or using Global Positioning Systems (GPS; Garmin Ltd., Olathe, KS) during field surveys. To maximize the chance of encounter, surveys were usually conducted between dawn and dusk from late March through early November. The number of sites surveyed and the number of VES surveys per site differed between years, depending on conservation initiatives, site location, budget, and environmental or weather conditions. This variation in sampling effort and distribution across time and space introduced potential biases which precluded simple analysis of the entire dataset. An epidemiologic case-control study design, with density sampling across time from among sites within the AZGFD study base (Rothman and Greenland 1998), was used to minimize effects of these biases.

3.2.2 Classification of case and control sites

Data from all surveys through December 2002 were examined to determine whether at least one adult ranid frog from the list of target species was detected at that site during a given survey. Surveys were eliminated if target species could not be adequately distinguished from other introduced ranids (*Rana catesbieana and Rana clamitans*). Surveys during which frogs were not detected were omitted if conditions were unfavorable for finding frogs (dried ponds, cool temperature, hibernation season, or land development). Surveys were also excluded after any conservation releases of native frogs occurred at that site or at a site within the same canyon.

Surveys were then grouped by site and ordered by date. Sites were excluded if they were not located on government, public or private land in Arizona or if native ranid frogs were not present at least once during the site's survey history. Sites were also limited to locations where AZGFD biologists conducted at least one VES between 1993 and 2002 to provide contemporary confirmation of site characteristics, location and other data. If target species were not detected in any survey in a given calendar year, the site was classified as "absent" for that year. If target species were detected during at least one survey in a given calendar year, then the site was classified as "present" for that year. If no surveys were conducted during the calendar year of interest, the site was classified as "unknown" for that year.

The pattern of present and absent classifications across time was used to determine whether each site demonstrated evidence of local extinction or population persistence. Sites were classified as potential "cases" if they exhibited two or more consecutive absent year classifications. Sites were classified as potential "controls" if they had histories of either all "present" year classifications or single, embedded absent classifications preceded and followed by "present" year classifications. Sites where the last observation was a single "absent" year classification could have represented either a new case or a single, isolated survey where frogs were undetected. Sites where the first observations were two or more consecutive "absent" year classifications could have disappeared any time during the past and conditions at the time of disappearance could not be ascertained. Sites that fell into one of these categories were not included as case sites. Two additional sites were removed because their spatial distribution was substantially different from other study sites and would unlikely provide a good source of comparison. These eligibility criteria are summarized in Table 1.

Table 1. Criteria used to identify sites eligible for inclusion in the case-control study

Eligibility criteria	**Number ineligible**
Target species must be present in at least one survey	1224 sites
Site must be surveyed at least once between 1993 and 2002	225 additional sites
Site must be located on governmental, public or private land	1 additional site
Classification criteria	**Number not classified**
Identified frogs were indistinguishable from bullfrogs	55 additional sites
Post conservation release at site or nearby location	18 additional sites
Local drought	32 additional sites
Winter visit (absent survey in Dec.-Feb.)	1 additional site
Single trailing absent year	116 additional sites
Multiple leading absent years	8 additional sites
Cases and controls must represent similar spatial distributions	2 additional sites

Density sampling was used to randomly select an equal number of cases and controls that would have a similar distribution of time-related confounding and heterogeneity. For each year beginning in 1986 (the first case occurrence) and moving forward through 2002, the status of each site in the cohort was determined. Case sites were sampled at the time that frogs first disappeared (when a site was first classified as a case). An equal number of control sites were randomly selected from all surveyed sites at risk during the same year in which frogs were detected. If there were more case than control sites in a given year, the desired 1:1 case-control ratio was preserved by randomly selecting case sites. Thus, the final data set used for analysis consisted of one or more cases from each year and an equal number of controls that had the same distribution of sampling over time.

3.2.3 Risk factors

Potential risk factors were drawn from habitat variables collected in survey records in the AZGFD database and from spatial variables developed by overlaying coordinates from survey sites with attribute maps using a geographic information system (GIS; ArcView 8.1, ESRI, Redmond, CA).

Presence or absence of non-native species, including bullfrogs, fish and crayfish, at each study site was determined from site-specific AZGFD surveys. If any of these non-native species were recorded in any survey during a site's survey history, they were coded as a risk and compared to sites where introduced species were never identified.

Two hydrological characteristics were evaluated for associations with case/control status of sites. Median water pH was calculated for each site from serial pH readings taken during surveys. A variable was created to compare sites where median pH levels were between 4 and 8, the necessary pH for growth of *B. dendrobatidis* in vitro, to sites with pH values outside this range. Water type, whether lentic or lotic, was determined during AZGFD surveys. Still or slow moving water was classified as lentic, and flowing water, including streams containing natural pools that flowed at least some time during the year, were considered lotic. Lentic water systems were hypothesized to have higher risk as compared to lotic systems.

Effects of nearby disappearances and nearby extant populations were evaluated to examine patterns related to metapopulation movement and disease spread. To create a set of variables reflecting potential effects of nearby disappearances, each site was evaluated at every time step from 1986 thru 2002. Sites were categorized at each time step as a “current case” (became a case that year), a “recent case” (became a case within the past 2 years), a “future case” (became a case on the next future survey), or “other” (i.e. controls sites; sites that became cases longer than 2 years before or after the current year). All study sites were examined in the year they were selected into the study to determine whether another site classified as a current, recent or future case was within 2, 4, 6 or 8 kilometers. Having at least one nearby disappearance was compared to no nearby disappearance in the univariate and multivariable analyses separately for all 4 distances. The best distance was selected and reported based on the magnitude and associated variance estimates of the odds ratios.

The effect of nearby extant populations was evaluated in a similar manner. All sites were categorized at each time step as having “frogs currently present” (frogs were present during that year), “frogs recently present” (frogs were present the previous year), “frogs present in future” (frogs were present in the next future survey) or “other” (sites with no frogs reported to be present during the current, previous or subsequent year). Current, recent and future frog observations were considered to represent an extant population. Again, GIS was used to draw 2, 4, 6, and 8 kilometer circular buffers around all study sites for the year they were selected to determine whether a nearby site could potentially serve as a source for repopulation. Presence of a nearby extant population was assessed separately for each of the four distances in univariate and multivariable analyses and the most predictive distance selected.

Elevation was determined from 7.5 minute United States Geological Survey (USGS) topographical maps with dates ranging from 1948-1991. Global Positioning Systems and 7.5 minute Digital Elevation Model (DEM) files were used to verify elevation among recently surveyed sites. Risk was assessed as a linear effect per meter increase in elevation.

The aspect of the slope of each study site was determined from 7.5 minute DEM files dated from the 1990’s. Each site was overlaid on the digital aspect maps using the GIS and given the corresponding aspect value of its specific location. Southern aspect values (135 to 224 degrees) were coded as a risk and compared to data with all other aspect values (225 to 359 degrees, 0 to 223 degrees). Northwestern aspect values (270 to 359 degrees) were also evaluated and compared to data with all other aspect values (0 to 269 degrees).

Two soil characteristics were evaluated using digital soil maps from the 1994 State Soil Geographic (STATSGO) database developed and maintained by the United States Department of Agriculture, Natural Resources Conservation Service (USDA, NRCS). For each site, the underlying soil polygon was determined by overlaying site locations on the soil maps using the GIS. Each soil polygon was composed of several different soil profiles (U.S. Department of Agriculture 1995). Available water capacity (AWC), the amount of water a soil can store for use by plants, was calculated for each soil profile by summing weighted averages of AWC for each soil layer (weighted by layer depth). All AWC-per-profile calculations were normalized to the percent composition of the particular polygon to identify the best indicator of approximate site AWC (U.S. Department of Agriculture 1995). Standard USDA published AWC classification tables (U.S. Department of Agriculture 1993) were used to divide data into relevant groups. Higher AWC values (≥3.1 inches per square inch of soil) were compared to very low soil AWC (0-3 inches per square inch of soil). Soil organic matter (SOM), the percent composition of organic matter contributing to the soil, was determined using similar methodology. The average organic content for the top layer of soil was calculated and normalized according to the percent composition of the polygon. SOM values were divided into two groups, based on whether the data fell below or above the median (1.2). High SOM values (>1.2%) were compared to lower SOM values.

3.2.4 Statistical methods

Univariate analyses were used to screen for factors that differed between cases and controls. Odds ratios (OR) and Fisher's exact tests (for categorical variables) or Wald statistics (for continuous variables) were used to evaluate the magnitude and significance of risk factors. Significant associations at the $P < 0.25$ level were further examined in multivariable analyses.

Stratified contingency table analyses were used to check for plausible effect modifiers and confounders. The Breslow-Day test was used identify common odds ratios between strata. Presence of effect modifiers was also evaluated by including first order interaction terms in a model and forcing main effects in whenever dependent interatctions were included.

Multivariable, unconditional logistic regression was used to identify concurrent effects of spatial and environmental risk factors. The best subsets method of model selection (Hosmer and Lemeshaw 2000) was used to identify key variables for the adjusted model. The importance of each variable, in combination with other variables, was evaluated using the estimated coefficients, their respective standard errors, Wald statistics and the effect on other coefficients as variables were added and removed. Nested models were compared using likelihood ratio tests. Effect modification was evaluated by including first order interaction terms in each model and forcing main effects in whenever dependent interactions were included. The final model was selected for biological plausibility, strength of associations and the Hosmer-Lemeshaw goodness-of-fit χ^2 statistic. Influential observations were evaluated. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated for the final model. For elevation, risk was reported per 500 meter increase using the equations $e^{500(\beta)}$ and $e^{500(\beta)\ \pm 1.96(SE)(500)(\beta)}$ to determine the OR estimate and associated 95% confidence intervals, respectively. Univariate and multivariate analyses were conducted using EpiInfo 2002 (Centers for Disease Control and Prevention, Atlanta, GA) and SAS (version 8.1, SAS Institute; Cary, NC).

3.3 Results

The AZGFD Survey of Native Ranid Frogs Database included a total of 2007 sites with 7007 surveys conducted between 1884 and 2002. Each site was surveyed between 1-48 times over a period of 1-24 years. Native ranid frogs were never observed at 1224 of these sites, 225 other sites were never visited after 1993, and 1 additional site was located on native lands. The 556 confirmed sites where frogs had been observed at least once and surveyed during recent years included 2646 surveys conducted between 1891 and 2002. Two hundred thirty-two of these sites were unable to be classified as a case or control due to incomplete identification of frogs, too few site visits or visits during inclement weather, or reintroductions at the sites (Table 1). A total of 36% (117/324) of all classified sites became cases during the study period. New cases occurred in new localities every year from 1986 thru 2001, except 1988. Using density sampling, 105 of these cases and an equal number of controls were sub-selected for analysis. Both classified and study sites were located throughout Central and Southern Arizona, and generally reflected the geographical distribution of sites surveyed in the AZGFD database (Figures 2).

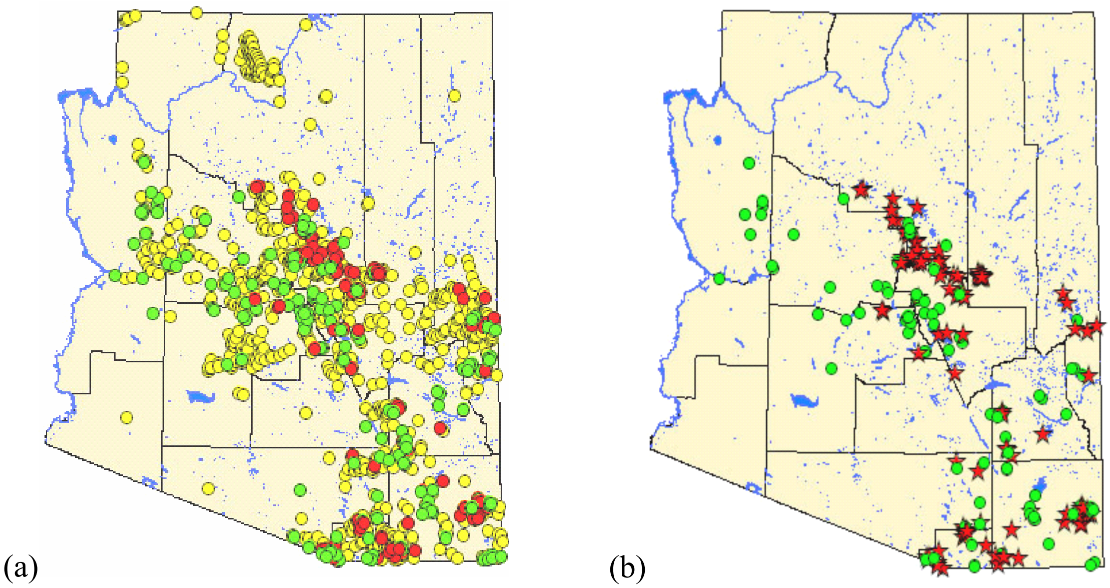

Figure 2. Distribution of localities surveyed by Arizona Game and Fish Department. (a) All 2007 sites in AZGFD survey of native ranid frogs' database. Eligible case sites (red), eligible controls (green), and ineligible sites (yellow). (b) Case sites (red stars) and control sites (green circles) selected for univariate and multivariate analyses.

Nine of the 12 variables examined using univariate analyses met screening criteria (Table 2), and were included in the multivariable analyses. Only four of these variables were statistically significant ($P<0.05$) when adjusted for cofactors in the logistic regression model (Table 3). Significantly higher risk for disappearance of frogs was observed for sites at higher altitude (OR=2.7 for every 500m increase in elevation), sites with a nearby disappearance (OR=4.3), and sites with crayfish (OR=2.6). In contrast, nearby source populations offered a substantial 85% reduction in risk of being a case (OR=0.15). No significant interactions were identified using stratified contingency table analysis or forcing main effects and their respective interaction terms into the logistic regression model. Other variable combinations did not improve the fit of the model or indicate additional sources of confounding. The *P* value for Hosmer-Lemeshaw goodness-of-fit statistic of the final model was 0.86, indicating an overall good fit.

Table 2. Risk factors for disappearance of frogs based on univariate contingency tables and univariate logistic regression analyses

	Cases		Controls					
Variable	Exposed	Unexposed	Exposed	Unexposed	OR	95% CI[a]	*P* value[a]	
Crayfish present	32	73	12	93	3.40	1.64 , 7.06	<0.01	b c d
Non-native fish present	45	60	32	73	1.71	0.97 , 3.02	0.09	c d
Bullfrogs present	15	90	8	97	2.02	0.82 , 4.99	0.18	c d
Lentic water system	57	48	41	64	1.85	1.07 , 3.21	0.04	b c d
Median pH range 4-8	29	70	26	59	0.94	0.50 , 1.77	0.87	
Extant population nearby at 8 kilometers	59	46	77	28	0.46	0.46 , 0.83	0.01	b c
Extant population nearby at 6 kilometers	52	53	68	37	0.54	0.54 , 0.93	0.04	b c
Extant population nearby at 4 kilometers	40	65	62	43	0.43	0.43 , 0.74	<0.01	b c
Extant population nearby at 2 kilometers	18	87	46	59	0.27	0.14 , 0.50	<0.01	b c d
Disappearance nearby at 8 kilometers	55	50	24	81	3.70	2.05 , 6.70	<0.01	b c
Disappearance nearby at 6 kilometers	51	54	17	88	4.89	2.56 , 9.32	<0.01	b c d
Disappearance nearby at 4kilometers	44	61	8	97	8.70	3.90 , 20.00	<0.01	b c
Disappearance nearby at 2 kilometers	14	91	1	104	16.00	2.00 , 124.00	<0.01	b c
Northwest aspect (270-359 degrees)	58	47	42	63	1.85	1.07 , 3.20	0.04	b c d
Southern aspect (135-224 degrees)	19	86	20	85	0.94	0.47 , 1.88	1.00	
High soil available water capacity (AWC) (>3 " per square inch)	84	20	65	40	2.58	1.38 , 4.84	<0.01	b c d
Soil organic matter (SOM) > 1.2	53	51	53	52	1.02	0.59 1.75	1.00	
Elevation (range 171-2524 m)[e]					2.89[f]	2.88 , 2.89[f]	<0.01	b c d

OR= odds ratio; CI=confidence interval
a=exact confidence intervals and p values
b=Significantly ($p<0.05$) associated with disappearances
c=met screening criteria $p<0.25$ for multivariable analyses
d=examined in multivariable analysis
e=results based on univariate logistic regression results

f=Statistic calculated for risk associated with every one meter increase in elevation with a beta coefficient of 0.00212. Odds ratio and associated 95% CI are reported to reflect risk associated with every 500 meter increase in elevation, using the formulas $e^{500(\beta)}$, and $e^{500(\beta) \pm 1.96(SE)(500)(\beta)}$, respectively, where SE (standard error) =0.000405.

Table 3. Multivariable model of factors associated with ranid frog disappearance

Variable	**Beta Coefficient**	**SE**	**Adjusted OR**	**95% CI**	***P* value**
Intercept	-3.086	0.6247			
Elevation	0.002	0.0004	2.66[a]	2.66 , 2.67[a]	<0.01
Extant population nearby	-1.885	0.4248	0.15	0.07 , 0.35	<0.01
Disappearance nearby	1.463	0.4096	4.32	1.94 , 9.64	<0.01
Crayfish present	0.960	0.4602	2.61	1.06 , 6.44	0.04

Model reflects adjusted risk factors among 105 cases and 105 controls. Variables are listed in descending order of significance. SE=standard error; OR=odds ratio; CI=confidence interval

a=Statistic calculated for risk associated with every one meter increase in elevation with a beta coefficient of 0.00196. Odds ratio and associated 95% CI are reported to reflect risk associated with every 500 meter increase in elevation, using the formulas $e^{500(\beta),}$ and $e^{500(\beta) \pm 1.96(SE)(500)(\beta)}$, respectively, where SE (standard error) =0.000405.

3.4 Discussion

Studies of amphibian declines in naturally occurring populations have been limited by scarcity of quantitative data, repeatedly measured in multiple populations over time (Pechmann 1991; Blaustein et al. 1994). The AZGFD database provides longitudinal information over many years on a wide variety of frog populations. However, it does not represent a complete inventory or truly random sample of native Arizona ranid frogs. Survey effort has also varied over time. These imperfections are characteristic of observational data collected for real populations. Theory and methodology to deal with observational data and make appropriate conclusions about population health has been extensively developed in the field of epidemiology, primarily for humans (Rothman and Greenland 1998; Gordis 2000; Nelson et al.

2001), but also for animals (Martin et al. 1987; Thrusfield 1995). These methods provided a means to design an epidemiologic study based on the AZGFD surveys and identify factors associated with local frog disappearances.

The simplest epidemiologic study involves assembling a representative sample from those in the population without the disease of interest and following them forward over time to observe the incidence of a health outcome (Gordis 2000). Risk factors can be examined to see which best predict disease occurrence (Gordis 2000). While the AZGFD database does represent such a cohort of sites across Arizona, the periods of observation are not complete enough to have detected all local disappearances and intensity of observation differed between types of sites over time (Sredl 1997). In the future, additional data collected from this cohort may allow such an analysis. For existing data, a nested case-control study was used to select sites within the longitudinal database where a disappearance occurred and to then randomly select an equal number of controls from among sites with extant frog populations visited during time periods when case sites were detected (density sampling). This method controls for bias due to variation in monitoring intensity by balancing effects on cases and controls (Rothman and Greenland 1998) and allowed identification of risk factors for disappearances. By examining environmental and spatial variables individually and then in combination, the relationships between these variables as well as their associations with disappearances were explored. Multivariable logistic regression was used to allow simultaneous evaluation of several different potential risk factors (Hosmer and Lemeshow 2000).

Using this approach, risk of having a disappearance was significantly and substantially associated with increasing elevation, in both the univariate and multivariate analyses. Elevation was important in explaining the variation between cases and controls in an adjusted model, even when the status of nearby populations and presence of crayfish were included. Strong altitudinal associations are consistent with patterns of worldwide amphibian declines (Carey 1993; Berger et al. 1998; Collins and Storfer 2003; Lips et al. 2003a, 2004). Carey (1993) proposed that declines at higher elevations may be due to suppression of the frog's immune system at cooler temperatures, while simultaneously promoting growth of the infectious agent *B. dendrobatidis*. Alternative hypotheses for observed declines at high elevations include increased exposure to harmful ultra-violet rays that have detrimental effects on amphibian embryos or cause other sublethal effects (Blaustein 1994; Jablonski 1998; Blaustein and Belden 2003). Although the effects of ultra-violet radiation may be more significant on microhabitat than on landscape levels, no significant association between risk of disappearance and having a southern aspect, the slope receiving the most sunlight throughout the year, was found.

Three variables found to be significant in the univariate, but not in the adjusted analysis, were also associated with elevation. Although elevation better explained the pattern of disappearances in this dataset, aspect of slope, soil AWC and water type may offer insight into the complex mechanisms through which elevation-related declines operate. High elevation sites were most likely to have a northwestern aspect (270 to 359 degrees), the direction associated with increased risk in univariate results. All aspects were not equally represented in this dataset; most aspect data ranged from 1 to 40 degrees and from 280 to 360 degrees. Thus, sites that faced a northwestern direction were predominately compared to sites facing south. The aspect of a slope influences mesoclimate by affecting sunlight (heat and ultraviolet radiation) and wind exposure (Howland et al. 1997). Sites facing northwest may receive less radiant energy during winter and more northwesterly winds that occur in Arizona throughout most of the year (Douglas et al. 1993; Adams and Comrie 1997). Cooler temperatures may suppress the frog immune system (Carey 1993) and wind may further contribute to wind chill and evaporation and drying of sites (Sredl 1997). Prevailing wind patterns were linked to the decline of the Tarahumara frog (*R. tarahumarae*) in Southern Arizona (Hale et al. 1995).

Soil AWC, the amount of water available for plant use, was also associated with elevation. High AWC is a general indicator for sandy soils, which are less likely to retain standing water than clay-based soils (Lyon and Buckman 1950). Soil plays an important role in determining hydrological characteristics and may influence survival of pathogenic agents. Although environmental preferences of *B. dendrobatidis* are not yet known, distribution of other chytrid species is related to organic matter, which probably reflects the kinds of nutrients found in the soil (Lee 2000). Other disease causing pathogens are distributed according to soil type, including agents responsible for anthrax, botulism, clostridia, histoplasmosis, leptospirosis, and lyme disease (Martin et al. 1987; Guerra et al. 2002). Association of certain soil types geologically with elevation may have contributed to the strong risk associated with elevation.

At high elevation sites, water type was more likely to be slow moving/still water than flowing water (i.e. lentic as opposed to lotic). Lentic water type was only marginally associated with risk of disappearance in the univariate analysis. Thus, when controlling for elevation in the adjusted model, water type was no longer statistically significant. Strong representation of lentic habitat as human-made ponds and cattle tanks may represent lower quality, warm water habitat that dries quickly (Rosen et al. 1995; Belsky et al. 1999) may account for the univariate findings. As the volume of water decreases, frogs risk desiccation and come into closer contact with one another, increasing opportunity for disease spread. Lentic sites also reflect pockets of isolated, unconnected habitat that may have low immigration success and be prone to local extinctions (Harrison and Quinn 1989; Hanski and Gilpin 1991).

Another water characteristic, pH, which has been shown to affect growth of *B. dendrobatidis* in laboratory experiments (Piotrowski et al. 2004), was not significantly associated with disappearances. Water pH was evaluated using the median of multiple surveys. Since water pH may fluctuate from rainfall and snowmelt (Wigington 1990), soil pH from the STATGO data was also tested as a proxy for baseline water pH. These values did not correlate well with site-specific readings. This might be attributable to the poor resolution of STATGO soil data. Variation in soil and water pH for all sites through differing seasons should be determined to effectively evaluate associations between pH and risk of decline.

Elaborate, spatially-explicit frog population models, linking patterns of disappearances to metapopulation movements, incorporating effects of geographic barriers and watershed corridors, would provide a powerful means of testing hypotheses about disease transmission between sites. However, little is known about local and landscape-level metapopulation structures of these frogs, and higher resolution digital spatial data are needed to incorporate effects of local landscape characteristics. Using simple circles around disappearance sites to represent reported migration distances (Frost and Bagnara 1977; Rosen et al. 1996; Sredl and Jennings 2005), nearby disappearances and nearby extant frog populations were both independently associated with local disappearances, exerting opposite effects in the model. This pattern is consistent with a metapopulation framework of disease spread and animal movements. Experimentally, *B. dendrobatidis* has been transmitted between infected and susceptible frogs through direct physical contact and through contact with infected water (Nichols et al. 2001; Rachowicz and Vredenburg 2004). Increased dispersal ability between close patches of habitat (Lidiker and Caldwell 1982) would facilitate direct spread of pathogens. Spacing of habitat patches has been found to significantly affect infection prevalence in other species, with highest prevalence infections found among sites located close together (Grosholz 1993). Although data were sparse, the univariate results suggested that nearby disappearances at closer distances may substantially increase risk for a local disappearance.

Conversely, once a local disappearance has occurred, presence of a nearby extant population could be critical as a source for site repopulation. Metapopulation models predict breeding ponds will blink in and out of existence with colonization rates related to spatial arrangement of habitat patches (Marsh and Trenham 2001). In experimental pond-recolonization studies, sites closer to the initial source of amphibians maintained larger populations (Halley et al. 1996). These results suggest conservation efforts may need to focus on groups of populations that can infect or recolonize each other, rather than on individual sites.

Globally, introduced predators, competitors or disease reservoirs have been of concern as a factor in amphibian declines (Collins and Storfer 2003; Kats and Ferrer 2003; Mazzoni et al. 2003; Weldon et al. 2004). Crayfish were found to be an important risk factor for disappearance of native ranid frogs in both univariate and multivariate analyses, and inclusion of this variable greatly improved model fit. The importance of crayfish in the adjusted model indicates that the negative effects of crayfish persist, regardless of elevation, or the status of nearby populations. The spread of crayfish is newly recognized threat facing Arizona's aquatic systems. Crayfish are not native to Arizona, but two species, the northern crayfish (*Orconectes virilis*) and the red swamp crayfish (*Procambarus clarkii*), have been successfully introduced since the 1970's to aquatic systems through stocking with game fish, use as fish food, or dumping of bait buckets (Fernandez and Rosen 1996, Gamradt and Kats 1996, Taylor et al. 1996, Kats and Ferrer 2003). Native leopard frogs have been completely eliminated from some habitats when crayfish were introduced (AZGFD unpublished data, Fernandez and Rosen 1996). While acting as direct predators and competitors of frogs (Fernandez and Rosen 1996, Gamradt and Kats 1996), crayfish also reduce aquatic vegetation, habitat heterogeneity and protective cover (Fernandez and Rosen 1996). It is uncertain whether crayfish are implicated in disease spread. It has been proposed that *B. dendrobatidis* could persist or disperse on non-amphibian hosts, including crayfish (Daszak et al. 1999, Longecore et al. 1999). *B.*

dendrobatidis has shown an affinity for growth on keratin (Berger et al. 1998; Daszak et al. 1999; Longecore et al. 1999), a nutrient that is also found in crayfish exoskeleton. Additional research is needed to determine whether non-amphibian hosts serve as pathogen reservoirs.

The role that introduced, non-native crayfish may play in worldwide declines has received less attention than other introduced predators. The negative impact of introduced fish and bullfrogs has been well-documented in amphibian decline literature (Hayes and Jennings 1986; Rosen and Schwalbe 1995; Gambradt and Kats 1996; Lawler 1999; Kiesecker 2000; Matthews et al. 2001; Kats and Ferrer 2003), but their role in large scale disappearances is less clear (Clarkson and Rorabaugh 1989; Hedges 1993; Mahoney 1996, Hayes and Jennings 1986). Neither fish nor bullfrogs were significantly ($p<0.05$) associated with frog disappearances in this study, but did meet screening criteria for the adjusted analysis.

Among the 50 species of introduced fish in Arizona (Minckley 1973, Rinne and Minckley 1991), centrarchid fish (family *Centrarchidae*), mosquitofish (*Gambusia affinis*), trout (*Oncorhynchus mykiss* and *Salmo trutta)* and fathead minnows (*Pimephales promelas)* have been widely reported to negatively impact amphibians. These species of fish have been linked to decreased survival of amphibians (Goodsell and Kats 1999; Adams 2000; Gillispie 2001) through direct predation (Needham and Vestal 1938; Mullaly and Cunningham 1956; Rosen unpublished data), competition (Lawler et al. 1999) and may alter amphibian assemblages and abundance (Smith et al. 1999) and change ecological characteristics of the aquatic environment (Zimmer et al. 2001). Information on which species of introduced fish were present in a habitat was not always available in every survey. Therefore, all non-native species were evaluated as a single group. This may have affected the ability to differentiate between introduced fish that negatively impact amphibian assemblages versus those that have no effect. Occurrence of fish may also have been less important for disappearances in this study because presence of fish may represent an indicator variable of water permanency in Arizona. Thus, detrimental effects of introduced fish may be balanced against beneficial effects of permanent water habitat.

Assessment of the relationship between bullfrogs and risk of native ranid frog disappearance was hampered by the small number of sites with bullfrogs (only 23 sites in the final dataset had bullfrogs). Fifty-five total sites were not included in the final dataset because the site failed to meet eligibility criteria after surveys that failed to indicate the species of ranid were excluded. Some of these sites may have been inhabited by target species, but introduced bullfrogs, which are also classified as ranids, were known to populate the area. If these sites were not excluded from analysis, then factors associated with successful and unsuccessful bullfrog colonization, which may be very different than factors associated with native ranid disappearance, may have overshadowed the importance of risk estimates for native species and/or may have produced spurious associations. Exclusion of these sites, on the other hand, may have decreased the power to detect bullfrogs as a risk factor for native ranid disappearances. Additionally, 142 surveyed sites where bullfrogs were present during recent VES visits lacked historical records to determine if native ranids species were ever present. Although these sites were not included in the present study, they may represent historical native ranid frog localities now inhabited by bullfrogs.

Case-control studies are a widely used approach and offer many advantages in investigating disease risks. However, there were a number of challenges in applying these methods to an existing amphibian population database. Cases were characterized by at least two successive survey-years, under favorable conditions, where no native ranids were found. Control sites could have a single year when frogs were undetectable. While this was designed to separate "normal" fluctuations from more serious declines, disappearances where at least one frog migrated in quickly would be missed. This would, in general, misclassify some cases as controls and bias the associations towards the null. However, it could also emphasize factors that caused short-term disappearances to persist, for example, increasing the significance of a recolonization source. On the other hand, this case definition was conservative with regard to sites where no frogs were ever found, but which appeared to be suitable habitat. For more than half of AZGFD VES sites, frogs were never found during any visit. It is unknown whether these sites represent localities that were never inhabited or localities where disappearances occurred prior to VES surveys. In fact, Clarkson and Rorabaugh (1989) documented that 94% of historical *R. chiricahuensis* localities were uninhabited by the mid-1980s. Density sampling was used to assure that cases and controls were contemporaneous, but quantitative data from initial, early disappearances and control sites may have identified risk factors different than those that led to disappearances after the early 1990's.

Another limitation was that all native ranids were analyzed as single complex. These species are similar in appearance and ecologic needs and all except for one, *Rana tarahumarae*, were classified together as the *Rana pipiens* complex until the late 1960's when scientists subdivided this group into multiple species based on slight variations in genetics, vocalizations, appearance and breeding characteristics (Hillis 1988). However, differences in risk factors among species, which could be useful in conservation planning, could not be assessed.

3.5 Conclusions

This epidemiologic study found environmental and spatial variables associated with naturally occurring population declines of amphibians in Arizona. Results not only implicate the importance of one factor in the disappearance of ranid frogs, but emphasize the concurrent effects of four distinct factors. The multivariable model approach describes the relationship between risk factors by estimating the magnitude of effect of each individual variable, when the other factors are also included in the model. In addition to measuring the magnitude and significance of risk factors explored by other studies, the findings support metapopulation effects on both disease spread and subsequent repopulation. The association of crayfish with sites where frogs have disappeared further substantiates this species as a newly recognized threat, adding it to those considered as potentially harmful or as a source of infectious disease.

Findings from this study have increased our understanding of the risk factors associated with frog disappearances and will help guide future wildlife conservation management decisions. Results also illustrate the value of using epidemiologic methods to investigate population losses, providing an important foundation for future studies aiming to examine multiple risk factors associated with amphibian declines.

Chapter 4: Expanded Discussion

This study required adaptation of methods traditionally used in epidemiology to analyze data collected to describe amphibian host ecology and population dynamics. The simplest analysis, using all available data would lead to considerable biases due to the high degree of data censoring and irregular survey distribution through time. The use of a case-control study design allowed for examination of hypotheses that these data were not specifically designed to test. Case-control definitions had to be developed, criteria for inclusion in this study had to be defined, and variables had to be coded to specifically examine the hypotheses of interest. In this section these items along with study design rationale will be discussed in more detail to facilitate understanding of underlying assumptions and reasoning behind data adjustments and interpretation. Discussions are supplementary to the previously examined topics previously examined in section 3.4.

4.1 Study design rationale and limitations

The AZGFD amphibian survey provided unique historic documentation of the occurrence of ranid frogs in Arizona. It was designed as an inventory with the goal of visiting and describing as many sites as possible where frogs may live. When the first amphibian disappearance occurred in 1986, most sites had not been visited in several years, often a decade or more. During these early surveys disappearances may have gone undetected at some sites due to long time intervals between surveys. As knowledge of amphibian declines surfaced in the early 1990's AZGFD started intensifying survey efforts and visiting more localities to determine the current distribution of native ranids in Arizona. More complete coverage of the surveyed population between 1997 and 2002 captured shorter time intervals when sites converted from having known extant populations to becoming cases. Lack of uniform population monitoring through time introduced considerable irregularities in survey distribution and variations in the lengths of time that each site remained in the cohort.

Epidemiologic methods were designed, and are continually being refined, to enhance study of observational data from real populations. A case-control study, one of the most commonly used epidemiologic designs, was nested within the large, long-term AZGFD study. This design provided a means to equalize effects of non-uniform population monitoring using well-characterized quantitative methods for controlling confounding and biases (Rothman and Greenland 1998).

The confounding effects of time were controlled through density sampling. Frequency matching (the same ratios of cases to controls from a given year) was chosen over individual matching because AZGFD site selection methods were similar for sites visited in the same year. Exclusions of sites and surveys (further discussed in section 4.3) also facilitated with controlling biases. Finally, an adjusted analysis was used to identify multiple risk factors associated with disappearance, each controlled for the effects of the others.

Density sampling of cases and controls in this dynamic longitudinal dataset may have limited detection of time-related predictors of risk. However, many of the factors of interest in this study (i.e. elevation, water type, aspect, and soil variables) were not time dependent, while many of the potential biases were related to time (i.e.

changes in data collection frequency and general sampling locations). This was a challenging problem, making it more practical to use a case-control study design. Matching allowed these biases to be minimized.

Power limitations precluded evaluation of species-specific data. Compiling all species into one analysis enabled investigators to achieve maximum power to detect differences between cases and controls. Examined variables were assumed to not vary significantly between species. However certain factors are known to be more prominent among some species than others (i.e. *R. subaquavocalis*, *R. chiricahuensis*, and *R. pipiens* usually inhabit high elevation areas and may be over-represented among cases, where *R. yavapaiensis* typically inhabit lowland areas and may be more represented among controls). With the exception of *R. tarahumarae*, target species are similar in appearance and ecologic needs, such that they were classified into the *Rana pipiens* complex until the late 1960's when scientists subdivided this group into multiple species based on slight variations in genetics, vocalizations, appearance and breeding characteristics (Hillis 1988). *Rana tarahumarae* has been considered extirpated since the early 1980's and is only represented in a few of the sites, imposing little impact on results. As this database continues to grow, similar methods may be used to evaluate risk factors associated with species-specific disappearances.

A final limitation to this study design is that associations cannot be translated in to cause and effect (Rothman and Greenland 1998). Additional studies need to be done to determine specific causes of range reductions and disappearances in Arizona. All risk factors identified should be considered associations to help guide conservation management and aid scientists in further exploring factors related to amphibian declines.

4.2 Rationale for case and control definitions

Global amphibian population declines have been repeatedly characterized by mysterious disappearances (Clarkson and Rorabaugh 1989; Carey 1993; Mahoney 1996; Berger et al. 1998; Daszak et al. 1999; Stallard 2001; Lips et al. 2004). Similar patterns have been observed in Arizona where ranid frogs have suddenly disappeared from known localities and have never returned (Clarkson and Rorabaugh 1989; Sredl 1997). This characteristic pattern was the foundation for the development of case and control definitions. Many of the cases in this dataset were characterized by localities where frogs disappeared at a specified point in time and never returned, while controls were characterized by localities where frogs were always observed. However, some sites departed from this pattern and showed variable absent and present year classifications through time. These variable patterns referred to as "recovery events" were used to further define and develop case-control definitions.

Published literature has stated that resurvey effort should occur for longer than the time to maturity before a population is determined extinct (cited in Marsh and Trenham 2001) and resurveying sites for greater than 1 or 2 years after amphibians disappear yields improved estimates of distributional change (Skelly et al. 2002). Thus, sites with 3 or more successive year absences could be rationally categorized as a case. However, concern resided with whether to consider sites with 1 or 2 embedded and/or trailing absences as a case or a control.

All embedded, leading and trailing absent observations were summarized for cases and controls (Table 4). A single embedded absent in a site history did not substantiate being classified as a case, because amphibian populations are often characterized by dynamic fluctuations (Pechmann et al. 1991). Therefore, either final status or multiple embedded absences were used to develop case and control definitions.

Table 4 . Summary of recovery events occurring with two different classification methods

Classification method	**% sites with recovery event**	**% cases with recovery**	**% controls with recovery**
Case = At least 1 trailing absence	18% (n=448)	14% (n=229)	23% (n=224)
Case = At least 2 successive embedded absences	15% (n=324)	15% (n=117)	15% (n=207)

The selected classification method was also consistent with the biology and reproduction cycle of these ranid species. Native ranids in Arizona usually mature in one breeding season (frogs breed in the early spring, March-June, and will metamorphose into adults by late summer or early fall, August-November), but may overwinter if metamorphosing conditions are inoptimal. If frogs are not observed for two or more seasons, then one may be more confident that findings are not simply an artifact of missing overwintering tadpoles, but that frogs may actually have declined or disappeared.

When sites were classified based on their final survey, 23% of controls and 14% of cases experienced recovery (Table 4). If sites were classified based on multiple successive absent years, then only 15% of controls and 15% of cases experienced a recovery event. The latter method was chosen to be the best method due to decreased numbers of consecutive embedded absences among control groups. A number of sites eliminated in this process would have been classified as cases using the final observation method due to single trailing absences. However, inclusion of these data may have led to increased biases due to misclassification.

Recovery events may indicate unstable populations or sites with moderations in risk factors that facilitate recoveries after disappearance events. To examine the effects of recovery events, an individual regression model was fit to the data using the same variables as the final model, but sites experiencing recovery events were removed (Table 5). While the effects of elevation and crayfish remained the same (although presence of crayfish was not statistically significant), the effects of nearby disappearances and nearby extant populations became more important. Thorough examination of factors associated with population recoveries was beyond the scope of this project, but may be an important future step in the conservation of Arizona ranids.

Case and control definitions were supported by results of a VES validation study conducted by the AZGFD. Howland et. al. (1997) found it rare to not observe frogs at a site that were known or suspected to have frogs during the activity season. Even when frog populations were relatively small, presence of target species were detected in 93% (93/100) of all surveys. The harsh, desert environment may enhance

Table 5. Multivariable model of factors associated with ranid frog disappearance, excluding sites with recovery events

Variable	Beta Coefficient	SE	Adjusted OR	95% CI	*P* value
Intercept	-2.940	0.6836			
Elevation	0.002	0.0005	2.65[a]	2.65 , 2.67[a]	<0.01
Disappearance nearby	2.256	0.5731	9.54	3.10 , 29.34	<0.01
Extant population nearby	-2.337	0.5517	0.10	0.03 , 0.29	<0.01
Crayfish present	0.960	0.5500	2.61	0.89 , 7.68	0.08

Model reflects adjusted risk factors among 90 cases and 84 controls.
Variables are listed in descending order of significance.
SE=standard error; OR=odds ratio; CI=confidence interval
a=Statistic calculated for risk associated with every one meter increase in elevation with a beta coefficient of 0.00195. Odds ratio and associated 95% CI are reported to reflect risk associated with every 500 meter increase in elevation, using the formulas $e^{500(\beta),}$ and $e^{500(\beta) \pm 1.96(SE)(500)(\beta)}$, respectively, where SE (standard error) =0.000450, .

the detectability of water-dependent target species, as they are rarely found far from a water source. From this validation study, it was concluded that zero counts were significant. Frogs are probably not present or only present in low numbers if the VES failed to detect them. Case definitions in this study required two distinct, successive years in which frogs were not detected in any survey. Development of these conservative case definitions enabled investigators to best capture disappearance events while substantiating confidence in case and control definitions.

4.3 Effects of data exclusion

Site eligibility was guided by the need to make valid comparisons (Rothman and Greenland 1998), which called for severe restriction of admissible sites. The best comparisons between cases and control and most appropriate statistical analyses were achieved by restricting included data to sites and surveys that formed a relevant, contemporary dataset with similar geographic distributions.

Many of the eliminated sites (1224/2007) were surveyed habitat that never had any historical record of target species. These sites may represent localities that have never been inhabited, in which case their exclusion would be inconsequential. These localities may also represent places where disappearances occurred prior to VES surveys. If they represent localities where declines recently occurred, then introduced selection bias would be non-differential (since a site may serve as either a case or a control and the effects of time were controlled in this analysis), which would bias estimates towards the null. If they represent localities where disappearances occurred in the distant past (pre-1990), including them in this more contemporary dataset may potentially obscure risk factors related to recently known declines. This reasoning also may be applied to the 225 sites that were eliminated from analysis due to lack of current surveys. Factors implicated in declines and disappearances long ago may be different, necessitating separate data analyses. The possibility that these sites represent localities where frogs were simply undetected during site visits can not excluded, however, it may be unlikely (see discussion in section 4.2).

Fifty-five sites were not included because surveys did not indicate the species of ranid observed. Some of these sites may have been inhabited by target species, but bullfrogs (also classified as ranids) had also been observed and were known to populate the area. If these sites were not eliminated, then factors associated with successful bullfrog colonization [i.e. warmer, permanent bodies of water (Hayes and Jennings 1986; Kiesecker 2000)], may have been represented in the control pool and factors associated with bullfrog disappearance (i.e. habitat that dries quickly) may have been represented among cases. Not only could presence of bullfrogs in the dataset lessen the importance of risk estimates, but spurious associations could be produced. Exclusion of these sites, on the other hand, may have lessened the ability to detect bullfrogs as a risk factor for native ranid disappearances.

Data from particular site visits were also excluded if weather (during local droughts or cool weather) or other conditions (such as post-conservation releases) precluded reliable assessment of native frog presence. If these surveys were used to classify sites as cases or controls, then misclassification would likely have led to diminished odds ratio estimates and biases towards the null. Reduction in misclassification error can improve validity and efficiency of comparisons. Additional variables with weaker associations may surface or may become more important as misclassification is controlled (Rothman and Greenland 1998).

4.4 Rationale for variable coding

The best coding for variables in univariate and multivariable analyses was determined by identifying relationships and coding schemes that made statistical and biological sense, based on contrasts of potential options. All univariate results discussed in this section are summarized in Table 6.

4.4.1 Elevation

Elevation was examined as a categorical variable and a continuous variable. High versus low ($\geq$ 1525 meters versus < 1525 meters; OR= 6.55, $p<0.01$) elevation was examined with univariate techniques using the overall median for data division. Low, medium, and high elevations were also compared. Data were divided into three

Table 6. Univariate exploratory analyses used for variable coding

	Cases		Controls						
Variable	Exposed	Unexposed	Exposed	Unexposed	N total	F	OR	95% CI[a]	*P* value[a]
Elevation (meters)									
continuous					210	50.4			<.01 [b]
2 level variable:									
high (≥ 1525) v. low (< 1525)	75	30	29	76	210		6.55	3.59 , 11.96	<.01 [b]
3 level variable:									
high (≥1800) v. medium (1301-1799)	51	38	16	34	139		2.85	1.38 , 5.90	<.01 [b]
medium (1301-1799) v. low (≤1300)	38	16	34	55	143		3.84	1.86 , 7.92	<.01 [b]
high (≥1800) v. low (≤1300)	51	16	16	55	138		10.96	4.97 , 24.16	<.01 [b]
AWC (inches per square inch of soil)									
continuous					209	1.1			0.30
2 level variable									
high (>5) v. low (< or = 5)	55	49	52	53	209		1.14	0.66 , 1.97	0.68
3 level variable									
high (6.64 -10.37) v. medium (3.33 – 6.58)	33	41	32	33	139		0.83	0.43 , 1.62	0.61
medium (3.33 – 6.58) v. low (0.74 – 3.24)	41	30	33	40	144		1.66	0.86 , 3.20	0.14
high (6.64 -10.37) v. low (0.74 – 3.24)	33	30	32	40	135		1.38	0.70 , 2.71	0.39
3 level variable (*using USDA classification*)									
moderate (6.1-9) v. low (0-3)	42	42	44	21	149		0.48	0.24 , 0.94	0.04 [b]
low (3.1-6) v. very low (0-3)	42	20	21	40	123		4.00	1.89 , 8.47	<.01 [b]
moderate (6.1-9) v. very low (0-3)	42	20	44	40	146		1.90	0.96 , 3.78	0.08

Table 6. Univariate exploratory analyses used for variable coding (*continued*)

	Cases		Controls						
Variable	**Exposed**	**Unexposed**	**Exposed**	**Unexposed**	**N total**	**F**	**OR**	**95% CI**[a]	***P* value**[a]
SOM (% composition of organic matter)									
continuous					209	1.00			
3 level variable									
high (>1.5) v. medium (1-1.5)	30	39	33	39	141		0.91	0.47 , 1.77	0.87
medium (1-1.5) v. low (<1.5)	39	35	39	33	146		0.94	0.49 , 1.8	0.87
high (>1.5) v. low (<1.5)	30	35	33	33	131		0.86	0.43 , 1.7	0.73

OR= odds ratio; CI=confidence interval
a=exact p values and confidence intervals
b=Significantly (p<.05) associated with disappearances

approximately equal groups, representing high, medium, and low elevation values (≥1800m, 1301-1799 m, ≤ 1300 m). Contingency tables and odds ratios were used to examine the effects between high versus medium elevation (OR=2.85, $p<0.01$), and medium versus low elevation (OR=3.84, $p<0.01$), and high versus low elevation (OR=10.96, $p<0.01$). Although all categorical comparisons were highly significant, elevation was ultimately used as a continuous predictor, supported by a highly significant Analysis of Variance (ANOVA; F statistic=50.36, $p<0.01$). Evaluation as a continuous predictor conserved degrees of freedom in the final model and provided a referent comparison for future research in other geographic areas.

4.4.2 Water pH

Water pH was examined using both soil pH values derived from STATSGO soil data and serial pH readings from site visits. It was hypothesized that sites with a pH value between 4 and 8, the ideal pH for growth of the infectious pathogen *B. dendrobatidis* (Piotrowski et al. 2004), would be associated with an increased risk of disappearance compared to sites with pH values outside this range (higher pH values). Because site-specific data were sparse (i.e. missing data, few readings) and pH values can fluctuate during rainfall and snowmelt (Wigington 1990), using an alternative data source with standardized pH readings was sought. However, little overlap in pH values occurred between STATSGO digital soil pH and site-specific ranges. Therefore, data from site visits were used. To reduce the effects of outliers, median pH was calculated using the serial pH values recorded during each site visit to serve as an indicator for site pH. This variable was coded dichotomously according to whether it fell within or outside of the 4 to 8 range.

4.4.3 Soil available water capacity

Available water capacity (AWC), the amount of water a soil can store for use by plants, was examined using four different methods: 1) AWC as a continuous variable, which was not significant when evaluated with ANOVA (F=1.09, p=0.30). 2) High AWC was compared to low AWC by dividing data into two groups, above and below the median (5.16). Odds of disappearing did not significantly differ between areas of high and low AWC exposure (OR= 1.14, p=0.68). 3) Three approximately equal groups representing high, medium, and low AWC values were compared. Risk for disappearance was not significant in any of the comparisons. 4) three groups divided according to standard USDA published AWC classification tables (U.S. Department of Agriculture 1993; very low=0-3 inches per square inch, low= 3.1-6 inches per square inch and moderate= 6.1-9 inches per square inch), which was highly significant when comparing low versus very low AWC (OR=4.0, $p < 0.01$).

Dividing the data into groups by the USDA standard, published AWC classification tables was a better predictor of risk according to odds ratio estimates and provided easier interpretation of AWC values. To conserve degrees of freedom in the final model, AWC was collapsed into two groups, comparing higher AWC values (≥ 3.1 inches per square inch of soil) to very low soil AWC (0-3 inches per square inch of soil).

4.4.4 Soil organic matter

Soil organic matter (SOM), the percent of organic matter contributing to the soil, was first calculated for the entire soil profile and then normalized according to the percent composition of each polygon (U.S. Department of Agriculture 1995). However, this method yielded little variation in SOM values (0% to 1% soil organic matter content). Average soil organic content was recalculated for each site for only the top layer of soil and normalized according to the percent composition of the polygon. This method seemed more pertinent for the hypotheses of interest. If the pathogenic fungus, *B. dendrobatidis,* is distributed by organic matter, then only fungi in the top layer of soil where it could interface with frogs would be important. It also yielded sufficient variation in SOM across sites to test hypotheses of interest. However, it is important to note that the top layers of soil for which SOM was calculated were not all equal and ranged from 1 inch to 60 inches.

Dividing SOM data according to the standard USDA published classification tables created extremely uneven groupings. Therefore, associations were tested using two groups by median [>1.2 or < 1.2 (OR=1.2, p=0.47)] and three approximately equal groups comparing high to medium SOM (>1.5 to 1-1.5; OR = 0.91, p=0.87), and medium to low (1-1.5 to <1.5; OR=0.94, p=0.87), and high to low (>1.5 to <1; OR=0.86, p=0.73). SOM as a continuous predictor of risk was also evaluated, but was not significant. Since SOM was not a significant predictor of case-control status using any method, it was reported (and further checked in univariate analysis) as a two-level low versus high categorical variable to conserve degrees of freedom.

4.4.5 Aspect of slope

Aspect of slope was examined from several perspectives to identify any obscured relationships, however specific hypotheses of interest consisted of examining the role of sun and wind on the risk of declines. Each major direction (N, NE, E, SE, S, SW, W, NW) was assigned its appropriate aspect value (0, 45, 90, 135, 180, 225, 270, 315, respectively). Forty-five degrees was added and subtracted to values from each major direction to define a direction range. Each range was compared to all other aspect values. Concatenations of data were also compared (0-179 compared to 180-359 aspect values, 91-270 compared to 0-90 and 271-359). Additional relationships were not found. Southern and Northwestern aspect values were ultimately used to examine the role of sun and wind on the risk of declines. Southern aspect values (135 – 224 degrees) were coded as a risk and compared to data with all other aspect values (225-360 degrees, 1-223 degrees). Northwestern aspect values (270 – 359 degrees) were also evaluated and compared to data with all other aspect values (0-269 degrees).

4.5 Variables not evaluated

Several variables of interest were not evaluated due to data limitations. Some have reported declines most often occurring during cooler times of the year (Carey 1993; Sredl 2000; Bradley et al. 2002). Longitudinal studies, with repeated observation of the same extant populations through time, would be needed to capture these seasonal effects. Time-related, directional patterns of spreading have been reported for some declines (Laurance et al. 1996; Lips 1999, Lips et al. 2004). Potential time-related confounders in survey intensity prevented evaluation of time as a risk factor and necessitated control of this variable in the study design.

Additional soil parameters may be involved in the growth and persistence of chytrid or other pathogen species (Powell 1993). Limitations in availability of high-resolution digital datasets deterred evaluation of additional soil-related factors. Site-specific soil analysis is needed to draw better conclusions on how variations in soil patterns relate to disappearances of frogs.

Vegetation may be related to habitat quality and characteristics, such as ambient ground and water temperature, and exposure to sun and wind (Beschta et al. 1987, Platts 1991, Howland et al. 1997). Percent floating and percent perimeter vegetation (the two vegetation variables most often recorded during surveys that would impact frog habitat) were further evaluated to identify categorical variables characteristic of site vegetation. Vegetation was highly variable through time (greater than 50% changes in composition were often observed from survey to survey) and vegetation information was infrequently recorded during site visits, making it difficult to identify vegetation site characteristics.

Temporary water habitat may protect native species from introduced predators (Sredl and Wallace 2000). Temporary habitat may be approximated by lentic habitat, as many of the lentic sites are earthen cattle tanks that are known to periodically dry. Relationships between soil AWC and ability for soil to hold water are not completely clear, but low soil AWC may be considered a general indicator for clay-based soils (Lyon and Buckman 1950) which typically pool water as compared to sandy soils. Additional soil and weather variables are needed to compare with site-specific surveys to predict temporary and permanent ranid frog habitat.

There was considerable interest in examining the role of nearby disappearances and nearby extant populations along a spatial gradient, related to stream migration corridors and geographic barriers. It is hypothesized that sites along the same stream corridor would have a greater impact on each other than sites outside of the stream corridor. The development of elaborate, spatially-explicit frog population models are needed to incorporate the effects of geographic barriers and watershed corridors. High-resolution digital maps and knowledge of Arizona site-specific geography are needed to further evaluate these hypotheses.

Chapter 5: Conclusions

Epidemiologic research methods were useful for drawing conclusions from an amphibian monitoring database. These included methods to minimize inherent biases posed from irregular survey distributions and the high degree of data censoring. These types of methodologies will be useful for additional studies relating to amphibian declines as more datasets begin to lend themselves to dynamic analyses.

The observational methods used were meant to provide a “broad-brush” approach to identifying important risk factors associated with the decline of these amphibians. Important factors may be highlighted from multivariate analyses of large datasets that may not be realized in smaller studies. Results from multivariate and univariate analyses can be used to refine hypotheses about disappearances and in return focus future research, which may include examining risk factors among sites where known decline events have occurred, comparing factors between populations experiencing recovery events to populations that disappear and do not return, and further exploring spatial patterns between cases and controls.

One of the most important aspects of this study was obtaining useful information for the conservation of these protected, yet disappearing species of frogs. This is one of the first studies in amphibian decline research that will use findings to directly develop and refine conservation plans. It is hoped that more studies will follow this example and develop proactive strategies for large-scale population management. Specific plans are to be developed by AZGFD, but findings suggest that conservation efforts should focus on groups of populations at higher elevations. In addition, results support the hypothesis that crayfish have significantly impacted ranid populations in Arizona.

5.1 Conservation and management implications

The Arizona Game and Fish Department continues to actively monitor suitable frog habitat throughout the state in order to re-establish and protect native ranids. Findings from this study will benefit the conservation of these species by enabling wildlife managers to prioritize threats that were implicated in ranid disappearances. The degree to which these different factors work together in synergistic and antagonistic ways to produce widespread disappearances was also clarified.

Emphasis should be placed on focusing conservation efforts on a broad array of sites that may act as a metapopulation as opposed to a single site of interest. Dispersal facilitation between patches of habitat may promote beneficial repatriation of sites that previously went extinct. However, a cautious approach should be taken in facilitating dispersal, as findings also support the notion that nearby populations may have substantial negative effects on local populations. If neighboring sites are harboring diseases or accommodate non-native species that are known to negatively affect amphibian populations, such as crayfish, then care and vigilance are advised to ensure that the recognized threats do not spread. Thus, conservation plans should be developed with knowledge of the status of nearby populations that may either benefit or threaten the local population of interest. Wildlife managers may need to consider establishing populations in alternative localities to avoid any potential harmful effects of neighboring sites.

Re-introducing populations to historic distributions at lower elevations may offset the strong negative correlations between disappearances and altitudinal increases. If altitudinal effects are a result of decreased immune function or increase in pathogen virulence (Carey 1993), then establishment of populations in localities that lack these risk factors may benefit the overall population.

Crayfish continue to be an insidious threat facing Arizona aquatic systems and pose many challenges for conservationists. Mechanical removal of these species has not been an effective means of long-term exclusion (Sredl, pers. comm.). Current management practices by AZGFD include efforts to slow the rate of crayfish spread by installing barriers and trapping (Sredl, pers. comm.). Legislation has also been passed that makes it illegal to possess and transport live crayfish. However, additional methods of crayfish eradication may need to be developed and considered in order to prevent further spread into ranid habitat.

5.2 Suggestions for improving AZGFD surveys

Adding a variable indicative of site drying is one suggestion for facilitating future data retrieval. Drying of sites was documented in survey notes, but was not specifically summarized in the dataset. Pond size and drying may affect food supply, density of tadpoles, size at metamorphosis, (Carey and Alexander 2003) number and efficiency of predators (Semlistch 2000) and population stability (Sredl 1997). Specific documentation of this variable may reduce surveyor error and aid in developing quick summaries of non-permanent habitat.

An indicator variable identifying sites in the same canyon would be helpful for developing future spatial models. Geographic Information Systems' maps were not specific enough to permit easy identification of groups of frogs that may commonly intermingle as a metapopulation.

High-resolution digital soil data are needed statewide to more effectively evaluate soil characteristics. Alternatively, examining site-specific soil characteristics may be important for predicting environments favored by the chytrid fungus. Many chytrid species live in aquatic environments and moist soils (Powell 1993). Although *B. dendrobatidis* has not yet been isolated from the environment, it may grow saprophytically according to microhabitat characteristics (Daszak 1999). Understanding site-specific soil characteristics across a large geographic landscape could be useful in describing in microhabitat that may favor chytrid growth.

Focusing efforts on sites that do exhibit some degree of variation in frog populations through time may provide valuable information regarding factors pertaining to repopulation of sites after experiencing disappearance events.

5.3 Final Comments

This study would not have been possible without the diligent efforts of the Arizona Game and Fish Department in reviewing and inputting information from historical literature into a dynamic database, and continuously resurveying ranid frog habitat to update present distribution and monitor populations. The documenting of natural population disappearances and associated factors may continue to provide valuable insight as researchers learn more about the complex processes involved in worldwide amphibian declines.

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