PERSISTENCE AND PREVALENCE OF THE ENZOOTIC CHYTRID FUNGUS, *BATRACHOCYTRIUM DENDROBATIDIS*, IN RELATION TO AMPHIBIAN POPULATION DECLINE IN PANAMA

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ABSTRACT

The pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, causes population decline and extinction of numerous species of tropical, principally montane, amphibians. Is *B. dendrobatidis* an enzootic pathogen emerging as a disease, or rather a novel invasive pathogen infecting naïve amphibians? Are only high-elevation amphibians susceptible to the pathogen? If the chytrid is enzootic, then it may be in the environment before or after epidemic decline and may infect both montane and lowland amphibians as well as other organisms. To determine distribution of the pathogen and corresponding anuran abundance, I established eight research sites of varying elevations and stages of epidemic infection from west to east, ranging from 45 m to 1215 m elevation throughout Panama west of the Canal. Differential infection susceptibility among anurans was addressed in relation to three ecological factors: anuran body size, season and habitat. Prevalence and infection intensity of the chytrid were determined at all sites and for all factors using sensitive DNA-based RT-qPCR amplification. Amphibian populations at all elevations and stages of decline showed at least some degree of chytrid infection, and the chytrid was found on reptiles. In addition to presence of the pathogen, effects of the disease chytridiomycosis were variably seen at all elevations. Habitat and season did not seem to have a strong effect on infection prevalence and/or intensity, but frogs did appear to show greater infection at smaller anuran body sizes. All of the above results are suggestive of an enzootic pathogen and perhaps only the current epidemic of chytridiomycosis disease is novel. Since the infection can remain in frog communities at any elevation, habitat and season, can persist for long periods of time (up to 11 years), and can survive on non-amphibian hosts, the eventual reintroduction of captive-bred amphibians as a plausible management plan for conservation should be carefully examined.
RÉSUMÉ

Le champignon pathogène chytrid, *Batrachochytrium dendrobatidis*, cause la diminution et l’extinction de nombreuses populations d’amphibiens tropicaux, principalement dans les régions à haute altitude. Si le champignon est endémique, il peut rester dans l’environnement après le passage d’une épidémie, contaminant les amphibiens des hautes et basses terres, ainsi que les autres organismes. Les sites de recherche étaient établis à diverses élévations et à différents stades de l’épidémie, à l’ouest du canal de Panama, où l’état des populations d’amphibiens pouvait être examiné. L’hypersensibilité différentielle des grenouilles à la maladie était adressée pour trois facteurs: la taille des grenouilles, la saison et l’habitat. La prévalence et l’intensité de l’infection étaient déterminées pour chaque facteur à tous les sites en utilisant la technique du RT-qPCR. Les amphibiens de toutes les élévations et de tous les stades de l’épidémie ont montré au moins un niveau d’infection, indiquant la présence d’un pathogène endémique. Le chytrid a aussi été trouvé sur les reptiles. En plus de la présence du champignon, les symptômes de la maladie chytridiomycosis ont même été remarqués sur les grenouilles des terres basses. La prévalence de l’infection est restée similaire pour les forêts et les ruisseaux, et aussi pour les deux saisons, et plus élevée pour les petites grenouilles que pour les plus grosses. Puisque l’infection peut rester dans les communautés amphibienne à toutes altitudes, habitats et saisons, qu’elle peut persister pour longtemps (jusqu’à 11 ans), et qu’elle peut survivre sur d’autres organismes, la réintroduction éventuelle des amphibiens élevés en captivité n’est pas un moyen crédible pour leur conservation.
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PREFACE AND CONTRIBUTION OF AUTHORS

During my MSc. degree I studied many facets of the biology and epidemiology of the chytrid fungus, *Batrachochytrium dendrobatidis*, which variably causes the lethal disease chytridiomycosis in amphibians in Panama. Many elements of this thesis constitute original scholarship and advancement in the domain of chytridiomycosis research; because these elements are able to stand alone in separate publications I have chosen to write this thesis in manuscript-style. All novel findings are clearly stated in the final discussion.

For all manuscripts I am the first author, having done all field collection, data analysis and writing for the three manuscripts appearing in this thesis. Co-author contribution is identical for all manuscripts. Dr. David Green has financed the field work and RT-qPCR laboratory analyses done by Genome Quebec on all samples. Dr. Roberto Ibáñez was instrumental in helping me with experimental design of the field work conducted in Panama. In addition both Dr. Green and Dr. Ibáñez rigorously edited all manuscripts. Dr. Oris Sanjur provided academic advice for all preliminary laboratory work done in Panama and Dr. Eldredge Bermingham provided financial support for this work. I have not submitted any manuscripts to date and will only do so after final thesis submission. Manuscripts are thus currently organized for the thesis rather than for publication, and at this time are not as concise as they will appear in publications. Materials and Methods sections of each manuscript are only as detailed as necessary for each study; for publication these sections will be elaborated upon. References used in each manuscript are combined into one Literature Cited section at the end of the thesis but will be separated into individual manuscripts for publication.
CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW
INTRODUCTION

There is compelling quantitative evidence for a global decline of amphibian populations (Houlahan et al. 2000, Green 2003, La Marca et al. 2005, Lips et al. 2005b). Although habitat loss is the primary cause of amphibian declines in many parts of the world, amphibians are also declining in presumably pristine habitats where other “enigmatic” factors, like climate change, disease, and contamination, must also be at work (Stuart et al. 2004). Experimental data are limited with respect to the exact role played by these enigmatic factors, but complex synergistic interactions among the varying factors are suspected (Lips et al. 2005a).

The recently identified pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, has been implicated in amphibian declines, particularly those in high elevation riparian habitats in the Neotropics (Berger et al. 1998). *Batrachochytrium dendrobatidis* is an amphibian chytrid fungus that causes the disease chytridiomycosis and is widespread, having been found in captive and wild populations of amphibians on all continents (Morgan et al. 2007). Undoubtedly chytridiomycosis limits anuran survival as some amphibian species have suffered great declines in chytrid infected areas. Yet, other sympatric species have not (Lips et al. 2005a), indicating that certain species of amphibians may be more susceptible to the pathogen than others (Stuart et al. 2004). It has been argued that differential susceptibility may be due to amphibian immunosuppression caused by extraneous environmental factors (Daszak et al. 2004), including global warming (Pounds et al. 2006). There may be other complicating factors; for example, Burgin et al. (2005) found that frog mortality due to chytridiomycosis-like symptoms was not directly related to the chytrid infection but rather another unidentifiable pathogen, or pathogens. Differential host response to the chytrid makes it impossible to quantify a standard level of infection as the disease chytridiomycosis (Smith 2007). It has been suggested that *B. dendrobatidis* may exist as a non-lethal pathogen at low levels without causing the disease chytridiomycosis (Pounds et al. 2006), but much remains unknown about *B. dendrobatidis*, and this apparent feature of the pathogen requires further investigation.

Is *B. dendrobatidis* a novel pathogen impacting naïve amphibians, or an endemic pathogen that has become fatal to amphibians as a result of changing environmental
conditions? Why does it appear to more negatively impact highland amphibians? Exactly how is it transmitted? The current lack of robust information on the ecology of the chytrid, the epidemiology of the disease, and the ecologies and phylogenies of the amphibian species affected (Lips et al. 2005a) hampers attempts to answer these questions. We know little about post-epidemic persistence of the chytrid, including the extent to which it resides as a soil saprobe, although we do know that it can survive on non-amphibian hosts in the laboratory (Longcore et al. 1999, Johnson and Speare 2005). Recent evidence suggests that the ecology of the chytrid is complex and that the disease it causes may not be limited to cooler, high-elevation habitats in the tropics (Pounds et al. 2006, Woodhams et al. 2008a). Understanding these issues is critical for implementing conservation management plans for declining amphibian populations.

DISTRIBUTION OF B. DENDROBATIDIS

In Central and South America, B. dendrobatidis is causing widespread amphibian decline and extinction in a number of genera, including Dendrobates spp. and Atelopus spp. (La Marca et al. 2005, Lips et al. 2003, 2004, 2005a,b). In North America, chytridiomycosis is causing decline in populations of Bufo boreas (Muths et al. 2003) and Rana muscosa (Fellers et al. 2001). In Europe, chytrid is the prime agent of decline in the common midwife toad (Alytes obstetricans) of Spain (Bosch et al. 2001). Decline and local extinction of a number of Litoria spp. and Taudactylus spp. in Australia are also associated with B.dendrobatidis infection (Woodhams and Alford 2005). Common to all chytrid-induced amphibian declines worldwide is their occurrence in mountainous areas from middle (200m) to high elevation (up to 5348m) (Seimon et al. 2007).

Ron (2005) modeled the fundamental niche of B. dendrobatidis in the New World and predicted that its likely occurrence in any habitat type is extensive. Within the predicted regions, most habitats are in the highlands, with an annual precipitation of between 1500 and 2500 mm, and a mean annual temperature range between 20ºC and 25ºC (Ron 2005). Despite extensive distribution of suitable B. dendrobatidis habitat, the disease occurs in only a limited number of predicted localities, either because the pathogen has not yet colonized all areas, because sampling is poor or non-existent, or because pathogen presence does not always translate to presence of chytridiomycosis.
Using histology on museum specimens, researchers have dated the arrival of *B. dendrobatidis* to South America in 1980, in Ecuador (Ron et al. 2003), although amphibian declines were first documented from the “mid 1970’s” in Venezuela (Bonaccorso et al. 2000). In Central America, *B. dendrobatidis* was first detected on museum specimens from Costa Rica dating from 1986 (Puschendorf et al. 2006a), one year before any major catastrophic declines were noted for Costa Rican amphibians (Pounds and Crump 1994). In Panama, the disease has spread in a southeastward “wave” of infection at a rate of 30 km/year since 1996 (Lips et al. 2006). During the epizootic in 2004 in El Copé, presence of the chytrid increased rapidly to very high prevalence and was associated with massive die-offs of amphibians; amphibian density and species richness declined abruptly in 48 species of both diurnal and nocturnal stream communities following six consecutive years of high population abundances, and disease prevalence was reaching 50% in a year (Lips et al. 2006).

**ORIGIN OF *B. DENDROBATIDIS* AND CHYTRIDIOMYCOSIS**

There are currently two competing hypotheses regarding the emergence and origin of *B. dendrobatidis* as an amphibian pathogen: the novel pathogen hypothesis and the endemic pathogen hypothesis (Rachowicz et al. 2005). While the novel pathogen hypothesis suggests that the chytrid has recently spread into new geographical regions and is impacting naïve populations of amphibians, the endemic pathogen hypothesis suggests that *B. dendrobatidis* has always been present in amphibian populations but is only now developing as a pathogenic disease, likely as a result of compounding environmental factors (Rachowicz et al. 2005). Both hypotheses are accompanied by supporting evidence but no formal scientific consensus has been reached. Supporters of the novel pathogen hypothesis suggest that *B. dendrobatidis* originated in Africa where it existed as a stable endemic disease for 23 years before spreading to new areas through the world trade in *X. laevis* in the 1930’s (Weldon et al. 2004).

Suggestive of a recently emerged disease agent, population genetic studies of 35 different fungal strains found worldwide showed very little genetic variation (Morehouse et al. 2003). Despite genetic similarities, however, pathogenic virulence varies greatly with fungal strain, suggesting species divergence and likely particular genes coding for
virulence (Berger et al. 2005). Fortunately, recent completion (2007) of the *Batrachochytrium dendrobatidis* 7x Genome Assembly by the Fungal Genome Initiative at the Broad Institute has begun to shed some light on the chytrid’s origin as a pathogenic disease. For example, Morgan et al. (2007) found support for the novel pathogen hypothesis in the form of low genetic diversity and little correlation between fungal genotype and geography. However, some diverse, recombining populations of *B. dendrobatidis* were detected at a local scale (Morgan et al. 2007), suggesting that the chytrid may be enzootic at least in some areas.

Certainly there has been a recent push for substantiating the novel pathogen hypothesis (Skerratt et al. 2007, Lips et al. 2008), and although there appears to be more evidence for this hypothesis, many authors continue to dispute the claim that *B. dendrobatidis* is a novel pathogen. According to Alford et al. 2007, the novel pathogen hypothesis ignores the potential that chytrid persists in frog populations at very low levels as a natural non-lethal parasite, emerging as a lethal pathogen only in the advent of shifting environmental conditions. McCallum (2005) argues that the novel pathogen hypothesis is too simplistic and that likely *B. dendrobatidis* is an endemic pathogen that has become pathogenic due to co-interaction between one or more factors. McCallum (2005) also argues that it is implausible that a water-borne disease could variably affect such a large proportion of amphibians when so many potential competitors of *B. dendrobatidis* exist in a natural ecosystem. Since the low innocuous levels of infection characteristic of an endemic pathogen might easily be overlooked in an amphibian community due to error in infection diagnostic tools, some authors suggest that Real-Time quantitative PCR should be adopted as the primary tool for pathogen diagnostics since it can reliably detect pathogen intensities of less than 1.0 zoospores per sample (Kriger et al. 2007a).

According to disease dynamic theories, an African origin fails to address why there have been a number of recent outbreaks of chytridiomycosis in African anuran species sympatric with *X. laevis*, since they should have co-evolved successfully with the fungus (Rachowicz et al. 2005). Furthermore, the pathogen should have appeared in a deadly context many decades earlier when world trade of *X. laevis* first began (Rachowicz et al. 2005). In a study completed using museum specimens from the Canadian Museum of
Nature and the Redpath Museum, it was discovered that many amphibians from the Canadian provinces in as early as 1961 were infected with the chytrid, even though this disease had not yet been identified and frog declines had not been detected (Ouellet et al. 2005).

*Batrachochytrium dendrobatidis* can exist in a stable host-parasite relationship without causing pathogenic effects, since amphibians with no clinical signs of disease frequently carry light infections in the wild (Hanselmann et al. 2004, Retallick et al. 2004, McDonald et al. 2005). Thus, perhaps the chytrid is an enzootic pathogen while the disease chytridiomycosis is indeed novel and invasive. Or, perhaps the more virulent strains of the chytrid have emerged and spread invasively as a novel disease into areas where more benign strains have always existed (Morehouse et al. 2003). Despite genetic similarities in *B. dendrobatidis* strains, it is likely that chytrid effects would vary geographically; differential confounding environmental factors might cause an enzootic *B. dendrobatidis* strain to emerge as an epizootic in some areas, and be introduced as a novel pathogen in others (Wright et al. 2001). Perhaps alone neither emergence hypothesis can explain chytrid-related amphibian decline.

**ECOLOGY OF B. DENDROBATIDIS**

*Batrachochytrium dendrobatidis* was discovered in 1998 as the first vertebrate chytrid fungus (phylum Chytridiomycota), and was initially misidentified as a protozoan as it lacks the typical branched, filamentous structure characteristic of most fungi (Halliday 1998). The fungus is a water-borne pathogen with motile, flagellated zoospores, which make up the infective stage (Berger et al. 1999). Asexual reproduction results in the growth of a thallus and the production of a single zoosporangium (Berger et al. 2005b). The contents of the zoosporangia cleave into zoospores, which exit the zoosporangia through one or more discharge papillae (Berger et al. 2005b). Zoospores are nearly spherical, or somewhat ovate, and vary in size between 3-5 μm in diameter (Longcore et al. 1999). Under laboratory conditions, *B. dendrobatidis* is found to grow best at temperatures of approximately 23°C or less, which likely explains its prevalence in higher elevation habitats (Longcore et al. 1999). Chytrid zoospores have been shown to
die at incubation temperatures of 32º C and higher (Speare et al. 2004), and at 4ºC or lower (Longcore et al. 1999).

For many fungi, including other chytrids (e.g., Chytriomyces hyalinus), a sexual phase precedes the production of resistant sporangia, which aid in persistence and dispersal (Morgan et al. 2007). Because B. dendrobatidis has not been known to reproduce sexually, it has generally been assumed that spores resistant to freezing or desiccation are not produced (Berger et al. 1999). However, recent evidence suggests that a resistant stage may exist since sexual reproduction appears to occur (Morgan et al. 2007). Furthermore, researchers from the University of Perugia in Italy have recently reported a finding of potentially encysted zoospores that may embody a resting spore (Rosa et al. 2007). Under experimental conditions zoospores live for up to 4 weeks in tap water and as long as 7 weeks in lake water (Johnson and Speare 2003). More recently, Johnson and Speare (2005) found that the fungus could survive for 3 months in sterile moist river sand with no added nutrients. The zoospores can also attach and grow on moist bird feathers (Johnson and Speare 2005). The above findings, in combination with the fact that the fungus can be cultivated on boiled snake skin, suggest that the fungus most likely survives as a saprobe in the environment and can be transferred to new areas in the absence of an amphibian host (Longcore et al. 1999, Johnson and Speare 2005). However, as of yet there has been no documented evidence of such processes occurring under natural field conditions.

Although many aspects of chytrid ecology and pathology remain unclear, the fungus appears to prefer cooler, high-elevation, moist, riparian habitats (Berger et al. 1999, Longcore et al. 1999). The fungus invades and probably feeds on the keratin of amphibian skin (Daszak et al. 2003). Chytridiomycosis becomes fatal to amphibians either by interfering with cutaneous respiration and osmoregulation, by releasing toxic proteolytic enzymes or other active compounds that are absorbed through the permeable skin of the amphibian, and/or through facilitation of other microbial infections (Berger et al. 1998; Pessier et al. 1999). The disease is highly contagious and can be transmitted through physical contact with infected amphibians or water (Berger et al. 1999). Zoospores enter the animal's skin and grow in diameter and complexity to form new zoosporangia, which release zoospores into the environment after 4 or 5 days (Berger et
Zoospores are most commonly distributed intracellularly on the digits, venter and pelvic patch since these areas are commonly in contact with water and remain moist (Berger et al. 2005b).

Chytridiomycosis usually becomes severe only in adult amphibians, although larvae can harbour the chytrid in their keratinised mouthparts (Halliday 1998; Berger et al. 1999). Superficial diagnosis of chytridiomycosis is problematic as symptoms are numerous and variable and resemble symptoms of other amphibian diseases. Generally, amphibians showing severe infection exhibit a great deal of epidermal hyperplasia and hyperkeratosis which results in the sloughing of skin, lethargy, hyperpigmentation, extreme buoyancy, loss of slime layer, and often death (Berger et al. 1999). Death from *B. dendrobatidis* occurs within 10 – 47 days following initial infection (Berger et al. 1999). Chytridiomycosis does not always lead to death, however, and infected individuals in laboratory experiments have shown full recovery (Davidson et al. 2003). Amphibians severely infected with the chytrid often show signs of other bacterial invasions as well; additional pathogens may arrive due to initial infection by the chytrid (Bradley et al. 2002), or the presence of other pathogens may encourage *B. dendrobatidis* growth (Burgin et al. 2005). The severity of infection is known to vary from species to species, and even individuals within a species often show a high variation in pathology and susceptibility, at least under laboratory conditions (Davidson et al. 2003). This topic will be explored in greater detail under the subheading Host Responses to *B. dendrobatidis*.

**INFECTION DIAGNOSIS**

Diagnosis of *B. dendrobatidis* is done via a variety of laboratory techniques. Histology, originally the widespread tool for pathogen diagnosis, has since been replaced by more sensitive techniques, in the form of DNA-based assays using Conventional PCR (Annis et al. 2004), and even more recently, Real-Time Quantitative PCR (Boyle et al. 2004). Histology is done through the examination of tissue under a light microscope using transmission electron microscopy, whereby the appearance of chytrid thalli and zoospores denotes a positive diagnosis (Longcore et al. 1999). Although this technique is not as sensitive as either of the DNA-based assays, it is unlikely to detect light infections and so
may be better at diagnosing the chytrid as a disease (Smith 2007). Conventional PCR, although significantly more sensitive than histology, still only detects a baseline of 10 zoospore equivalents per skin swab sample (Annis et al. 2004), and low-level non-lethal infections are entirely overlooked. Kriger et al. (2007a) argue that the most reliable technique of \textit{B. dendrobatidis} diagnosis is Real-Time Quantitative PCR, since detection sensitivity is very high (1+ zoospores per skin swab sample) and zoospore numbers can be quantified. Because RT-qPCR analysis is done on epidermal skin swabs, it can be considered non-invasive and thus addresses ethical issues associated with toe-clipping for histology (May 2004: McCarthy and Parris 2004). Recently Kriger et al. (2006b) presented a more cost-efficient protocol; until then, this technique had been financially inaccessible to many researchers.

**HOST RESPONSES TO \textit{B. DENDROBATIDIS}**

\textit{Batrachochytrium dendrobatidis} is a generalist pathogen, potentially infective to all species of amphibians (Woodhams et al. 2006a). In both Panama and Australia, declining species have a high aquatic index (aquatic larvae, living in riparian habitat) whereas persisting species have lower aquatic indices (direct-developing, living in terrestrial leaf-litter habitat) (Lips et al. 2003, Woodhams and Alford 2005). In Eastern Australia, prevalence of the fungus is also known to be much higher in stream-breeding amphibians than in terrestrial or ephemeral breeders (Kriger and Hero 2007). Conversely, in one study conducted in Central Spain, the species most affected by chytridiomycosis were the most terrestrial of species (Bosch et al. 2007). Elevational range also contributes to the probability of decline, but is as much an aspect of host response as chytrid ecology since amphibian endemism is highest at higher elevations (Campbell 1999). Endemic species usually show less genetic variation (Waller et al. 1987), and are more likely to decline in the advent of environmental change because they are more specialized and more ecologically restricted than widespread species (Lips et al. 2003). Certainly some species of amphibians are more susceptible to environmental contamination and anthropogenic change than are others, and these factors influence disease susceptibility (Blaustein and Belden 2003). Large species are more likely to decline than small species at high elevations and less likely to decline at lower elevations; amphibians of all sizes with
broad elevational ranges do not decline (Lips et al. 2003), likely due to greater genetic variation.

Although the effects of host genetic variation on susceptibility to chytridiomycosis remain relatively unknown, genetic diversity buffers invertebrate and vertebrate populations against many widespread epidemics (Altizer et al. 2003). Pathogens represent powerful selective agents in natural populations, and can cause major shifts in the genetic composition of their host population on short time scales, as seen in invertebrates (Little and Ebert 1999, 2001). Among vertebrates, the major histocompatibility complex (MHC) is one of the most important determinants of immune defence, and patterns of extreme polymorphism at MHC Class I and II provide strong evidence for balancing selection mediated by infectious agents (Altizer et al. 2003). The implications of amphibian population genetics and phylogenetics are great with respect to conservation of amphibians in the face of this pathogen, and will be discussed in more detail under the subheading Conservation Implications.

Differential behaviours between species might also affect the prevalence of the chytrid (Retallick et al. 2004; Woodhams and Alford 2005). Some amphibians engage in lengthy amplexus or other social activities that promote physical contact, which may also increase the probability of chytrid infection and transmission (Green, Pers. Comm., 2005). In at least one species, *Eleutherodactylus coqui*, subadults are found to perch closer to the forest floor than are adults (Beard et al. 2003, Beard and O’Neill 2005), which may make them more susceptible to a water-borne pathogen like *B. dendrobatidis*. Irrespective of behavioural differences between life stages, body size may play a role in differential chytrid infection; indeed Kriger et al. (2007b) found that small *Litoria lesueuri* in Australia showed a higher prevalence and carried more intense infections than larger frogs, suggesting that frogs either outgrow infection, have infection-stunted growth, or succumb to pathogen-induced mortality.

Woodhams and Alford (2005) found that some species show differential use of habitats depending on the time of year, increasingly choosing aquatic habitats over other habitat types in drier months in Australia. Congregation of anurans in wet areas during the dry season could potentially facilitate disease spread (Ibáñez, Pers. Comm., 2006). Seasonality in itself has been shown to affect the prevalence and pathogenicity of
chytridiomycosis in tropical regions, with cooler temperatures causing increased mortality (Aplin and Kirkpatrick 2000, Berger et al. 2004) and warmer temperatures ridding amphibians of infections (Krige and Hero 2006).

There is recent evidence that co-interaction between two or more pathogens can have major effects on the host response to a particular pathogen by causing cross-regulation, where there is a trade-off in immune response (Ezenwa, Pers. Comm., 2007). Although a very new concept which has not been applied to many vertebrates, the potential for cross-regulation in amphibians infected with multiple pathogens seems likely and certainly many researchers have noted the presence of a number of other pathogens in frogs infected by *B. dendrobatidis* (Burgin et al. 2005). It logically follows that infections by other pathogens in such individuals could cause differential immune responses to *B. dendrobatidis*.

Amphibians have effective immunities against *B. dendrobatidis* in the form of antimicrobial peptides that combat against bacterial, viral and fungal infections (Rollins-Smith et al. 2003, 2005; Rollins-Smith and Conlon 2005). However, the number and type of antimicrobial peptides present in the dermal granular glands of amphibians differ between species (Amiche et al. 1999) and can help to further explain how disease effects caused by the chytrid are so widely variable in amphibians. Under laboratory conditions, most amphibian species are capable of ridding themselves of at least low concentrations of *B. dendrobatidis* following experimental injection, and Ranid frogs are particularly successful (Rollins-Smith et al. 2003, 2005; Rollins-Smith and Conlon 2005).

A recent laboratory study by Rollins-Smith and Conlon (2005) showed that *Litoria spp.* in Australia have potent antimicrobial peptides but are still experiencing massive chytrid-induced decline. Thus environmental factors likely have a profound effect on the synthesis and secretion of antimicrobial peptides, as exemplified in *Rana sylvatica*, which does not produce any antimicrobial peptides at very cold temperatures (Rollins-Smith et al. 2005). Unfortunately, to date all studies on the effectiveness of antimicrobial peptide defences in combating *B. dendrobatidis* have been done *in vitro*, and the link between *in vitro* peptide effectiveness and protection in the wild is tentative (Woodhams et al. 2006b). Recently, Woodhams et al. (2006a) found that they could predict disease susceptibility of certain species of anurans based entirely on the composition of their
antimicrobial peptides. Interestingly, individuals within a species showed huge variation in their antimicrobial peptide mixtures (Woodhams et al. 2006a).

The differential possession of certain high potency antimicrobial skin peptides should be highly selected for in the wild, and indeed numerous authors have reported apparent co-evolution between host and pathogen (Retallick et al. 2004, McDonald et al. 2005, Briggs et al. 2005). Retallick et al. (2004) presented the first quantitative evidence for endemic infection in *Taudactylus eungellensis* 5 years post-decline, McDonald et al. (2005) documented chytrid enzootism in populations of *Litoria genimaculata* in Australia post-decline, and Briggs et al. (2005) noted a similar phenomenon in *Rana muscosa* in North America. In all of these cases, chytridiomycosis resulted in near disappearance of the amphibian species but the surviving remnant populations successfully returned to pre-decline numbers with individuals harbouring stable chytrid infections. Co-evolution has yet to be detected in Neotropical amphibian populations.

RESERVOIR HOSTS AND CARRIERS

According to the Food and Agriculture Organization of the United Nations, a reservoir host is defined as: “an animal species which carries a pathogen without detriment to itself and serves as a source of infection”. There may be a number of reservoir hosts that play important roles in the dissemination of chytridiomycosis, and certainly many amphibian species are known to carry the infection without succumbing to disease. The rapid rate of disease spread in Panama (30 km/year) suggests that non-amphibian vectors or reservoir hosts are likely; regardless, many amphibian reservoir hosts have still been posited. Amphibian dispersal in itself is poorly understood, although it is known that some anurans in temperate regions disperse up to 13 km/year and may disperse even further (Smith and Green 2005). In Australia the dispersal rate of *Bufo marinus* is about 27 km/year (Freeland and Martin 1985), similar to the rate of chytridiomycosis disease spread in Panama. Although dispersal rates of most Neotropical frog species still remains a mystery, dispersal of the pathogen by amphibians, and in particular *B. marinus*, must not be ruled out. However, *B. marinus* in Australia has not reached its ecological limits and has no predators, and so likely its dispersal is faster and further in Australia than in Central America (Green, Pers. Comm., 2007).
Indeed, the bullfrog (*Rana catesbeiana*), marine toad (*Bufo marinus*), African clawed frog (*Xenopus laevis*) and most recently, *Eleutherodactylus coqui*, have all been suggested to be potential reservoir hosts for the disease in Neotropical America. All of these species are known to harbour high infections of *B. dendrobatidis* but are not experiencing any related decline (Berger et al. 1999; Daszak et al. 2004; Weldon et al. 2004; Beard and O’Neill 2005), with the exception of *E. coqui* at high elevations in its native range in Puerto Rico (Burrowes et al. 2004). *Rana catesbeiana, B. marinus, X. laevis* and *E. coqui* are all broadly ranging species that inhabit a wide-range of habitat types, from pristine, undisturbed habitats to habitats that have been highly disturbed by human activity. Moreover, humans have either advertently or inadvertently introduced all four of these species globally to non-native parts of the world through commercial activities. Low elevation anurans may also be culpable, harbouring infection and subsequently transporting it to susceptible higher elevation populations through dispersal (Puschendorf et al. 2006a).

Aquatic frog larvae have been implicated as important reservoir hosts by a number of scientists (eg. Daszak et al. 2003, Blaustein et al. 2005). In fact, larvae may exist as a bridge between seasons in more temperate regions where some tadpoles over-winter (Bradley et al. 2002). Although chytrid was known to cause deformities in keratinised mouthparts of larvae, it was only recently that Blaustein et al. (2005) discovered that the larvae of some species exhibit increased mortality due to chytridiomycosis. However, there are many species of anurans that have direct development and therefore no aquatic larvae stage but are still known to succumb to chytrid infection (Burrowes et al. 2004, Lips et al. 2003); chytrid-induced decline in such species are usually less than those in species having aquatic larvae (Lips et al. 2005a).

Davidson et al. (2003) demonstrated that the infection could be transmitted between different orders, like Ambystomatid salamanders and Ranid frogs, alluding to the potential of other aquatic invertebrates and vertebrates as potential hosts. Aquatic cold-blooded vertebrates with keratinised structures, such as fish or reptiles, have been suggested as potential carriers, particularly introduced exotic fish species such as rainbow trout (*Oncorhynchus mykiss*) or goldfish (*Carassius auratus*) (Berger et al. 1998; Lips et al. 2003). Experimental and genetic evidence show that the transfer of pathogens
between fish and amphibians is possible (Mao et al. 1999; Kiesecker et al. 2001a). Certainly some researchers have hinted to the possibility that fish may serve as reservoir hosts of the chytrid as well (eg. Gillespie and Hero 1999, Lips et al. 2005a). Freshwater shrimp may harbour *B. dendrobatidis* infection and maintain the pathogen in an aquatic environment (Rowley et al. 2006), and it follows that a wide range of aquatic organisms might be capable of such.

**ENVIRONMENTAL FACTORS**

Human-induced environmental changes have been occurring at an unprecedented rate over the last century and are likely impacting amphibians, perhaps in combination with disease effects. According to Rachowicz et al. (2005), changes in environmental factors could have two outcomes: 1) provide a more favourable environment for the pathogen itself, perhaps through the elimination of a previous competitor, or 2) cause the host species to be more susceptible to the disease because environmental conditions are acting as sublethal stressors that are compromising amphibian immunities. Certainly in the lab experiments have shown that amphibians show increased susceptibility to disease due to immunosuppression (eg. Kiesecker et al. 2001b).

Among the most often cited environmental problems are global climate change, contamination via air and/or water pollution, and increased UV-B radiation. All of these factors have been suggested as potential amphibian stressors that could result in immunosuppression and consequently *B. dendrobatidis* infection (Bosch et al. 2001; Lips et al. 2003; Muths et al. 2003). Global climate change has been a research focus, as its effects on organisms are being seen worldwide, especially in the form of changing weather patterns such as El Niño (Glynn 1990). Nicholls (1993) found that the incidence of a number of both vector-borne and water-borne diseases increased following the 1982-83 El Niño-Southern Oscillation event. Pounds and Crump (1994) and Pounds et al. (1999) proposed that the chytrid-induced extinction of several species of frogs and toads in Monteverde, Costa Rica, was a result of abnormal climate patterns following the 1986-87 El Niño event that resulted in dry-season mist reduction causing stress and/or behavioural changes in amphibians that increased their infection susceptibility. However, El Niño events do not explain why chytridiomycosis is most severe in high-elevation
cloud forests, because these habitats rarely experience extreme reduction in dry season mist. Recently Pounds et al. (2006) elucidated this phenomenon by presenting evidence that increased cloud cover due to global warming encourages the spread and pathogenicity of *B. dendrobatidis* through a cycle of daytime cooling and nighttime warming that creates a very favourable climatic environment for the fungus (the “chytrid-thermal-optimum” hypothesis). Bosch et al. (2007) substantiated this hypothesis through their identification of a link between climate change and chytridiomycosis outbreaks in Central Spain. There has been some recent criticism of the hypothesis proposed by Pounds et al. (2006), as some authors question the likelihood of climate-related declines occurring solely through a single pathogen disease outbreak (Alford et al. 2007, Rosa et al. 2007).

Amphibians at lower elevations should also experience increased stress levels due to climatic changes, but there is no evidence for this in the literature, even though the chytrid is now known to be present at lower elevations and may be lethal to at least some individuals or species there (Oliveira de Queiroz Carnaval et al. 2006, Puschendorf et al. 2006a, 2006b, Woodhams et al., 2008b). Climatic changes that result in exposure to low humidity and higher temperatures may be beneficial to lowland amphibians in areas of infection since infections are lethal only if there is enough moisture and appropriate temperatures for zoospores to survive and re-infect their hosts (La Marca et al. 2005, Pounds et al. 2006). However, recent evidence suggests that *B. dendrobatidis* has a more complex ecology than was originally thought and appears to do well at a variety of temperatures and thus elevations; the effects of variable environmental temperatures are likely to be complex (Pounds et al. 2006, Woodhams et al., 2008a). Furthermore, all research into ecology of the chytrid has been done in the laboratory, and the role played by temperature in limiting the chytrid’s distribution in the field requires re-evaluation. The conclusion that *B. dendrobatidis* does not cause declines at lower elevations is not justifiable since disease outbreaks in the lowlands could be easily overlooked due to other anthropogenic decline factors present (Wright et al. 2001). Inherently there is a scientific bias, as declines in “pristine” areas are easier to quantify (Wright et al. 2001).

Environmental contamination and its influence on disease pathogenicity are even more obscure. In Spain, prevalence of the chytrid increased in aquatic areas where lower
than usual water pH was detected, perhaps because the change acted as a stress factor and increased amphibian susceptibility (Bosch et al. 2001). A change in water pH could actually make an environment more favourable to the fungus, as water pH is known to affect the epidemiology of chytrid blooms (Sparrows 1968; Berger et al. 1999). Chemical contamination in the form of pesticides also causes increased susceptibility to disease in some amphibians, including Woodhouse’s toads (*Bufo woodhousii*) (Taylor et al. 1999). Indeed many chemicals are widely used in agriculture and mining in Latin America (La Marca et al. 2005), and although contamination might be expected to be greater at lower elevations, contaminants transported atmospherically at a local scale would have the potential to affect amphibians in remote, relatively undisturbed environments of high precipitation (Pounds and Crump 1994; Blaustein et al. 2003). However, no authors have reported abnormal levels of contamination in high elevation streams in the Neotropics where chytrid-induced decline is occurring, and research is just beginning on the deleterious effects of these chemicals on amphibian populations over the long-term (Izaguirre et al. 2000; Blaustein et al. 2003; Young et al. 2004).

Increased UV-B radiation due to ozone depletion has been implicated in amphibian declines, and in particular is known to increase mortality and deformities at embryonic stages (Lizana and Pedraza 1998). Kiesecker and Blaustein (1995) also found that UV-B damage could act synergistically with other factors to weaken amphibian immune systems. Information from remote sensing indicates that levels of UV-B radiation have risen significantly (especially since 1979) in both tropical and temperate regions, although information regarding changes in UV radiation at the local-scale cannot be gleaned using this method (Middleton et al. 2001). It is unclear whether UV-B damage could have an effect on the susceptibility of high-elevation amphibians to chytridiomycosis since the almost continuous cloud and canopy cover characteristic of their habitat should limit penetration of the sun’s damaging UV-B rays. However, some tropical high-elevation species do frequent light gaps (Blaustein and Kiesecker, Pers. Obs., 2002), and Lips (Pers. Obs. 2002) reports that egg clutches of *H. calypsa* in Costa Rica are exposed to sunlight and incidental radiation during their developmental period. Chytrid-infected amphibians often exhibit unusual sun-basking behaviour, perhaps as an adaptation to infection, and although this may decrease zoospore load (Woodhams et al. 2003,
Retallick et al. 2004), it could potentially worsen the infection if UV-B damage indeed decreases the immune response of amphibians.

QUANTIFYING AMPHIBIAN DECLINES

Our lack of knowledge about the animals succumbing to decline hampers our understanding of the global amphibian population decline phenomenon (Lips et al. 2005b). Very few long-term population studies of any species have been done, thus compromising our ability to accurately detect declines since confident detection of a “decline” depends on the intensity and duration of investigation (Wright et al. 2001). Especially in tropical, pristine areas, a lack of complete biological surveys (Lips et al. 2005a) could result in unreported or over-reported decline. In fact, numerous authors have noted that amphibians often exist as metapopulations and show a dynamically variable pattern of occupancy across different patches (e.g. Green 2003, Storfer 2003). Metapopulation dynamics suggest that populations in a given patch are more likely to decline in a given period of time than increase (Alford and Richards 1999), and traditional monitoring programs are more likely to detect patch extinction than patch colonization (McCallum 2005). Due to the ecologically inherent instability of amphibian populations, controversy often abounds regarding whether or not an amphibian decline is underway (Wright et al. 2001). Because of variable species responses to the same decline factors, it is not only hard to report on declines but also to identify causative agents (Blaustein and Kiesecker 2002). Our inability to quantify declines and concretely identify decline agents makes it difficult to implement proper conservation management programs.

CONSERVATION IMPLICATIONS

Like many environmental and conservation-based issues, the amphibian population decline phenomenon has received much scientific attention and debate, but the development of management programs has lagged behind. Lips et al. (2005a) outline a number of options for prioritizing conservation efforts, and many of these are in the form of increased research into the area of ecology of both the chytrid and its hosts. We must gain a greater understanding of the ecology of the fungus under natural conditions,
including its genetics, distribution along elevational and latitudinal gradients, how and where it survives, how long it persists in the environment, and how it moves between populations (Lips et al. 2005a). Complete amphibian biotic surveys are required for virtually all amphibian species in the Neotropics in order to increase our knowledge of population size, fluctuations and demography (Lips et al. 2005a). Special attention is needed to manage and decrease the spread of exotic species and pathogens from infected to non-infected areas (Ron 2005). Park wardens should develop educational programs to prevent the spread of chytridiomycosis by residents and tourists (Lips et al. 2005a), and scientists can do the same by disinfecting all field equipment with a 10% Bleach solution when moving between research sites.

*Ex situ* captive breeding of amphibians is currently underway in many parts of the world, and the endangered golden frog (*Atelopus zeteki*), endemic to Panama, is being bred successfully at The National Amphibian Conservation Centre at the Detroit Zoo (Lips et al. 2005a). Currently the only option believed available for amphibian conservation in the short-term is in the form of *ex situ* captive breeding programs, and experts from the CBSG/WAZA are promoting this worldwide initiative (Zippel et al. 2006). *Ex situ* breeding of amphibians has been criticized because it is unclear whether future reintroduction is possible given our current lack of knowledge about chytrid ecology. Unnatural size reduction of wild populations through removal for such programs will decrease genetic variability and hence disease resistance in the wild, and animals bred in chytrid-free areas in captivity will be bred without disease resistance (Altizer et al. 2003). Under these circumstances reintroduction will surely fail if the pathogen remains in the environment. Captive breeding should be supplementary to increasingly rigorous scientific research into additional conservation programs.

**THESIS OBJECTIVES**

Numerous gaps exist in the literature regarding the pathogenic chytrid fungus and its variable effects on anuran hosts. In particular, much debate abounds regarding the mechanism by which the pathogen has emerged as an epidemic disease, and we are still not sure to what extent the pathogen exists and causes host damage at lower elevations. Conflicting results are present in the literature regarding the effects of season, habitat and
body size on pathogen prevalence and intensity, and it is not even clear whether presence of the pathogen *B. dendrobatidis* invariably translates to presence of the disease chytridiomycosis. Furthermore, a great deal of evidence points to the possibility of non-amphibian hosts or vectors playing a role in the dissemination of the pathogen and/or disease, but this has not been substantiated under natural field conditions. The overall goals of this thesis are thus to put together some important pieces of this conservation puzzle, including: further substantiating the novel or endemic pathogen hypothesis through a thorough quantification of *B. dendrobatidis* occurrence throughout Panama; determining the extent to which pathogen and disease are present at lower elevations; addressing the roles that habitat, season and anuran body size play in pathogen and disease prevalence; and determining the extent to which the chytrid is present on non-amphibians. There is an urgent need for more research and a better understanding of how the above factors relate to amphibian decline, since the efficacy of conservation efforts are highly dependent on a greater understanding of these issues.

To address these overarching goals, I surveyed abundance of all species of frogs and reptiles of eight sites of varying elevations and stages of epidemic infection in Panama, during both wet and dry seasons, in both forest and stream habitats, and for all body sizes, and took swab samples for infection diagnosis. Using this study design I address a series of hypotheses in this thesis, and have organized my results into three separate manuscripts that appear as Chapters 3 through 5.

**Chapter Three:**

- **Hypothesis No. 1:** If there is an elevational component to the distribution of the chytrid and/or the prevalence of chytridiomycosis, then both the occurrence of *B. dendrobatidis* and prevalence of the disease will differ among amphibians at low, middle and high elevations.

To address this hypothesis, I sought to determine the prevalence of the pathogen *B. dendrobatidis* along an elevational gradient in Panama as determined by RT-qPCR analysis. If there is no elevational component, then the prevalence of *B. dendrobatidis* and disease effects (as seen through abundance and species richness) will be similar among amphibians at all elevations. If chytridiomycosis is affecting lowland populations,
then anuran abundance would differ between years at any one site in the lowlands. I analyzed changes in anuran abundance between sampling years (post-highland infection 2006 and pre-highland infection 2001) for lowland sites in Panama expecting that if the chytrid is not affecting lowland populations, then anuran abundance would be similar between years at any one site. For comparative purposes, I also analyzed changes in anuran abundance between sampling years at a middle and high elevation site where disease outbreaks had not yet occurred (2006 and 1995/96). To control for potential intrinsic variation in anuran abundance and chytrid prevalence and intensity, I collected data from both seasons and habitats and compared between similar data.

- **Hypothesis No. 2**: If *B. dendrobatidis* prevalence depends on length of time incurred since initial epidemic decline, then highland amphibian communities at varying stages of epidemic infection will show differential infection prevalence and intensity.

To examine the degree of prevalence, intensity and persistence of the chytrid in amphibian communities at varying stages of epidemic infection in the highlands of Panama, I used sensitive RT-qPCR techniques to detect the chytrid in skin samples of anurans from pre, during and post-decline sites. If infection prevalence is not related to length of time incurred since initial epidemic decline, then all highland amphibian communities will show similar degrees of infection. Disease effects at each site were measured through anuran abundance and species richness, and prevalence of the pathogen and corresponding disease effects were determined by season and habitat to control for intrinsic environmental variation.

- **Hypothesis No. 3**: If *B. dendrobatidis* is indeed a novel pathogen, sites that have not yet undergone epidemic decline in Panama will show no evidence of the chytrid.

I used sensitive RT-qPCR techniques to detect the chytrid in skin samples of anurans from pre, during and post-decline sites and sites of all elevations in Panama to determine if any sites could be deemed chytrid-free, a result that would be in support of the novel pathogen hypothesis and the spreading disease wave theory currently proposed for chytridiomycosis in Panama. Finding the infection to some degree in all sites would provide support for, although not necessarily confirm, the endemic pathogen hypothesis.
Chapter Four:

- **Hypothesis No. 4:** If there is a seasonal aspect to the occurrence of *B. dendrobatidis*, then prevalence and intensity of *B. dendrobatidis* and anuran abundance will differ between seasons at any one site at all elevations.

To evaluate the effect of seasonality on chytrid prevalence and intensity, I sampled sites at varying elevations and stages of epidemic infection in Panama during both wet and dry seasons for the presence of the chytrid in amphibians through RT-qPCR analysis. If there is no seasonal aspect, prevalence and intensity of the chytrid and anuran abundance will be similar between seasons at any one site.

- **Hypothesis No. 5:** If chytrid prevalence/intensity and disease effects on anuran abundance and species richness are habitat specific, then anurans of different ecological guilds at all elevations and stages of decline will show differential *B. dendrobatidis* susceptibility depending on habitat occupancy.

I used RT-qPCR to determine *B. dendrobatidis* prevalence and intensity in epidermal skin swabs for amphibians of different ecological guilds as a function of habitat type (forest or stream) at varying elevations and stages of epidemic decline in Panama. If chytrid prevalence and its effects on anuran abundance are not habitat specific, then *B. dendrobatidis* occurrence and its effects on anuran abundance will be the same for anurans of all ecological guilds, regardless of habitat occupancy, at all elevations and stages of epidemic infection.

- **Hypothesis No. 6:** If there is a relationship between *B. dendrobatidis* prevalence and anuran body size, then within a species individuals of a certain body size will show greatest infection.

I measured the snout-vent length (SVL) of frogs caught along an elevational gradient and an epidemics gradient in Panama, and used RT-qPCR to determine chytrid prevalence for all samples. If there was no relationship between prevalence of the chytrid and anuran body size, then *B. dendrobatidis* infection would vary randomly within a given species with respect to body size.
Chapter Five:

- **Hypothesis No. 7:** If *B. dendrobatidis* is resident in non-amphibian vectors, then it should be detectable in other organisms that could spread the disease among amphibians.

Reptiles may be reservoir hosts for the chytrid, harbouring infection without succumbing to disease and thereby maintaining the infection in amphibian communities. I took epidermal skin swabs from all lizards and snakes caught in eight sites of varying elevations and stages of epidemic decline in Panama and used RT-qPCR analysis to determine the presence of the chytrid. If *B. dendrobatidis* is not carried by reptiles, it should not be detectable.
CHAPTER 2: GENERAL MATERIALS AND METHODS

Combinations of the same sites as well as common survey, sampling, and laboratory techniques were used in all manuscripts appearing as Chapters 3-5 in this thesis. To avoid excessive repetition in my thesis I present these general methods here so that the Materials and Methods sections of each chapter are only as detailed as necessary for that particular study. All georeferences for Study Sites are UTM data, Zone 17P, WGS84 datum, determined with a Garmin E-Trex Legend™ handheld GPS unit. The vegetation of each site is classified using Holdridge’s Life Zones.
STUDY SITES

**Fortuna** (FOR) is a Hydrological Forest Reserve located at 1215 m elevation and covers 19,500 ha of land in the Cordillera Central in Chiriquí Province of western Panama (Figure 1). I surveyed forest habitat in secondary growth premontaine rain forest in the small village of Hornito, off the main highway that runs through Fortuna about 8.7 km south of the STRI research station (UTM: E365679 N958816). Quebrada Aleman was surveyed as the stream site, and is located in secondary growth premontane wet forest off the main Fortuna road about 2 km south of the research station (UTM: E364382 N962562). For Chapters 3 and 4, this site was used as the long post-epidemic site of the epidemics gradient.

**Altos del María** (AM) was located within a gated retirement community in primary and secondary growth tropical wet forest in the El Valle region of Coclé Province, central Panama in the Cordillera Central (Figure 1). Surveys were done in the stream known as Rio María and in surrounding forest at 890 m elevation with UTM coordinates E602033 N955520. For Chapters 3 and 4, this site was used as the during-epidemic site of the epidemics gradient.

**Altos de Campana** (AC) was within Altos de Campana National Park in primary and secondary growth premontane wet forest in Panama Province of central Panama (Figure 1). The park encompasses an area of 4816 ha at 860 m elevation in the continental divide of the Cordillera Central, and is located in the western portion of the Panama Canal Watershed. All streams surveyed in the area were within the park’s main trail system (UTM: E617846 N959851). For Chapters 3 and 4, this site was used as the pre-epidemic site of the epidemics gradient, and the high elevation site of the Campana elevational gradient.

**Cerro Trinidad** (CT) was located near a small village called Lidice within the boundaries of Altos de Campana National Park on the Atlantic Versant, at the base of Cerro Trinidad (540 m elevation), with an entrance via the town of Capira on the Pan-American Highway in Panama Province (UTM: E615346 N966692, Figure 1). For Chapters 3 and 4, this site was used as the middle elevation site of the Campana elevational gradient.
El Copé (EC) was located just north of the town of El Copé (UTM: E544757 N958286) within the boundaries of the Parque Nacional General de Division Omar Torrijos Herrera (PNGDOTH), a National Park which straddles the continental divide of the Cordillera Central in Coclé Province, central Panama, with an area >25,275 ha (Figure 1). Forest in the park is characterized as mostly primary or secondary growth premontane wet forest. This site was located at 760 m elevation in the main visitor area of PNGDOTH. In this site I sampled within the streams known as Río Guabal, Quebrada Silenciosa, Quebrada Cascada and Loop Stream, as well as along the marked forest trails Loop Trail and Sendero de Ranas. Although within PNGDOTH, this site will be referred to as El Copé throughout the thesis. For Chapters 3 and 4, this site was used as the recent post-epidemic site of the epidemics gradient, and the high elevation site of the El Copé elevational gradient.

La Rica (LR) is a small village at 250 m elevation within PNGDOTH boundaries on the Atlantic versant, about 12 km north of the main visitor area of the park, along the Río Guabal (UTM: E544474 N932600, Figure 1). Tributaries of the Río Guabal and Río Blanco as well as surrounding secondary growth tropical wet forest were surveyed in this area. For Chapters 3 and 4, this site was used as the middle elevation site of the El Copé elevational gradient.

Palmarazo (PAL) is on the Atlantic versant, at 135 m elevation along the Río San Juan (UTM: E538038 N965388) within PNGDOTH boundaries (Figure 1). Sampling was conducted in two streams, Quebrada Varona and Quebrada Guabalito, and in surrounding secondary growth tropical wet forest. For Chapters 3 and 4, this site was used as one of the two low elevation sites of the El Copé elevational gradient.

Cuatro Callitas (CC) is on the river Coclé del Norte on the Atlantic versant, at an elevation of 45 m with UTM coordinates of E548703 N992176 (Figure 1). Three streams and surrounding secondary growth tropical wet forest in Cuatro Callitas were sampled, including Quebrada Cuatro Callitas, Quebrada Peñalosa and Quebrada Caucho Blanco. For Chapters 3 and 4, this site was used as one of the two low elevation sites of the El Copé elevational gradient.
SURVEYS

Herpetofauna (frogs, lizards and snakes) were surveyed in both stream and forest habitat by day and night. Diurnal forest surveys were done by searching through an area of forest and looking for animals at ground level by overturning leaf litter, rocks and branches. Diurnal streambed surveys were conducted by walking upstream alongside the stream edge and searching along the edge as well as on rocks in the streambed. At night animals were found mainly on low to mid-level vegetation along forest trails and streams, although I searched the streambed as well for nocturnal aquatic species. Nocturnal surveys were conducted using Princeton Tek® Switch Back headlamps with halogen lights. All searching was quantified using Search Effort Hours (SEH) in order to maximize the number of individuals sampled and minimize the chances of counting and/or collecting skin swab samples from the same individuals more than once. I did not survey the same area twice over a given sampling interval.

All of the above methods were chosen so that I could sample the greatest number of animals possible, thus improving upon the accuracy of infection prevalence estimates that could be obtained through Real-Time Quantitative PCR analysis. Total Search Effort Hours and the timing of search periods over the course of this study were constrained by the ability to find field help during certain periods and explain any differences in the search period or Search Effort Hours between sites. Wherever possible I tried to equalize the amount of surveying and sampling time spent at each site. I standardized SEH by multiplying the number of people searching by the number of hours searched in the particular site at the given time interval. No searches were conducted without my presence and supervision.

In Panama in 2006, the dry season (lower temperatures and minimal rainfall) extended from January 13th through April 28th and was subsequently followed by a distinct wet season (higher temperatures and increased precipitation), according to the Smithsonian Tropical Research Institute’s Environmental Science Program (ESP). I attempted to conduct nocturnal and diurnal surveys and sample collection at all sites in stream and forest habitat during both the dry and wet seasons in order to sample the largest proportion of species and individuals as possible. However, both sites from the Campana elevational gradient were sampled only during the wet season, as was Cuatro
Callitas from the El Copé elevational gradient. Cuatro Callitas and the Campana elevational gradient were added as sites later in the 2006 field season. Because there is considerable evidence that season and habitat can affect the prevalence and intensity of *B. dendrobatidis* (Aplin and Kirkpatrick 2000, Lips et al. 2003, Stuart et al. 2004, Berger et al. 2004, Woodhams and Alford 2005), I organized data by habitat and season, and analyzed between habitats and seasons accordingly. So as to compare amphibian community composition between sites and years, I grouped frog species into eight different ecological guilds since different species were present depending on the site or year (see Appendix 0). Since data collection for this study was completed, the majority of amphibian species have undergone name changes to reflect new taxonomic groupings (Frost et al. 2006). Species names have not been changed for this thesis and remain according to Savage (2002).

Depending on frog abundance in a given habitat type (stream or forest) at a given search period (day or night), the length of time spent searching was adjusted accordingly to maximize the number of frogs found, particularly in sites where chytridiomycosis had taken its toll. Nocturnal stream searches were conducted more frequently than nocturnal forest searches; many stream-dwelling species are active only at night (e.g. frogs from the “leaf frogs” and “treefrogs” guilds), whereas most terrestrial species present can be found during diurnal forest surveys (e.g. frogs from the “leaf-litter frogs” and “terrestrial diurnal frogs” guilds), a trend noted by other researchers (Ibáñez, Pers. Comm., 2006). Frogs may move between forest and stream habitats over short time periods, especially in Neotropical environments where streams or other small bodies of water tend to be frequently distributed throughout the environment. Because it was impossible to say what habitat type individual captured frogs most regularly frequented, frogs were categorized as being forest or stream habitat dwellers depending on the habitat type in which they were captured.

**SAMPLE COLLECTION**

For each 2-hour survey period, animals were caught and restrained in clean, unused, 20 x 25 cm individual clear plastic bags until the survey was completed and sample collection could begin. Due to the potential of the chytrid spreading among individuals,
all researchers were careful to touch the animals only with plastic bags during capture and to avoid cross-contamination by disallowing direct animal-to-animal contact, except when amplexant anuran pairs were caught together. Juveniles and adults of both sexes were sampled from all species.

I examined all animals for visible symptoms of chytridiomycosis (lethargy, sloughing skin). Epidermal skin swabs were taken from the animals using sterile autoclaved Q-tips® cotton swabs following a procedure developed by Lauren Livo at the University of Colorado (Livo, unpublished, 2004) for chytrid diagnosis using PCR. I wore clean unpowdered latex gloves when constraining each animal, and rubbed the cotton-tipped portion of the swab slowly and gently about 25 times over the ventral surface, with particular attention paid to the pelvic area, venter, toes and tails (lizards and snakes only) since infection is known to be most prevalent in these areas (Hyatt et al. 2007). Shedding skin was also collected from individuals when available. Latex gloves were changed between individuals to avoid cross-contamination. Swabs were stored cotton-tip down in 70% ethanol in individually labelled 2.0 ml screw-capped microcentrifuge tubes, with O-ring seals (Fisher catalogue #s 02-681-343 tubes & 02-681-358 caps). In addition to swab samples, I also took snout-vent-length (SVL) measurements of all individuals to the nearest 0.1 mm using Spi 2000 callipers. All amphibians and reptiles were swabbed, measured and released on site immediately following sampling, except for two individuals of each species of the frog families Eleutherodactylidae and Centrolenidae (according to Savage 2002), which were kept as voucher specimens. These animals were humanely killed through submersion in a 20% ethanol solution and later fixed in 10% formalin. Upon return from the field, samples were stored at -20°C.

CHYTRID DIAGNOSIS

Preliminary infection diagnosis was conducted between June and September 2006 on a portion of the samples in the Smithsonian Naos Laboratory in Panama City, Panama. DNA extraction and PCR amplification were done using a protocol adapted by Corinne Richards (Richards, unpublished, 2004) from Annis et al. (2004). I later optimized this protocol for use in the Naos laboratory (Appendix 1). Preliminary results from conventional PCR allowed me to obtain presence/absence data from various sites so that I
could modify my fieldwork accordingly. Real-Time Quantitative PCR was then used for all critical analyses as RT-qPCR is known for its high sensitivity, accuracy and ability to quantify low levels of chytrid infection (Kriger et al 2006b). Internal Positive Controls (IPCs) are run on every individual sample so that researchers are aware of PCR inhibited samples; hence the obtainment of false negatives is not a problem as it is in conventional PCR (Hyatt et al. 2007). DNA from all samples was extracted at McGill University using PrepMan Ultra™ extraction kits from Applied Biosystems (ABI) optimized for *B. dendrobatidis* extraction. DNA from those samples already extracted in Panama was re-extracted from the original samples using PrepMan Ultra™. All DNA extractions were done according to Hyatt et al. 2007.

Preparation of field samples for DNA extraction began with centrifugation at 13,000 rpm in a microfuge for 5 min in order to settle the cotton swabs, zoospores and debris to the bottom of the tubes. Being careful not to dislodge the cotton swab, I first decanted the ethanol from the sample tubes. Because it has been found that most zoospores remain embedded in the cotton of swabs (Hyatt et al. 2007), I then removed the swabs from the tubes with forceps, and holding the paper stick portion, I ripped the cotton portions of the swabs from their sticks with tweezers and placed the cotton back in their respective original sample tubes to dry overnight in a fume hood. To avoid contamination, I sterilized all instruments using ethanol and flame from a Bunsen burner between samples. To each sample I added 50 μl of PrepMan Ultra™, as well as 30–40 mg of 0.5 mm diameter zirconium/silica beads from Biospec Products; to minimize zoospore loss, all extractions were done in the original sample tubes containing the dry cotton swab. Samples were homogenized with the beads and PrepMan solution for 1 min. in a Vortex Genie2® (VWR Scientific) with a TurboMix® attachment (Scientific Industries) and after brief centrifugation to settle all material to the bottom of the tube (1 min. at 13,000 rpm), homogenization and centrifugation were repeated as before. The homogenized samples were then placed in a heat block at 100°C for 10 min. as outlined in the PrepMan Ultra™ protocol, and then allowed to cool for 2 min. Finally, samples were centrifuged in a microfuge for 6 min. and 20+ μl of supernatant was recovered from each sample and stored in new, labelled 0.3 ml PCR tubes at –20°C.
In order to minimize the effect of PCR inhibitors, extracted DNA samples containing yellow or brown extracted DNA (260 samples) were cleaned according to the Qiagen DNeasy mini spin column extraction kit protocol for purification of total DNA from animal blood or cells (Spin-Column protocol Steps 1-7). This protocol has been known to be effective at getting rid of at least some PCR inhibitors (Pessier, Pers. Comm., 2007).

To begin, I added 200 ml of 100% ethanol to each sample and mixed thoroughly by vortexing. This mixture was pipetted into a Qiagen spin column nested in a 2 ml Qiagen collection tube (provided with the kit) and centrifuged for 8,000 rpm for 1 min. The collection tube was discarded and the spin column was placed in a new collection tube where 500 ml of Buffer AW1 was added and centrifuged for a further minute at 8,000 rpm in the microfuge. Again the collection tube was discarded and the spin column was placed in a new collection tube, but this time I added 500 ml of Buffer AW2 and centrifuged for 3 min. at 14,000 rpm in order to dry the membrane on the spin column. In the final step the collection tube was discarded and the spin column was added to a sterilized 1.5 ml microcentrifuge tube, not provided with the kit. Two hundred ml of Buffer AE was pipetted directly onto the membrane of the spin column, incubated at room temperature for 1 min. and eluted through a final centrifugation at 8,000 rpm for 1 min. The eluted DNA was then stored in new, labelled 0.3 ml PCR tubes at -20°C with the other extracted DNA samples.

REAL-TIME QUANTITATIVE PCR ANALYSIS

All optimization and Real-Time Quantitative PCR analysis was conducted at the Rnomics Platform of the Genome Quebec laboratory in Sherbrooke, Quebec using an EPPENDORF® Mastercycler© ep Realplex² sequence detection system following a protocol developed by Boyle et al. (2004) and later adapted for singlicate assays by Kriger et al. (2006b). The final qPCR amplification conditions used for this study were: 5 min at 50°C and 2 min at 95°C, followed by 15 sec at 95°C and 1 min at 60°C for 50 cycles.

The general RT-qPCR techniques applied by the EPPENDORF® Mastercycler© ep Realplex² system are essentially the same as those used by the ABI Prism 7700 sequence detection system detailed by Boyle et al. (2004), where the instrument software calculates
and plots the change in fluorescence signal from the fluorescent labelled probe during the PCR cycling reaction versus the cycle number. The Ct value (threshold cycle) corresponds to the cycle number where the fluorescence curve reaches the threshold set up for the assay. This threshold is determined using the standard curve which is obtained by 5 points of dilution (1000, 100, 10, 1, 0.1) using a known quantity of chytrid zoospores. If chytrid is detected in the assay, the software calculates quantification of the sample as a function of where on the standard curve the Ct value lies. For large amounts of DNA (~ 10000 zoospores/µl) a Ct value of around 20 is expected, with lower amounts of DNA yielding a much greater Ct value (Figure 2). Ct values of 33 and above typically correspond to an amount of ~ 1 zoospore/µl or less of DNA, and at these small quantities quantification is generally considered to be inaccurate. To quantify zoospore amounts to whole samples I multiplied the zoospore concentration detected per well (1 µl) by the total amount of extracted DNA (50 µl) for all samples except for the 260 samples that were cleaned up. Because those samples were essentially diluted ¼ from the original DNA extraction concentration I multiplied the amount of zoospores by 200 to get the final zoospore equivalent for these samples. For analyses, samples were deemed positive only if the zoospore equivalent was one zoospore or higher.

The chytrid detection protocols of Boyle et al. (2004) and Kriger et al. (2006b) were optimized for Genome Quebec. The reaction components (in 10 µl) were as follows: 5 µl of 2x Perfecta Multiplex qPCR Supermix from QUANTA BIOSCIENCES INC (cat# 95063-050), 1 µl of undiluted DNA, PCR primers at a final concentration of 0.9 µM (forward sequence: CTG CTG CCC GAC AAC CAC and reverse sequence: AGC CAA GAG ATC CGT TGT CAA A), 0.25 µM concentration of the Chytrid Taqman MGB probe (CHYTRMGB, sequence: 6FAM CGA GTC GAA CAA AATMGBNFQ) and 1µl of dH2O.

Following Hyatt et al. (2007), we attempted to control for the obtainment of false negatives through potential PCR inhibition that could be present in some samples. To do this we designed specific internal positive controls (IPCs) for this study in the form of: 0.25 µM of the GFP-JOE Taqman probe (sequence (3’ Iowa Black FQ): (JOE) CCA GTC CGC CCT GAG CAA AGA CC), GFP Primers (0.1 µM of the forward sequence: CTG CTG CCC GAC AAC CAC and 0.04 µM of the reverse sequence: TCA CGA ACT CCA...
GCA GGA C) and 10 pg of the GFP vector (pCMS-eGFP Maxi prep). In our duplex reactions, we detect both our interest gene (CHYTRID probe coupled with FAM) for quantification, and amplification of the GFP to determine whether inhibition is present. Knowing the input amount of GFP vector we put in the reaction, we always expected a Ct value of about 20 for the IPC. If there was some inhibition, the Ct value would shift towards 23 and more, or result in no amplification and thus no Ct value. In this study, if samples showed an IPC value of 23 or more they were considered to be inhibited at least to some extent and were excluded from all analyses.

Prior to qRT-PCR analysis, I prepared a positive control of known quantity of extracted chytrid DNA so that a standard curve could be made for quantification purposes. Cultured chytrid zoosporas for this standard curve were obtained dead and sterilized in 70% ethanol, from a zoospore culture grown by Joyce Longcore at the University of Maine in Orono. First, zoospore numbers were obtained by counting in quadruplicate in a haemocytometer, using 100 μl samples each time. I used a hand-held counter to count the absolute numbers of zoospores in the sample by moving in a zigzag pattern across 4 large squares, each containing 16 smaller squares. This was repeated three more times and the mean of all four counts was used in the haemocytometer formula to obtain the most accurate count possible. According to the formula the culture contained 1,262,500 cells/ml and there were 2 ml of cultured chytrid for extraction available, for a grand total of 2.5 x 10⁶ zoospores. Before the chytrid DNA could be extracted I centrifuged the zoospore culture until there was a pellet (14,000 rpm for 1 min.), decanted the ethanol, and resuspended the pellet in 200 μl of PrepMan Ultra™ with 30-40 mg of zirconium/silica beads. The DNA for the positive control was then extracted as previously described for the epidermal skin swab samples.

DATA ANALYSIS OF TRIPLICATE VS. SINGLICATE STUDY

An initial pilot study using triplicate RT-qPCR analysis was performed on 100 randomly chosen samples from three known positive sites. Samples were deemed initially positive if the total amount of zoospores in the sample was 1.0 or higher for all three wells. Following the reasoning of Kriger et al. (2006b), all ‘suspicious’ samples (those that yielded a positive for only one or two wells out of three in the triplicate
analysis) were re-analyzed in triplicate and samples still showing at least one positive well were deemed positive while those with no positive wells deemed negative. To ensure that singlicate analysis would be sufficient for the purposes of my study, I determined the probability of infection prevalence for a hypothetical singlicate assay with the assumptions that all suspicious samples from the initial triplicate study containing only 1 out of 3 positive wells would have a 1/3 probability of being positive in a singlicate assay, and those with 2 of 3 positive wells would have a 2/3 probability of being positive in a singlicate assay. For the two cases of suspicious samples, I multiplied the probability by the respective number of suspicious samples in each case to determine the total number of positive samples for a singlicate analysis. Infection prevalence was determined as a function of the number of positive samples divided by the total number of samples. Prevalence between the observed triplicate assay and the theorized singlicate assay was compared using a Pearson chi-square test (Table 1).
Figure 1: Map of Panama showing the location of all study sites and their respective elevations and epidemic status. The El Copé elevational gradient of Chapter 3 and 4 was comprised of El Copé, La Rica, Palmarazo and Cuatro Callitas, and the Campana elevational gradient of these studies encompassed Altos de Campana and Cerro Trinidad. The epidemics gradient used in these same studies comprised Fortuna, El Copé, Altos del María and Altos de Campana. All sites were used in Chapter 5, without organization by transect.
Figure 2: Standard curve showing quantification of zoospore amounts using RT-qPCR. Five points of dilution in addition to the original known chytrid zoospore quantity is shown on the standard curve. The Ct [cycle] value corresponds to the cycle number where the fluorescence curve reaches the threshold set up for the assay. If chytrid is detected in the assay, the software calculates quantification of the sample as a function of where on the standard curve the Ct value lies.

Slope: -3.605
Y-Intercept: 33.26
Efficiency: 0.89
R^2: 0.997
Table 1: Summary of results for the pilot study. Using the same reasoning as Kriger et al. (2006b) the theorized results of a singlicate assay were determined from the analysis of 100 samples in triplicate using RT-qPCR. A Pearson chi-square test was used to determine that the two types of assays did not differ in their ability to determine chytrid prevalence. The formula for determining theorized singlicate assay prevalence was: 53 (pos from initial triplicate) + 2/3*7 (suspicious) + 1/3*5(suspicious).

<table>
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<td>Final</td>
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\[ X^2 = 0.085 \]
\[ p = 0.771 \]
MANUSCRIPT ONE EXPLANATIONS

My first manuscript, which appears as Chapter 3 in this thesis, pursues the question of the comparative prevalence and pathogenicity of the chytrid fungus, *Batrachochytrium dendrobatidis*, at varying elevations and stages of epidemic infection in Panama. Many authors assert that *B. dendrobatidis* is a novel invasive pathogen causing the disease chytridiomycosis. Alternatively, other authors suggest that *B. dendrobatidis* is an enzootic pathogen and changing extraneous environmental factors have caused the chytrid to emerge as a disease. If the pathogen is enzootic, the disease chytridiomycosis could still be novel. Alternatively the disease itself might be enzootic, arising when conditions are right; this is occurring presently and may have also occurred in the past. More work is needed in Panama to determine if the chytrid is in the environment prior to an epizootic disease outbreak, and if it persists in amphibian communities post-decline.

Although it is known that the chytrid fungus causes pathological effects at higher elevations in the Neotropics, the extent to which it is distributed and causes disease at lower elevations is poorly understood. Research done in Panama to date has focused on the chytrid fungus’ observed preference for, and fatal effects at, higher elevation habitats. Disease effects are thought to be greater at higher elevations because lower temperatures are optimal for the growth and survival of *B. dendrobatidis* zoospores, at least under laboratory conditions. However, recent scientific evidence suggests that temperature plays a more complex role than previously thought, and it may simply be an artefact of sampling that only high elevation “pristine” habitats appear to support chytridiomycosis.

Substantiating either the novel or enzootic pathogen scenarios is important since the implementation of conservation efforts in response to amphibian decline would have to vary accordingly. Currently *ex situ* breeding and subsequent re-introduction is the only conservation action in place, but re-introduction may not be possible if the pathogen is present pre-decline and remains post-decline. I find and discuss the likelihood that *B. dendrobatidis* is an enzootic pathogen in central Panama from the discovery that it exists and persists at all elevations and stages of epidemic infection, including pre-decline. Disease effects and decline were noted for lowland amphibians, and I propose that chytridiomycosis is a disease also affecting lowland amphibians.
CHAPTER 3: PREVALENCE AND PATHOGENICITY OF THE PATHOGENIC CHYTRID FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS*, IN ANURAN COMMUNITIES IN PANAMA

Kilburn, V.L., Ibáñez, R., Sanjur, O., Bermingham, E. and Green, D.M.

*Batrachochytrium dendrobatidis* has been implicated as the main driver of many enigmatic amphibian declines in Neotropical sites at high elevation. The chytrid is thought to be a water-borne pathogen with a thermal optimum of about 23°C, but the extent to which it persists and causes disease in amphibians at lower elevations in the Neotropics is not known. It is also unclear by what mechanism(s) *B. dendrobatidis* has emerged as a pathogenic organism. To test whether the chytrid is limited by temperature and/or elevation in the Neotropics, we sought to determine the prevalence and intensity of *B. dendrobatidis* at eight sites of varying elevations (45 m to 1215 m) and stages of epidemic amphibian decline (pre, during, recent-post and long-post epidemic) in central Panama using RT-qPCR analysis. We analyzed anuran abundance and species richness at all sites and compared between two sampling years to determine disease effects at varying elevations. The chytrid was found in all sites regardless of elevation or stage of epidemic decline. Save for the one site where a disease outbreak was occurring, pathogen prevalence and intensity was relatively low across sites. In addition to presence of the pathogen in frog communities up to 11 years post-decline, symptoms of chytridiomycosis and corresponding decline in amphibian populations were variably seen at all elevations. The results suggest that the chytrid may be an enzootic pathogen.
INTRODUCTION

There is compelling quantitative evidence for a global decline of amphibian populations (Houlahan et al. 2000, Green 2003, La Marca et al. 2005, Lips et al. 2005b). Although habitat loss is still the leading cause of amphibian declines worldwide, amphibians are also declining in relatively pristine habitats where other “enigmatic” factors, like climate change, disease, and contamination, must also be at work (Stuart et al. 2004). Experimental data are limited with respect to the exact role played by these enigmatic factors, but complex synergistic interactions among the varying factors are suspected (Lips et al. 2005a).

The recently identified amphibian disease chytridiomycosis, caused by the pathogenic chytrid fungus, Batrachochytrium dendrobatidis, has been implicated as a main driver of many of these so-called enigmatic declines (eg. Berger et al. 1998, Lips et al. 2003, 2006, 2008). The chytrid is widespread, having been found in captive and wild populations of amphibians on all continents (Morgan et al. 2007). It has water-borne, motile zoospores with a thermal optimum of about 23°C, does not resist freezing, and is known to die at temperatures above 30°C in the lab (Longcore et al. 1999). The chytrid has been implicated in amphibian declines particularly in high elevation riparian habitats in the Neotropics (Berger et al. 1998), and its ecology explains its appeared preference for this type of habitat. Because the immune response in amphibians is suppressed at lower ambient temperatures, some have suggested that amphibians living at higher elevations are naturally more susceptible to negative effects of potential pathogens like B. dendrobatidis (Carey et al. 1999). Endemism among amphibians is also known to be highest at higher elevations (Campbell 1999), and since these species usually show less genetic variation and are often more specialized and ecologically restricted (Waller et al. 1987), they show greater decline in the advent of environmental change (Lips et al. 2003). In Central and South America, B. dendrobatidis is thought to be an agent causing widespread amphibian decline and even extinction of species in a number of montane anuran genera, including Dendrobates and, most notably, Atelopus (La Marca et al. 2005; Lips et al. 2003, 2004, 2005a,b).

There are currently two competing hypotheses regarding the emergence and origin of B. dendrobatidis as an amphibian pathogen: the novel pathogen hypothesis and the
endemic pathogen hypothesis (Rachowicz et al. 2005). While the novel pathogen hypothesis suggests that the chytrid has recently spread into new geographical regions and is impacting naïve populations of amphibians, the endemic pathogen hypothesis suggests that \textit{B. dendrobatidis} is an enzootic pathogen in amphibian populations that is now developing as a novel pathogenic disease as a result of compounding environmental factors (Rachowicz et al. 2005). Supporting evidence is available for both hypotheses but no formal scientific consensus has been reached.

In support of the endemic pathogen hypothesis, a number of disease emergence theories have been proposed. Environmental change could cause an enzootic, potential pathogen to emerge as an epizootic, either via a shift towards the thermal optimum for the pathogen (Pounds et al. 2006) or by reducing host resistance and increasing disease susceptibility (Daszak et al. 2004). Pounds and Crump (1994) and Pounds et al. (1999) proposed that the chytrid-induced extinction of several species of frogs and toads in Monteverde, Costa Rica, was a result of abnormal climate patterns following the 1986-87 El Niño event that resulted in dry season mist reduction causing stress and/or behavioural changes in amphibians that increased their infection susceptibility. Recently Pounds et al. (2006) presented evidence that global warming increases cloud cover over tropical forest, thereby encouraging the spread and pathogenicity of \textit{B. dendrobatidis} at both high elevations and lower elevations through a cycle of daytime cooling and nighttime warming that creates a favourable climatic environment for the fungus (the “chytrid-thermal-optimum” hypothesis). Bosch et al. (2007) substantiated this hypothesis through their identification of a link between climate change and chytridiomycosis outbreaks in Central Spain.

Despite the claim made by Pounds et al. (2006) that global warming could increase chytrid infection at lower elevations, La Marca et al. (2005) argues that climatic changes resulting in exposure to low humidity and higher temperatures may actually be beneficial to lowland amphibians in areas of chytrid infection since infections are lethal only if there is enough moisture and appropriate temperatures for zoospores to survive and re-infect their hosts. Using histology on museum specimens, Puschendorf (2006a) found that \textit{B. dendrobatidis} was present in lowland anurans in Costa Rica at fairly high prevalence in both declining and persisting species. The chytrid is known to be broadly distributed at
low elevations throughout the Brazilian Atlantic rainforest as well (Oliveira de Queiroz Carnaval et al. 2006). In both studies, the chytrid was present in the lowlands but did not appear to harm its hosts, thus making them disease reservoirs. However, there was a lack of long-term population monitoring in these Neotropical lowland sites prior to this study, and the conclusion that the disease chytridiomycosis does not affect lowland amphibians is perhaps under false pretences given the difficult nature of quantifying amphibian declines; disease outbreaks at low elevations could remain unnoticed due to the occurrence of other decline factors like habitat destruction (Wright et al. 2001). In addition, amphibian declines may occur over a longer time scale in warmer climates and thus are more likely to be overlooked at lower elevations (Whitfield et al. 2008). Inherently there is a scientific bias, as declines in “pristine” areas are easier to quantify (Wright et al. 2001). Detailed studies on B. dendrobatidis distribution and disease-related effects have not been done at middle or low elevations in Panama.

Using histology on museum specimens, researchers have dated the arrival of Batrachochytrium dendrobatidis to South America in 1980, in Ecuador (Ron et al. 2003), although amphibian declines were first documented from the “mid 1970’s” in Venezuela (Bonaccorso et al. 2000). In Central America, B. dendrobatidis was first detected on museum specimens from Costa Rica dating from 1986 (Puschendorf et al. 2006a), one year before any major catastrophic declines were noted for Costa Rica (Pounds and Crump 1994). In Panama, chytridiomycosis has spread as a “novel” disease in the highlands in a southeastward “wave” of infection at a rate of 30 km/year since 1996 (Lips et al. 2006). During the 2004 El Copé epizootic, presence of the chytrid increased rapidly to very high prevalence and was associated with massive die-offs of amphibians; amphibian density and species richness declined abruptly in 48 species of both diurnal and nocturnal stream communities following six consecutive years of high population abundances, and disease prevalence reached 50% in a year (Lips et al. 2006).

Certainly there has been a recent push for substantiating the novel pathogen hypothesis (Skerratt et al. 2007, Lips et al. 2008), and although there appears to be more scientific evidence for this hypothesis, many authors continue to dispute the claim that B. dendrobatidis is a novel pathogen. According to Alford et al. 2007, the novel pathogen hypothesis ignores the potential that chytrid persists in frog populations at very low levels
as a natural non-lethal parasite, emerging only as a lethal pathogen in the advent of shifting environmental conditions. McCallum (2005) argues that the novel pathogen hypothesis is too simplistic and that likely *B. dendrobatidis* is an endemic pathogen that has become pathogenic due to co-interaction between one or more factors. McCallum (2005) also argues that it is implausible that a water-borne disease could variably affect such a large proportion of amphibians when so many potential competitors of *B. dendrobatidis* exist in a natural ecosystem. Since the low innocuous levels of infection characteristic of an endemic pathogen might easily be overlooked in an amphibian community due to error in infection diagnostic tools, some authors suggest that Real-Time quantitative PCR should be adopted as the primary tool for pathogen diagnostics since it can reliably detect pathogen intensities of less than 1.0 zoospores per sample (Kriger et al. 2007a).

Does the chytrid persist in amphibian communities post-decline, and can it be found at low innocuous levels pre-decline? Does the degree of prevalence differ depending on the length of time since initial infection, or as a function of elevation? Is chytridiomycosis really only a disease of highland tropical amphibians? If the survival of *B. dendrobatidis* is so temperature dependent, and climate change is a real phenomenon that results in unpredictable climate shifts throughout an altitudinal range, is it likely that the chytrid survives and negatively impacts only highland amphibians? Given the uncertainties presented in the literature regarding the emergence of chytridiomycosis and the effects of temperature on pathogenicity, can we assume that *B. dendrobatidis* is a novel pathogen in Panama and affects only high elevation hosts? The assumption that the pathogen is completely novel obviates additional research into environmental changes that could result in disease outbreak and spread, and recent evidence suggests that chytrid ecology may be more complex than previously thought. Thus, the prevailing idea that the chytrid fungus has emerged as a novel pathogen and is lethal only at lower temperatures requires further examination.

To answer our questions regarding elevational distribution of chytrid infection and disease, we sought to determine whether the pathogen was present in amphibian communities at all elevations of Panama west of the Canal Zone. Additionally we hoped to investigate how chytridiomycosis was affecting lowland amphibian community
composition by comparing frog abundance between sampling years. To address our questions regarding the competing disease emergence hypotheses, we sought to determine the distribution of the pathogen at high elevation sites along an epidemics gradient, from a long post-epidemic site to an alleged pre-epidemic site, as determined by previously completed work in Panama. If the pathogen was deemed absent from an amphibian community using sensitive RT-qPCR infection diagnosis, this would substantiate the novel pathogen hypothesis. Finding the pathogen in all sites regardless of elevation or stage of decline would yield support for the endemic pathogen hypothesis.

MATERIALS AND METHODS

STUDY SITES, SAMPLE COLLECTION AND SURVEYS

Sampling to compile anuran abundance and species richness surveys and assay for the presence of the chytrid and the disease occurred in Panama at 8 sites of varying elevation and stage of epidemic infection over a 9-month period in 2006, beginning February 18\textsuperscript{th} and ending October 28\textsuperscript{th}. All animals were examined for symptoms of chytridiomycosis (lethargy, sloughing skin). We collected epidermal skin swab samples from a total of 1,252 frogs over 530.25 Search Effort Hours. We sampled 59 different species of 15 genera and 6 families (according to Savage 2002) of both sexes and of both juvenile and adult age classes from a wide range of body sizes. Surveys were conducted by day and night in forest and stream habitat in the dry season and in the wet season (see Chapter 2) for all sites except Cuatro Callitas, Altos de Campana and Cerro Trinidad, which were surveyed in the wet season only (Appendix 2a). No forest surveys were done in the wet season at Altos del María (Appendix 2a). Combinations of these sites made up the combined elevational gradient (consisting of alleged “infected” El Copé and “non-infected” Campana transects) and epidemics gradient (pre, during, recent post and long post-epidemic) as follows (see also Chapter 2 of this thesis):

**Combined Elevational Gradient:**

**El Copé gradient (Coclé Province):** Cuatro Callitas at 45 m elevation, Palmarazo at 135 m elevation, La Rica at 250 m elevation, and El Copé at 760 m elevation. Of these sites, only Cuatro Callitas was outside of the boundaries of Parque Nacional General de
Division Omar Torrijos Herrera (PNDGOTH), a National Park which straddles the continental divide of the Cordillera Central. These four sites ran southwestward along an elevational gradient in continuous forest on the Atlantic versant for a distance of about 50 km.

**Campana gradient (Panamá Province):** Cerro Trinidad at 540 m elevation and Altos de Campana at 860 m elevation in Altos de Campana National Park boundaries of the Cordillera Central, located in the western portion of the Panama Canal Watershed. The two sites ran southwestward through continuous forest on the Atlantic versant, separated by a distance of about 25 km. Only two sites were surveyed along this transect because lowland forest on both versants has been decimated for agriculture, cattle ranching, and other land uses.

**Epidemics gradient: west (Chiriquí province) to east (Panamá Province):**
Fortuna (long post-epidemic), El Copé (recent post-epidemic), Altos del María (during-epidemic) and Altos de Campana (pre-epidemic) were sites of varying longitudes spanning southeastward at high elevations (>700 m) from western to central Panama along the Cordillera Central. Sites were chosen as such in order to follow the same pattern of disease spread as has been reported for chytridiomycosis in Panama (Lips et al. 2006). Altos del María was surveyed both before and during the inception of chytridiomycosis outbreak.

To describe *B. dendrobatidis* impacts on anuran communities at varying elevations and stages of epidemic infection, amphibian abundance and species richness as well as pathogen prevalence/intensity were compared across sites along the elevational transects and across sites along the epidemics transect. Because there were only two sites along the Campana elevational transect, sites from this transect and the El Copé elevational transects were merged into one combined elevational transect for all inter-site analyses in abundance and disease prevalence/intensity. To determine the extent to which *B. dendrobatidis* might be causing amphibian population decline, we compared anuran abundance and species richness between two sampling years for two low elevation sites (Palmarazo and Cuatro Callitas), one middle elevation site (Cerro Trinidad), and one high
elevation site (Altos de Campana). These inter-year comparisons were done using our current data from 2006, and past survey data from 2001 (Palmarazo and Cuatro Callitas) and 1995/96 (Cerro Trinidad and Altos de Campana) obtained by Dr. Roberto Ibáñez and colleagues. No data exists regarding the infection status of the lowland frog communities at Palmarazo and Cuatro Callitas from the 2001 sampling period, nor for the frog communities from the Campana elevational transect from 1995/96. For the purpose of our study we assumed that chytridiomycosis did not exist in the lowland populations along the El Copé elevational gradient at this time, as the chytrid did not impact the corresponding highland community of El Copé until 2004. Similarly, since the pathogen was not known to Altos de Campana at the time of this study in 2006, we assumed that frogs would have been free of the chytrid during the 1995/96 sampling period at these sites as well.

CHYTRID DIAGNOSIS

*Batrachochytrium dendrobatidis* prevalence and intensity were assayed using Real Time Quantitative PCR (RT-qPCR) of collected epidermal skin swabs. All DNA extractions and purifications of samples were done at McGill University according to the protocol outlined in Hyatt et al. (2007). After DNA extraction and purification was complete, samples were sent to the Rnomics Platform of Genome Quebec for RT-qPCR analysis, as developed by Boyle et al. (2004) and adapted for a singlicate assay by Kriger et al. (2006b). A thorough discussion of Chytrid Diagnosis procedures used prior to and for Real-Time Quantitative PCR Analysis of epidermal swab samples can be found in Chapter 2 of this thesis. A pilot study was done to ensure that singlicate analysis would be sufficient for this study (see Chapter 2). Samples deemed inhibited by qPCR analyses were omitted from all laboratory analyses. Along the El Copé elevational gradient, a total of 18 samples were inhibited. The Campana elevational gradient had 60 inhibited samples. A total of 52 samples were deemed inhibited along the epidemics gradient according to qPCR results and omitted from this study.
DATA AND STATISTICAL ANALYSES

Anuran survey data: Anuran abundance was calculated for every species (number of individuals/Search Effort Hour) and organized into stream/forest habitat and wet/dry seasons in all years for all sites, where data were available. Two-tailed randomization tests were used to determine statistical differences in anuran abundance between sites and years. Separate analyses were done on the combined elevational transect and on the epidemics transect. All tests were conducted using the Resampling Stats add-in for Microsoft Excel. Data permutations were done 10,000 times for all samples, with α = 0.05.

For inter-site analyses, we clumped all individual species’ abundances into one large grouping (“all frogs”) for each site for overall anuran abundance comparisons between sites. It was first necessary to account for inherent natural variation in site-to-site anuran abundance (due to elevation or otherwise) by normalization of the data. Species abundances along the elevational transects as well as the epidemics transect were normalized to the genus *Centrolenella* (“leaf frogs” guild) since species from this genus were common to all sites. Separate analyses were done on the elevational transects and the epidemics transect. The mean abundance of *Centrolenella* between all sites was calculated and site-specific scaling factors were determined for the separate elevational and epidemics transects. Species abundances at each site were scaled thusly. For the combined elevational gradients in 2001/1995 all data was normalized to the genus *Colostethus* (“diurnal stream frogs” guild). Because ecological guilds contained different species depending on the site, we grouped all species into their respective families (according to Savage 2002), calculated the mean “scaled” abundance for each family, and compared between sites for “all frogs” using the mean family abundances rather than individual species’ abundances.

For inter-year analyses, we addressed changes in lowland amphibian community composition between sampling years by grouping frog species into eight ecological guilds and comparing changes in abundance between them, as follows: “treefrogs”, “leaf-litter frogs”, “diurnal terrestrial frogs”, “diurnal stream frogs”, “leaf frogs”, “nocturnal stream frogs”, “riparian frogs” and “toads” (see Appendix 0 for guild membership). Ecological guild groupings were made based on the species’ usual life history traits; most guilds
contained species that were found in both stream and forest habitat at some point during our surveys. We did inter-year abundance analyses at only two lowland sites along the El Copé elevational transect (Cuatro Callitas and Palmarazo), but at both sites along the Campana elevational transect. We were not able to obtain past anuran survey data for the sites El Copé or La Rica and thus those sites are excluded from the El Copé transect inter-year analyses. Data was not available for inter-year analyses along the epidemics gradient.

A measure of relative species richness per site was obtained for each combination of habitat and season as a function of the number of species. Species richness differences are described as relationships in the form of percent differences. To ensure that species richness was dependent on the site and not on our search effort, we determined the cumulative number of species found as Search Effort Hours progressed and calculated what proportion of all species had been found by 75% of the total search period for each site.

To compensate for lack of data in certain seasons in certain sites, tests comparing sites or years were done on wet season and combined habitat data only. Since there is considerable evidence that habitat and season may affect chytrid epidemiology (Lips 2003, Berger et al. 2004, Woodhams and Alford 2005, Kriger and Hero 2006), we compared similar data to remove any discrepancies.

**Chytrid prevalence assays:** Chytrid prevalence was determined for every possible intra-site combination of season and habitat as a function of the number of positive samples/total samples, a mirror of the organization of abundance data. Multiple contingency Pearson chi-square tests were performed on raw binary infection status data (positive or negative) to test for inter-site differences in chytrid prevalence within the elevational and the epidemics transects separately ($\alpha = 0.05$). When sites were deemed statistically different in prevalence, Fisher’s exact tests between all possible pairs of sites were used to determine where the differences occurred. One-Way ANOVAs through randomization were used on log$_{10}$ transformed arithmetic mean zoospore data to test for differences in chytrid intensity between sites. We used One-tailed randomization tests to test for differences in intensity between Altos del María and three combined sites of the
epidemics gradient. Data permutations were done 10,000 times for all samples, with \( \alpha = 0.05 \). Confidence intervals (95%) based on a binomial distribution were calculated for all prevalence estimates, and 95% bootstrap confidence intervals (BCa) were calculated for all intensity estimates.

Prevalence was determined as a function of the number of positive samples/total number of samples. Analyses were done to test for inter-site differences in prevalence using samples of 1.0 or greater zoospores as positive for the first round of tests, but we re-ran the same tests using only samples of 10.0 or greater zoospores as positive, in order to emulate results that would have been attained using conventional PCR rather than the more sensitive RT-qPCR diagnosis that we used in this study. For tests of intensity, only positive samples (mean zoospore equivalents of \( \geq 1.0 \)) were included in analyses. Again, we conducted tests to determine inter-site differences in chytrid intensity using samples of \( \geq 1.0 \) zoospores, and then re-ran these tests using only samples where intensity was \( \geq 10.0 \) zoospores. All frogs were treated as one group to increase the sample size for analyses. Separate inter-site analyses were used for the elevational transects and the epidemics transect. Again, we compensated for lack of data in certain seasons in certain sites by comparing sites using only wet season and combined habitat data. Minitab 15.0 Statistical Software was used to test for differences in prevalence and to compute the confidence intervals for prevalence. We used the Resampling Stats add-in for Microsoft Excel to test for differences in pathogen intensity and to compute the bootstrap confidence intervals (BCa) for intensity.

**RESULTS**

**INTER-SITE DIFFERENCES IN CHYTRID PREVALENCE AND INTENSITY**

**Elevational gradients:** The chytrid fungus was detected in all sites along the combined elevational transects regardless of elevation or decline status (Appendix 2b). Visible signs of disease (lethargy, sloughing skin) were detected on at least 16 of all 321 sampled frogs from sites at all elevations along the El Copé elevational gradient, but no frogs along the Campana elevational gradient exhibited such signs of disease. Prevalence of the chytrid was fairly low for all sites, ranging from 9.6% at Palmarzo (CI: 4.5% to
17.4%, n = 94) to 22.2% at La Rica (CI: 8.6% to 42.3%, n = 27), and was not statistically different between the six sites (Fig. 3A).

Intensity of the chytrid was also not seen to be statistically different between the six sites (Fig. 3B), ranging from 3.1 (CI: 2.2 to 4.7, n = 4) mean zoospores at El Copé to 694.0 (CI: 9.4 to 2008.8, n = 12) mean zoospores in Cuatro Callitas. In Altos de Campana and El Copé, the vast majority of samples had mean zoospore equivalents of less than 10.0 (Fig. 3B). In fact, if site-specific prevalences were re-calculated to exclude samples whose mean zoospore equivalents were less than 10.0 (as in using conventional PCR), sites would show the following overall disease prevalence: Altos de Campana 0.1% (CI: 0.02% to 0.3%), Cerro Trinidad 0.6% (CI: 0.3% to 1.0%), El Copé 0.0% (CI: 0.0% to 1.1%), La Rica 1.1% (CI: 0.2% to 2.9%), Palmarazo 0.2% (CI: 0.02% to 0.7%), and Cuatro Callitas 0.6% (0.1% to 1.5%). When re-analyzing these inter-site differences in prevalence, sites were indeed deemed to be significantly different ($\chi^2 = 15.8, p = 0.008$, Pearson Chi-square test). Samples from Altos de Campana showed a significantly lower pathogen prevalence than samples from Cerro Trinidad ($p=0.003$, Fisher’s exact test), La Rica ($p=0.012$, Fisher’s exact test) and Cuatro Callitas ($p=0.032$, Fisher’s exact test). Chytrid intensity was still not deemed to be different between sites when samples with less than 10.0 mean zoospores were removed.

**Epidemics gradient:** Chytrid fungus was detected in all four sites along the epidemics gradient regardless of epidemic infection status (Appendix 2b). Visible signs of disease (lethargy, sloughing skin) were detected on a total of 14 of all 291 frogs sampled from El Copé and Altos del María, but no sign of disease was noted in frogs from Fortuna or Altos de Campana. There was a highly significant difference in chytrid prevalence between sites ($\chi^2 = 163.3, p<0.001$, Pearson Chi-square test, Fig. 4A). The highest prevalence was found in during-epidemic site Altos del María (78.3%, CI: 69.6% to 85.4%, n = 115) and the lowest was found at pre-epidemic site Altos de Campana (13.1%, CI: 9.3% to 17.8%, n = 267, Fig. 4A). Fortuna showed the second highest disease prevalence (27.0%, CI: 13.8% to 44.1%, n = 37) and recent post-epidemic site El Copé showed only a slightly higher prevalence (14.8%, CI: 4.2% to 33.7%, n = 27) than Altos de Campana (Fig. 4A). When Altos del María was removed from the transect and the three
other sites compared against one another, the difference in prevalence between those three sites (El Copé, Altos de Campana and Fortuna) was no longer highly significant. When these three sites were combined as one and tested against Altos del Maria, we again found a highly significant difference in prevalence (p<0.001, Fisher’s exact test).

Chytrid intensity was not found to be statistically different between the four sites, ranging from 3.1 (CI: 2.2 to 4.7, n=4) mean zoospores at El Copé to 2312.6 (CI: 694.2 to 4550.2, n=89) mean zoospores at Altos del Maria, a site of known disease outbreak during the time of study (Fig 4B). When the outlier site Altos del Maria was removed from the transect and the three other sites compared against one another, the difference in intensity between those three sites (El Copé, Altos de Campana and Fortuna) was non-significant. However, when these three sites were combined as one and tested against Altos del Maria, we found a highly significant difference in mean intensity (p<0.001, One-way ANOVA through randomization), with intensity at Altos del María found to be significantly higher than intensity at the three sites combined. In Altos de Campana, Fortuna, and El Copé, the vast majority of samples had mean zoospore equivalents of less than 10.0 (Fig 4B). In fact, if results were re-analyzed to exclude samples whose mean zoospore equivalents were less than 10.0 (as in using conventional PCR), sites would show the following overall infection prevalence: Altos de Campana 0.1%, El Copé 0.0%, Fortuna 0.8% and Altos del María 28.7%.

When re-analyzing the inter-site differences in prevalence with samples of less than 10.0 mean zoospores deemed negative, sites were still deemed to be significantly different ($\chi^2 = 79.4$, p<0.001, Pearson Chi-square test). With Altos del María removed, prevalence was still significantly different between the three sites, with Fortuna showing a higher prevalence than Altos de Campana (p=0.026, Fisher’s exact test). When these three sites were combined as one and tested against Altos del Maria, we again found a highly significant difference in prevalence (p<0.001, Fisher’s exact test). Chytrid intensity was still not deemed to be different between sites when samples with less than 10.0 mean zoospores were removed, nor was a difference in intensity found between Fortuna, El Copé, and Altos de Campana when Altos del Maria was removed from the analysis. However, when these three sites were combined as one and tested against Altos
del Maria, we found a weakly significant difference in intensity (p=0.057, Randomization test).

INTER-YEAR DIFFERENCES IN ABUNDANCE AND SPECIES RICHNESS
Along the El Copé elevational gradient, mean anuran abundance was found to be significantly lower in 2006 compared to 2001 for “all frogs” in the lowland site Cuatro Callitas (p=0.002, Randomization test, Fig. 5A). When frogs were analyzed as separate guilds, abundance of the “nocturnal stream frogs” was significantly lower in 2006 compared to 2001 (p=0.031, Randomization test), as was the abundance of the “leaf-litter frogs” (p=0.004, Randomization test, Fig. 5A). A similar trend was seen for many anuran guilds in Palmarazo, but this trend was not significant for overall anurans (p=0.666, Randomization test) or for any of the anuran guilds (Fig. 5A). Species richness was appreciably lower at these two sites in 2006 compared to in 2001; 40% lower in Palmarazo and 33% lower in Cuatro Callitas (Fig. 5B). Along the Campana elevational transect, a significantly higher abundance was seen in 2006 compared to 1995/96 for the “leaf-litter frogs” (p=0.044, Randomization test) in Cerro Trinidad but was masked for “all frogs” (Fig. 5C). No significant differences were found between anuran abundances in 2006 and 1995/96 in Altos de Campana (Fig. 5C). Compared to 1995/96 levels, species richness was slightly higher in 2006 for both sites (8% higher in Altos de Campana and 20% higher in Cerro Trinidad, Fig. D).

INTER-SITE DIFFERENCES IN FROG ABUNDANCE AND SPECIES RICHNESS
Elevational gradients: Overall anuran abundance did not differ significantly between any of the six sites along the combined elevational transects in 2006; overall abundance was lowest at El Copé and highest at Cerro Trinidad, but there were only slight variations in-between and high variance in abundance at each site (Fig. 6A). Species richness varied greatly between the six sites, with the lowest richness seen at El Copé, only 8% higher in La Rica, 24% higher at Cerro Trinidad, 43% higher in Palmarazo, 46% higher at Cuatro Callitas, and 51% higher at Altos de Campana (Fig. 6B). When all sites along both elevational transects in 2001/1995 were scaled and compared, no difference was found between any sites with respect to overall anuran abundance, but amphibian abundance
was highest and showed the most variance at Cuatro Callitas (Fig. 7A). Species richness was highly variable in 2001/1995, and highest in the lowland site Palmarazo, 11% lower at Cuatro Callitas, 38% lower in Altos de Campana and 68% lower at Cerro Trinidad (Fig. 7B).

**Epidemics gradient:** No significant differences in overall anuran abundance were found between sites along this gradient; anuran abundance at Altos del María was highest, with Altos de Campana abundance showing a close second, but all sites surveyed showed high variance in abundance (Fig. 8A). As shown in Figure 8B, species richness was found to be highest in the pre-epidemic site (Altos de Campana), 4% lower at the during-epidemic site (Altos del María), 56% lower at the recent post-epidemic site (El Copé) and 84% lower at the long post-epidemic site (Fortuna).

**DISCUSSION**

**EVIDENCE THAT B. DENDROBATIDIS IS AN ENZOOTIC PATHOGEN**

In order to conclude that the chytrid fungus was indeed a novel pathogen in Panama, we would have had to find at least one site free of the pathogen *B. dendrobatidis*. However, contrary to our expectations based on work done by Lips et al. (2006) in Panama, the chytrid fungus was detected in frogs at all sites examined, including all six sites along the two elevational transects, and all four sites along the epidemics gradient, including the pre-epidemic site. Infection prevalence and intensity were not found to differ between any of the sites along the elevational transects. However, both the prevalence of the chytrid and its intensity were found to vary significantly by site along the epidemics gradient. As would be expected, highest pathogen prevalence/intensity was seen in the site undergoing epidemic decline at the time of study, Altos del María. With the exception of this site, prevalence and intensity of the chytrid were low for the majority of anuran communities along both the elevational gradients and epidemics gradient; prevalence ranged from a low of 9.6% at the lowland site Palmarazo to a high of 27.0% at the long post-decline site Fortuna. Infection prevalence for these sites is within the range reported for enzootic chytridiomycosis in other regions (Alemu et al. 2008). Although we
cannot rule out that the novel pathogen hypothesis may explain chytridiomycosis emergence in Panama, our results do not support the novel pathogen hypothesis and provide evidence that the chytrid fungus is an enzootic pathogen.

The “novel pathogen hypothesis” (Lips et al. 2006, Skerratt et al. 2007) is problematic since reliable disease diagnostics cannot be done on the limited past data available to us, especially in Neotropical regions. Histology is the only way of diagnosing *B. dendrobatidis* on previously collected specimens, but with histology only high levels of infection can be detected, interpretable as representing the disease chytridiomycosis (Kriger et al. 2006a). To date, the majority of studies insisting that chytridiomycosis is a novel disease (e.g. Lips et al. 2006, Rachowicz et al. 2005) describe healthy amphibian populations when *B. dendrobatidis* is absent, and acute die-offs and subsequent population declines after detection of *B. dendrobatidis*. Generally the date of arrival of the chytrid into a given area is based on histological findings (e.g. Ron et al. 2003, Puschendorf et al. 2006, Lips et al. 2008). Studies using this method to date the arrival of the chytrid are thus only dating the incidence of the disease, chytridiomycosis, and not the pathogen *B. dendrobatidis*. By using conventional PCR techniques on swab samples obtained at El Copé prior to decline, Lips et al. (2006) presented better evidence that *B. dendrobatidis* was absent from an amphibian community prior to disease outbreak. However conventional PCR cannot detect less than a baseline of 10 zoospore equivalents per sample (Annis et al. 2004). Low, innocuous infections that do not exceed this baseline will remain undetectable with this method. Only Real-Time quantitative PCR is capable of detecting very low levels (1 zoospore) of *B. dendrobatidis* and, according to some authors, should be adopted as the primary tool for pathogen diagnostics (Kriger et al. 2007a).

Through our use of the highly sensitive detection technique of RT-qPCR, we determined mean infection intensity to be low across all sites not undergoing decline in our study, and the vast majority of anurans showed an infection of less than 10.0 mean zoospore equivalents. If we were to have relied upon conventional PCR instead of RT-qPCR for disease diagnosis, detection of chytrid prevalence would have dropped considerably for all sites in this study, and save for Altos del María, infection prevalence would have been close to 0.0% (between 0.0% and 1.1%, site depending). It is likely that
through the use of conventional PCR techniques for disease diagnosis, Lips et al. (2006) could have easily missed low, enzootic levels of *B. dendrobatidis* that were likely always present in the amphibian community of El Copé prior to the chytrid-induced decline. Instead, it was concluded that the infection was absent from the population prior to decline, when in fact the detection techniques used in this study were not adequate to make this conclusion. Indeed, when our infection data was re-analyzed for differences in prevalence among sites with only samples of 10.0 zoospores or greater deemed positive, we found that prevalence did indeed differ for the elevational gradient, in addition to the epidemics transect. As suspected, pathogen prevalence was significantly lower at the pre-epidemic highland site Altos de Campana, and this was the case along both gradients. This result is more in line with what we had predicted since we had not expected to find the pathogen at Altos de Campana.

We recognize that amphibian communities in the known “infected” study sites may be maintaining the chytrid as an enzootic infection post “novel” invasion (Woodhams and Alford 2005), and we do not rule out that amphibian communities in the “uninfected” sites may have only recently been hit by a strain of the “novel” pathogen and have not yet declined. However, we do agree with other authors that the novel pathogen hypothesis is oversimplistic (eg. McCallum 2005) and we speculate from our results that *B. dendrobatidis* is likely present at innocuous levels in amphibian communities long before disease outbreaks occur. It is known that *B. dendrobatidis* can exist in a stable host-parasite relationship without causing pathogenic effects, since amphibians with no clinical signs of disease frequently carry light infections in the wild (Hanselmann et al. 2004, Retallick et al. 2004, McDonald et al. 2005). Thus, the chytrid may still be an enzootic pathogen even if the disease chytridiomycosis is indeed novel and invasive. Or, perhaps a more virulent strain of the chytrid has emerged and spread invasively as a novel disease into areas where more benign strains have always been present (Morehouse et al. 2003). Despite genetic similarities in *B. dendrobatidis* strains, it is likely that chytrid effects would vary geographically; differential confounding environmental factors might cause an enzootic *B. dendrobatidis* strain to emerge as an epizootic pathogen in some areas, and be introduced as a novel pathogen in others (Wright et al. 2001). Perhaps alone neither emergence hypothesis can explain chytrid-related amphibian decline.
We therefore propose a third, neutral hypothesis, whereby the chytrid is an epizootic disease like any other (the flu, polio, plague, etc.), having multiple similar strains of varying virulency, and prone to outbreaks under the right conditions. Currently in the Neotropics conditions may be optimal for pathogenic outbreaks, but likely outbreaks occurred in the past and will continue to occur. Despite assertions from other authors that *B. dendrobatidis* is definitively novel (eg. Skerratt et al. 2007, Lips et al. 2008), our results do not support this conclusion. Chytridiomycosis may be moving in an observable “wave” of infection in Panama as Lips et al. (2006) propose, even if the pathogen is enzootic (Pounds et al. 2006). Further research is needed to substantiate our claim, and this could be accomplished with more intensive sampling in advance of the predicted disease wave in Panama using the highly sensitive RT-qPCR diagnostics on many samples. It is estimated that negative samples from at least 300 individual frogs are needed to be 95% confident that the infection is not present (Ibáñez, Pers. Comm., 2006).

**ELEVATIONAL DISTRIBUTION OF PATHOGEN AND DISEASE**

Our results prove that the chytrid fungus is present at low and middle elevations in central Panama. The impact on higher elevation amphibians in the Tropics has been the fundamental observation behind most research to date on the disease, and has been explained in terms of the chytrid’s proposed reliance on lower temperatures (~23°C) for survival. It has been thought that warmer climates in middle or lower elevation sites in the Tropics may limit chytrid distribution (Longcore et al. 1999). Regardless, we detected symptoms of chytridiomycosis (lethargy, sloughing skin) from at least 16 of 321 frogs sampled at all elevations along the El Copé elevational gradient. Anuran abundance was indeed significantly lower overall and for both “leaf-litter frogs” and “nocturnal stream frogs” in 2006 as compared to 2001 in the site Cuatro Callitas at 45 m elevation, but no significant inter-year differences in abundance were detected at Palmarazo at 135 m. Species richness was noticeably lower at both of these sites in 2006 compared to in 2001. Thus the *B. dendrobatidis* pathogen is not only present at low elevations but appears to be causing chytridiomycosis and may be causing subsequent amphibian population decline, at least in one locality. Although our results do not prove conclusively that chytridiomycosis is the agent of decline at Cuatro Callitas, the
invariable presence of the chytrid at low elevation sites in Panama and the visible symptoms of chytridiomycosis displayed by some frogs is adequate evidence that the chytrid is indeed having at least some adverse effects at lower elevations.

Thus the pathogen appears to be capable of inflicting damage on hosts at temperatures beyond the thermal optimum under natural field conditions. Perhaps then low humidity and higher temperatures at lower elevations may not limit the pathogenicity of the chytrid as some authors contend (La Marca et al. 2005). This would not be surprising since even under the most extreme temperature regimes there would always be microhabitats available to amphibians that are moist and cool enough for zoospores to survive and re-infect their hosts. Certainly climatic trends at the microhabitat scale can dwarf local ambient climatic trends, and in some places the trends might even be entirely opposing (Pounds et al. 2006).

The importance of an abiotic factor like temperature on pathogenicity means that environmental change may have drastic effects on the host-pathogen relationship. Global warming could feasibly cause a temperature-dependent enzootic pathogen to emerge as an epizootic (Pounds and Crump 1994, Pounds et al. 1999, Pounds et al. 2006 and Bosch et al. 2007). Increased cloud cover typical of climate change over mountainous tropical forest decreases daytime temperatures and increases night-time temperatures, promoting temperatures that shift the fungus towards maximal survival and reproduction at not only higher elevations but a variety of elevations (Pounds et al. 2006). There is recent evidence that *B. dendrobatidis* has a more complex ecology than was originally thought and appears to do well at a variety of temperatures (Woodhams et al., 2008a). Thus the effects of variable environmental temperatures are likely to be complex. Pounds et al. (2006) found that patterns of amphibian extinction varied over an elevational range, and extinctions actually peaked at middle elevations. They suggested that both warmer temperatures and colder temperatures may limit the pathogenicity of chytrid fungus. Rapid climate change via anthropogenic global warming is a double-edged sword with respect to the disease chytridiomycosis since it can both promote a more prosperous environment for the pathogen and simultaneously cause host immunosuppression. Because the disease is so highly contagious, a single frog already lightly infected with the
pathogen could quickly cause a community-wide epizootic if infection levels increased drastically through immunosuppression (Blaustein and Kiesecker 2002).

The observed differential disease effects in the lowlands are perhaps due to a number of factors intrinsic to the sites. The higher infection prevalence and intensity in Cuatro Callitas compared to Palmarazo perhaps led to the greater disease pathogenicity and subsequent decline at that site. Though at a lower elevation, Cuatro Callitas may have presented the chytrid with a more favourable environment than Palmarazo, according to the “chytrid thermal-optimum hypothesis” (Pounds et al. 2006). Additionally, Cuatro Callitas was the only site outside of the boundaries of Parque Nacional General de Division Omar Torrijos Herrera, and amphibians may have been more susceptible to disease at this site due to increased stress and decreased immune function resulting from other anthropogenic decline factors. Certainly increased forest extraction was noted at this site, and the close proximity of farmland to the survey streams may have resulted in water contamination, although the quantification of these factors were out of the scope of this study. Declines at lower elevations are more likely to be overlooked due to the myriad other decline factors present; this runs counter to declines in “pristine” higher elevation sites which are usually easier to quantify (Wright et al. 2001). Because of the potential for additional decline factors to have caused the observed population decline at this site, it would be very difficult to tease apart the effects of chytridiomycosis from the effects of the other factors. Furthermore, there is a strong likelihood that these factors were working synergistically to cause the decline and that any one factor alone may not have been able to result in the pattern we observed.

Finally, it is important to acknowledge that any apparent population decline observed in this study may not be indicative of overall trends given that only two sampling years of data were available with which to make comparisons. Our ability to accurately detect declines at many elevations in the Neotropics is compromised since a confident detection of a “decline” depends on the intensity and duration of investigation (Wright et al. 2001). This reality clearly signifies the importance of long-term studies on amphibians in areas infected with chytridiomycosis, and this is especially true in lowland areas when other decline factors must also be accounted for. Indeed Ibáñez et al. (2002) reported that high year-to-year variations in all populations of amphibians in Panama underscore the need
for long-term monitoring. Amphibian populations are known to be inherently variable, and evidence from one long-term amphibian population study proves that populations wax and wane significantly on an annual basis; conclusions about a population’s decline status from any one year may not be reflective of the overall population status (Green 2003). Furthermore, abundances of individual species in our study were highly variable and non-normal across sites, and in order to do non-parametric statistical analyses on abundance data we had to group species into ecological guilds, resulting in mean abundances confounded by very high variance. Therefore, most differences in abundance between sampling years were probably masked due to the high variance, and deemed insignificant as a result. This artefact was especially pronounced when comparing overall abundances of all anurans between sites, since post-decline sites were characterized as supporting very few but highly abundant disease-resistant species, and pre-decline sites supported many, less abundant species. From these results it is clear that chytridiomycosis does not completely obliterate anuran communities with respect to overall abundance and biomass. Moreso it poses a severe threat to biodiversity, as is clearly exhibited by the marked differences in species richness between years and between sites at varying elevations and stages of decline.

EVIDENCE OF HOST PATHOGEN-RESISTANCE

The continuous persistence and seemingly innocuous effects of the pathogen in Fortuna more than a decade post-decline is substantial evidence that the chytrid persists after major population crashes. One individual of Eleutherodactylus podiciferous, a forest-dwelling leaf litter frog that apparently does not succumb to decline, was found to maintain a high degree of B. dendrobatidis infection in Fortuna (12,850 mean zoospore equivalents) without showing any evidence of disease. Retallick et al. (2004) presented the first quantitative evidence for endemic infection in Taudactylus eungellensis 5 years post-decline, McDonald et al. (2005) documented chytrid enzootism in populations of Litoria genimaculata in Australia post-decline, and Briggs et al. (2005) noted a similar phenomenon in Rana muscosa in North America. In all of these cases, chytridiomycosis resulted in near disappearance of the amphibian species but the surviving remnant
populations successfully returned to preDecline numbers with individuals maintaining some degree of infection.

CONCLUSIONS AND CONSERVATION IMPLICATIONS

We suspect that *B. dendrobatidis* has always been enzootic throughout central Panama in Neotropical amphibian communities at all elevations. This speculation is at odds with some authors that maintain that the pathogen is novel in Panama, and reflects the importance of continued research in this area. Until very recently, pathogen diagnostic techniques were not adequately sensitive to confirm that the pathogen is definitively novel in Panama, and as a result we are not convinced of this conclusion. More work is needed in Panama to determine *B. dendrobatidis* distribution ahead of the predicted disease “wave” using sensitive RT-qPCR diagnostic techniques.

Very little research has been done at lower elevations in the Neotropics, and most authors maintain that lowland amphibians do not succumb to chytridiomycosis because warmer lowland climates limit the chytrid’s survival. However, we have found that *B. dendrobatidis* is distributed at all elevations and does indeed appear to be causing disease effects in lowland amphibians. Currently our understanding of chytrid ecology is hampered by a reliance on laboratory work to determine such key facets of the chytrid’s biology as its response to temperature, and laboratory results may not be indicative of responses under natural field conditions. Clearly the role of temperature on disease effects of chytrid fungus in the field needs re-evaluating. Current research in this area has focused on broad-ranging climatic trends, and more research is needed to determine abiotic and biotic factors that encourage pathogenicity and transmission of *B. dendrobatidis* in situ at the microhabitat scale.

In Panama, conservation efforts to thwart the destructive effects of chytridiomycosis on amphibian populations have been to track the spread of the disease and remove amphibians ahead of the infection wave for captive breeding. *Ex situ* breeding of amphibians is problematic because it is unclear whether future reintroduction is possible given our lack of knowledge about chytrid ecology. Unnatural size reduction of wild populations through removal for such programs could decrease genetic variability and hence disease resistance in the wild, and animals bred in chytrid-free areas in captivity
will be bred without disease resistance (Altizer et al. 2003). Host-parasite co-evolution between amphibians and the chytrid has highly significant implications for amphibian conservation. Since the chytrid is present at all elevations and stages of amphibian decline, removal of animals for captive breeding programs and subsequent re-introduction could fail without selective breeding for resistance. If host-parasite co-evolution is a real phenomenon and there exists such a wide range of poorly understood differential host responses to the chytrid, the removal of wild healthy animals from infected areas for captive breeding programs may be counter-productive. While captive breeding is an important “last-ditch” effort for amphibian conservation, it should be accompanied by rigorous scientific research into additional management programs for declining amphibians.
Figure 3. Chytrid prevalence (3A) and intensity (3B) by site along two elevational transects (post- and pre-decline) in the wet season in Panama in combined habitat in 2006. In Figure 3A, the two bars in each site panel represent frequency of samples positive and negative for chytrid, and the percent numbers are overall chytrid prevalence. Sites were not different with respect to either prevalence or intensity.
Figure 4. Chytrid prevalence (4A) and intensity (4B) by site along an epidemics transect (pre, during, recent post and long post-decline) in the wet season in Panama in combined habitat in 2006. In Figure 4A, the two bars in each site panel represent frequency of samples positive and negative for chytrid, and the percent numbers are overall chytrid prevalence. Sites were significantly different with respect to infection prevalence (Chi-square test, $\chi^2=163.3$, p<0.001) but not intensity.
Figure 5: Mean abundance (Fig. 5A and 6A) of anurans (+/- SD) and species richness (Fig. 5B and 6B) in the wet season for two sampling years along two elevational gradients in Panama (combined habitat). “All frogs” is the total # frogs found/SHE for each site, and the guild abundances are made up of the mean of all individual species’ abundances for each guild. Abundance was statistically higher in 2001 than in 2006 for “all frogs” (p=0.002), “nocturnal stream frogs” (p=0.031), and “leaf-litter frogs” (p=0.004) in Cuatro Calitas. Abundance was significantly higher in 2006 than in 2001 for the “leaf-litter frogs” in Cerro Trinidad (p=0.044).
Figure 6: Scaled mean family abundance (+/-standard deviation) and species richness of all frogs at sites of varying elevations along both elevational transects (post- and pre-decline) in the wet season in Panama in 2006. Scaled mean family abundances in Fig. 6A were normalized to the genus *Centrolenella* (“leaf frogs”). The inlay in Fig. 6A shows the unscaled mean abundances (+/- standard deviation) at each site. No differences in overall anuran abundance were found between sites in 2006, but variance was high. Species richness was highest at the pre-decline highland site Altos de Campana and lowest at post-decline highland site El Copé (Fig. 6B).
**Figure 7**: Scaled mean family abundance (+/-standard deviation) and species richness of all frogs at sites of varying elevations along both elevational transects (post- and pre-decline) in the wet season in Panama in 2001/1996. Scaled mean family abundances in Fig. 7A were normalized to the genus *Colostethus* (“diurnal stream frogs”). The inlay in Fig. 7A shows the unscaled mean abundances (+/- standard deviation) at each site. Overall anuran abundance in 2001/1996 was not different between sites. Species richness was highest at the El Copé elevational transect lowland site Palmarazo and lowest at the Campana elevational middle elevation site Cerro Trinidad (Fig. 7B).
Figure 8: Scaled mean family abundance (+/-standard deviation) and species richness of all frogs along an epidemics gradient (sites of varying stages of epidemic infection) in the highlands in the wet season in Panama in 2006. All scaled mean family abundances in figure 8A were normalized to the genus *Centrolenella* (“leaf frogs”), an anuran genus common to all sites in stream habitat. The inlay in figure 8A shows the unscaled mean abundances (+/- standard deviation) at each site. Overall anuran abundance was not different between sites when habitats were combined, but variance in abundance was high at each site. Species richness decreased with increasing length of time since infection (Fig. 8B).
MANUSCRIPT TWO EXPLANATIONS

This second manuscript (Chapter 4) pursues the question of differential disease effects among anurans infected with the chytrid fungus, *Batrachochytrium dendrobatidis*. In my first manuscript I find and discuss results that provide evidence that the chytrid fungus is present throughout central Panama at all elevations and stages of epidemic infection, including both post-decline and pre-decline sites. Despite extensive chytrid-induced decline that had occurred at some sites in this study, many amphibian species still remain, thus showing differential susceptibility to the disease, chytridiomycosis, caused by this generalist pathogen. In this manuscript I identify some ecological factors that contribute to differential pathogen presence that may contribute to differential disease effects. This is an important aspect of the host-pathogen relationship that could provide insight into improving conservation initiatives.

Many ecological factors have been cited in the literature that are thought to lead to differential pathogen susceptibility in anurans, notably: season, host body size, habitat requirements of host and pathogen, host behaviour, host population density, and immunosuppression in response to changing environmental conditions. Of these commonly cited factors, I sought to determine the extent to which habitat, season, and anuran body size affected the chytrid’s distribution and its effects on anuran abundance and species richness in Panama. I find that the degree of *B. dendrobatidis* prevalence and intensity does not necessarily dictate disease effects, since more aquatic species showed declines in abundance than terrestrial species, even though the pathogen was equally prevalent in both habitats and higher in intensity in the forest for some sites. Within two observed frog species, smaller individuals were more likely to show infection. Season may not play a role in differential infection in anurans, as pathogen prevalence was different between seasons for only two sites, and this difference was not the same for both sites.
CHAPTER 4: DIFFERENTIAL EFFECTS OF HABITAT, SEASON, AND ANURAN BODY SIZE ON INFECTION PREVALENCE AND INTENSITY OF THE CHYTRID FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS*

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Abstract: The chytrid fungus, *Batrachochytrium dendrobatidis*, has been implicated in many amphibian population declines globally, and most notably in the Neotropics. The chytrid is a generalist pathogen, potentially infective to all species of amphibian. However, certain species of amphibians show increased susceptibility to the disease caused by *B. dendrobatidis*: chytridiomycosis. If disease susceptibility in species differs as a function of ecology, then the degree of chytrid infection and disease may vary for species as a result of differing ecological factors. We determined pathogen prevalence and intensity and assayed the corresponding impacts on anuran abundance and species richness in relation to three ecological factors: habitat, season, and anuran body size. Eight sites throughout Panama were surveyed, and we used RT-qPCR for chytrid diagnosis of collected skin samples. Pathogen prevalence and intensity did not necessarily correlate with disease effects, especially with respect to habitat; aquatic frog species showed more declining trends in abundance and richness than terrestrial species, even though chytrid prevalence did not differ between habitats, and intensity was higher in the forest than in the stream at some sites. Smaller body sizes showed higher prevalence in some species, and season was a weak, and variable, predictor of chytrid presence.
INTRODUCTION

The global amphibian population decline phenomenon has sparked extensive scientific and media attention over the last decade. Initially, evidence for declines was anecdotal but by the early 2000’s quantitative studies had shown that amphibian populations were under siege (eg. Houlihan et al. 2000, Green 2003, La Marca et al. 2005, Lips et al. 2005b). There remains uncertainty about which factors contribute most to amphibian population declines (McCallum 2005). Although habitat loss is rated the primary cause of amphibian population decline, amphibian communities in pristine areas of the montane tropics are also experiencing die-offs, and thus other “enigmatic” factors, such as emerging diseases, global warming, and contamination, may be at work (Stuart et al. 2004). Unfortunately, our lack of knowledge about the animals in decline limits the conclusions we can make concerning the causes (Lips et al. 2005b).

Among the enigmatic factors influencing global amphibian population decline is the chytrid fungus, Batrachochytrium dendrobatidis. This pathogen of amphibians was first detected in 1998 in many declining amphibian populations in both Panama and Australia, and causes the disease chytridiomycosis (Berger et al. 1998). Batrachochytrium dendrobatidis can be considered as a generalist pathogen, and is potentially infective to all species of amphibians (Woodhams et al. 2006a). However, although many amphibian species in chytrid infected areas have suffered great declines, other sympatric species have not (Lips et al. 2005a), indicating that certain species of amphibians may be more susceptible to chytridiomycosis than others. This variable resistance may be related to the morphology, physiology, phylogeny and/or the ecology of the host amphibians (Stuart et al. 2004, Lips et al. 2003), including such factors as habitat (Lips et al. 2003, Woodhams and Alford 2005), body size (Lips et al. 2003), behaviour (Retallick et al. 2004; Woodhams and Alford 2005), population density (Lips et al. 2003), phylogenetics (Stuart et al. 2004), immunosuppression in response to changing environmental conditions (Daszak et al. 2004), and differences in the number and type of antimicrobial peptides present in the dermal glands (Amiche et al. 1999).

Host habitat, seasonality and anuran body size are three of the many ecological factors known to affect amphibian susceptibility to B. dendrobatidis and chytridiomycosis that
are most commonly cited in the literature (Aplin and Kirkpatrick 2000, Lips et al. 2003, Berger et al. 2004, Beard and O’Neill 2005, Woodhams and Alford 2005, Bosch et al. 2007, Kriger and Hero 2007, Kriger et al. 2007b). There are seemingly ecological correlates with chytrid infection and disease susceptibility for all of these factors in the Tropics, yet this does not appear to be universal among regions, altitudes, or species. For example, in both Panama and Australia, declining amphibian species tend to have aquatic larvae and live in riparian habitat, whereas persisting species generally have direct-development (no larvae) and inhabit terrestrial leaf-litter habitat (Lips et al. 2003, Woodhams and Alford 2005). In Eastern Australia, prevalence of the fungus is also known to be much higher in stream-breeding amphibians than in terrestrial or ephemeral pond breeders (Kriger and Hero 2007). Conversely, in Central Spain, those species most affected by chytridiomycosis are the most terrestrial of species (Bosch et al. 2007).

Seasonality in the Tropics influences the prevalence and pathogenicity of chytridiomycosis through intrinsic differences in environmental conditions (Kriger et al. 2007b), as well as through promoting differential behaviours in some amphibian species that may alter their susceptibility to disease (Woodhams and Alford 2005). Because the thermal optimum for *B. dendrobatidis* growth is approximately 23°C and less (Longcore et al. 1999), increased chytrid-induced mortality usually occurs in amphibians during seasons of lower temperatures (Aplin and Kirkpatrick 2000, Berger et al. 2004). During seasons of higher temperatures, amphibians may rid themselves of disease (Kriger and Hero 2006). Seasonal changes in precipitation might also contribute to pathogen seasonality, and certainly in Spain and Australia, *B. dendrobatidis* infections are more common during wetter periods (Bosch et al. 2007, Kriger et al. 2007b). On the contrary, Drew et al (2007) maintain that increased rainfall does not coincide with increased infection prevalence or intensity in amphibians. Instead, pathogenic effects might be heightened during drier months when the congregation of anurans in wet areas facilitates pathogen spread (Ibáñez et al. 2002).

Within a given anuran species, smaller body sizes show higher *B. dendrobatidis* infection rates than larger body sizes, and this is particular true for *Litoria lesueuri* in Australia (Kriger et al. 2007b) and *Eleutherodactylus coqui* in the Neotropics (Beard and O’Neill 2005). Hence, frogs may either outgrow infection, have infection-stunted
growth, or succumb to disease-induced mortality (Kriger et al. 2007b). Alternatively, anurans of different age classes may exhibit differential behaviours that alter disease susceptibility; in *E. coqui*, subadults are generally found to perch closer to the forest floor than are adults of some species (Beard et al. 2003, Beard and O’Neill 2005). This may make them more susceptible to a water-borne pathogen like *B. dendrobatidis* since the greater moisture content that is found in leaf litter is preferable to subadults (Pough et al. 1983). The relationship between body size and chytrid infection does not appear to hold true across species or altitude; large species are more likely to decline than small species at high elevations, less likely to decline at lower elevations, and amphibians of all sizes with broad elevational ranges generally do not decline (Lips et al. 2003).

Currently there is a profound lack of robust scientific data regarding the roles played by seasonality, habitat, and anuran body size on chytrid prevalence and pathogenicity in the Neotropics, which undoubtedly leads to contradictions in the literature. Not surprisingly, this greatly impedes our ability to properly manage declining amphibian populations. In the Neotropics, most of the detailed work done to date on chytrid-induced amphibian declines has occurred in Panama, where chytridiomycosis has spread in a southeastward “wave” of infection at a reported rate of 30 km/year since 1996 (Lips et al. 2006).

How can a single water-borne disease variably affect such a large proportion of amphibians? Within Panama, can we identify any ecological factors that increase disease susceptibility in amphibians? Do species sharing similar life history characteristics show similar trends in disease-induced decline? Are these species affected similarly across a range of elevations and stages of epidemic decline in Panama? Since chytridiomycosis is an agent of decline for many amphibian species, the degree of chytrid infection (prevalence or intensity or both) should have a bearing on disease-related decline. If this is true, anurans experiencing greater chytrid infection as a result of habitat preference, season, or body size should show decreases in anuran abundance and species richness. Drawing from current literature, we predicted that anurans highly associated with streams would show higher infection prevalence and/or intensity and lower abundance and species richness in response to disease-induced decline; that *B. dendrobatidis* would be more virulent in the wet season; and that anurans of smaller body sizes would show
greater infection prevalence and intensity. To comment on variations in abundance and species richness between species, we grouped frogs into ecological guilds based on natural history traits. Our goal was to determine if any of the identified ecological trends in pathogen prevalence and intensity could be applied throughout Panama at different elevations and stages of epidemic decline, and whether these trends were universally applicable outside of Panama as reported by other authors. Moreover we sought to determine whether *B. dendrobatidis* infection prevalence/intensity is a consistent predictor of disease effects in Panama.

**MATERIALS AND METHODS**

**STUDY SITES, SAMPLE COLLECTION AND SURVEYS**

Sampling to compile anuran abundance and species richness surveys and assay for the presence of the chytrid and the disease occurred at eight sites of varying elevations and stages of infection in Panama over a 9-month period in 2006, beginning February 18th and ending October 28th. All caught animals were examined for symptoms of chytridiomycosis (lethargy, sloughing skin). We collected epidermal skin swab samples from a total of 1,252 frogs over 530.25 Search Effort Hours. We sampled 59 different species of 15 genera and 6 families (according to Savage 2002) of both sexes and of both juvenile and adult age classes from a wide range of body sizes. Surveys were conducted by day and night in forest and stream habitat in the dry season and in the wet season (see Chapter 2) for all sites except Cuatro Callitas, Altos de Campana and Cerro Trinidad, which were surveyed in the wet season only (Appendix 2a). No forest surveys were done in the wet season at Altos del María (Appendix 2a). Combinations of these sites made up the combined elevational gradients (alleged “infected” El Copé and “non-infected” Campana) and epidemics gradient (pre, during, recent post and long post-epidemic) that were surveyed during this study: (see also Chapter 2 of this thesis):

**Combined Elevational Gradients:**

**ElCopé gradient (Coclé Province):** Cuatro Callitas at 45 m elevation, Palmarazo at 135 m elevation, La Rica at 250 m elevation, and El Copé at 760 m elevation. Of these sites, only Cuatro Callitas was outside of the boundaries of Parque Nacional General de
Division Omar Torrijos Herrera (PNDGOTH), a National Park which straddles the continental divide of the Cordillera Central. These four sites ran southwestward along an elevational gradient in continuous forest on the Atlantic versant for a distance of about 50 km.

Campana gradient (Panamá Province): Cerro Trinidad at 540 m elevation and Altos de Campana at 860 m elevation in Altos de Campana National Park boundaries of the Cordillera Central, located in the western portion of the Panama Canal Watershed. The two sites ran southwestward through continuous forest on the Atlantic versant, separated by a distance of about 25 km. Only two sites were surveyed along this transect because lowland forest on both versants has been decimated for agriculture, cattle ranching, and other land uses.

Epidemics gradient: west (Chiríquí province) to east (Panamá Province):
Fortuna (long post-epidemic), El Copé (recent post-epidemic), Altos del María (during-epidemic) and Altos de Campana (pre-epidemic) were sites of varying longitudes spanning southeastward at high elevations (>700 m) from western to central Panama along the Cordillera Central. Sites were chosen as such in order to follow the same pattern of disease spread as has been reported for chytridiomycosis in Panama (Lips et al. 2006). Altos del María was surveyed both before and during the inception of chytrid outbreak.

CHYTRID DIAGNOSIS
Batrachochytrium dendrobatidis prevalence and intensity were assayed using Real Time Quantitative PCR (RT-qPCR) of collected epidermal skin swabs. All DNA extractions and purifications of samples were done at McGill University according to the protocol outlined in Hyatt et al. (2007). After DNA extraction and purification was complete, samples were sent to the Rnomics Platform of Genome Quebec for RT-qPCR analysis, as developed by Boyle et al. (2004) and adapted for a singlicate assay by Kriger et al. (2006b). A thorough discussion of Chytrid Diagnosis procedures used prior to and for Real-Time Quantitative PCR Analysis of epidermal swab samples can be found in Chapter 2 of this thesis. A pilot study was done to ensure that singlicate analysis would
be sufficient for this study (see Chapter 2). Samples deemed inhibited by qPCR analyses were omitted from all laboratory analyses. Along the El Copé elevational gradient, a total of 18 samples were inhibited. There were 60 inhibited samples along the Campana elevational gradient. A total of 52 samples were deemed inhibited along the epidemics gradient.

DATA AND STATISTICAL ANALYSES

**Anuran survey data:** Anuran abundance was calculated for every species (number of individuals/Search Effort Hour) and organized into stream/forest habitat and wet/dry seasons in all years for all sites, where data were available. We tested within sites and between sites for differences in frog abundance by season and/or habitat, where data were available. For inter-site comparisons, we used separate analyses to compare sites along the combined elevational transects and sites along the epidemic transect. For all analyses, we determined seasonal and habitat differences in abundance for “all frogs” and between frogs composing eight different ecological guilds. To obtain the “all frogs” category, we clumped all individual species’ abundances into one large grouping. Ecological guild groupings were made based on the species’ usual life history traits; most guilds contained species that were found in both stream and forest habitat at some point during our surveys. Two-tailed randomization tests were used to determine statistical differences in anuran abundance by habitat and season for “all frogs” within each site, and One-way ANOVAs through randomization were used to compare differences in abundance between ecological guilds within each site and between sites for all available combinations of season and habitat. Separate analyses were done on the combined elevational transects and on the epidemics transect. All tests were conducted using the Resampling Stats add-in for Microsoft Excel. Data permutations were done 10,000 times for all samples, with \( \alpha = 0.05 \). To compensate for lack of data in certain seasons in certain sites, inter-site comparisons were made only on wet season and combined habitat data.
A measure of relative species richness per site was obtained for each combination of habitat and season as a function of the number of species. Species richness differences are described as relationships in the form of percent differences. To ensure that species richness was dependent on the site and not on my search effort, we determined the cumulative number of species found as Search Effort Hours progressed and calculated what proportion of all species had been found by 75% of the total search period for each site.

**Chytrid prevalence assays:** Chytrid prevalence was determined for all possible intra-site combination of season and habitat as a function of the number of positive samples/total samples. For pathogen prevalence and intensity tests between habitats and seasons, we only compared between “all frogs”, as sample sizes were not sufficient to test for inter-guild differences. Fisher’s exact tests were used for all prevalence tests, and we tested between seasons for each of the two habitat types, and between habitats for each available season using an alpha significance level of 0.05. We used the same sample combinations to test for differences in chytrid intensity using randomization tests (10,000 permutations, \( \alpha = 0.05 \)) on \( \log_{10} \) transformed arithmetic mean zoospores. Only positive samples (mean zoospore equivalents of \( \geq 1.0 \)) were included in analyses involving chytrid intensity. Raw binary disease status data (positive or negative) was used in all analyses involving prevalence. Confidence intervals (95%) based on a binomial distribution were calculated for all prevalence estimates, and 95% bootstrap confidence intervals (BCa) were calculated for all intensity estimates. Statistical tests and confidence intervals for prevalence were done using Minitab 15.0 Statistical Software. All analyses involving randomization, as well as calculations of the bootstrap confidence intervals for mean intensity, were calculated using the Resampling Stats add-in for Microsoft Excel.

To determine whether anuran body size predicts chytrid prevalence and/or intensity, we first identified six species of frogs where numbers of individual samples across sites were great enough for logistic and linear regression analyses using Minitab 15.0 (**Colostethus panamensis, Colostethus talamancae, Colostethus flotator, leutherodactylus crassidigitus, Eleutherodactylus cerasinus and Eleutherodactylus cruentus**). We pooled all samples from the various sites for each species and ran binary logistic regression on
infection status (positive/negative) as a function of Snout Vent Length in mm (SVL) for each species. Linear regression was run on infection intensity using rank transformed arithmetic mean zoospore data. Again, only positive samples were used in the chytrid intensity analyses. Again, tests of difference were rejected at an alpha level of 0.05.

RESULTS

OVERALL TRENDS IN ABUNDANCE AND SPECIES RICHNESS

**Elevational gradients:** Across the combined elevational gradients, anuran guilds showed differential abundance and highly variable species richness, but overall abundance differences between sites were not deemed statistically significant (One-way ANOVA through randomization, \(p = 0.206\)). Overall, the “leaf-litter frogs” showed the lowest mean abundance (n=18), and the “diurnal stream frogs” showed the highest mean abundance but with a high variance in abundance on the one species making up this group (Fig. 9). The “leaf-litter frogs” showed the highest species richness of all groups, but overall mean abundance was fairly low (Fig. 9).

**Epidemics gradient:** Across the epidemics gradient, anuran guilds were differentially abundant and showed highly variable species richness, but overall abundance differences between sites were not deemed statistically significant (One-way ANOVA through randomization, \(p = 0.829\)). Of all guilds, the “diurnal stream frogs” were the highest in mean abundance (represented by only one species), “treefrogs” second in abundance (n=7), and “leaf-litter frogs” third in abundance (n=16), but variance was high for all guild abundances (Fig. 10). The “leaf-litter frogs” showed the highest species richness of all groups, but overall mean abundance was fairly low (Fig. 10).

**SEASONAL AND HABITAT DIFFERENCES**

Significant seasonal and/or habitat differences in abundance, prevalence and intensity were seen in many sites for “all frogs” or the various guilds, but differences were highly variable and site-dependent (Table 2; Appendix 3). Search effort was sufficient for the majority of sites (La Rica, Palmarazo, Cuatro Callitas, Altos de Campana, Cerro Trinidad and Altos del María), with the cumulative number of species found over time levelling off.
by the end of the search period in both seasons (Fig. 11A), and the majority of all frog species found by 75% of the search period (between 82% and 100%, site and season depending, Fig. 11B). However, this trend was not seen for the post-decline sites El Copé or Fortuna, where the number of species increased steadily throughout the search (Fig. 11C), so that by 75% of the search period at El Copé only 67% of all species had been found in the dry season and 72% in the wet season (Fig. 11D). In Fortuna this trend was only noted for the wet season.

**Abundance and species richness**

*Seasonal differences*

In El Copé (760 m elevation, recent post-epidemic), a general trend towards higher anuran abundance in the wet compared to dry season was seen in forest habitat, and this trend was significant for abundance of “all frogs” (p=0.024, Randomization test, Table 2). Species richness was equal to or higher in the wet season for the majority of groups in both habitat types at this site (Appendix 3 Fig. B). In La Rica (250 m elevation), no seasonal differences in abundance were noted, but species richness was found to be slightly higher in the dry season than in the wet season for the majority of frog guilds in both habitat types (Appendix 3 Fig. D). In Palmarazo (135 m), a general trend towards higher anuran abundance in the wet season than in the dry season was seen in stream habitat only, and this trend was significant for abundance of “all frogs” (p=0.0004, Randomization test, Table 2). For the majority of anuran guilds at this site, species richness was found to be higher in the wet season (Appendix 3 Fig. F). Although no seasonal differences in abundance were noted for Altos del María (during epidemic), we noted that species richness was generally higher in the wet than in the dry season, particularly in stream habitat (Appendix 3 Fig. N). At Fortuna (long post-epidemic), species richness was generally higher in the dry season than in the wet season for frogs in both habitat types, with the exception of “treefrogs” which were only found in the wet season (Appendix 3 Fig. P).

*Habitat-specific differences*

Although no habitat-specific differences in abundance were noted for El Copé,
species richness was generally found to be greater in stream than in forest habitat, with the exception of the “leaf-litter frogs” (Appendix 3 Fig. B). In La Rica, a general trend towards higher anuran abundance in forest than in stream habitat was seen in the dry season only (Appendix 3 Fig. C), and this trend was significant for abundance of “all frogs” (p=0.035, Randomization test) and for the “leaf-litter frogs” (p=0.003, Randomization test). Conversely, species richness at La Rica was generally found to be higher in stream habitat than in forest habitat in both seasons, with the exception of the “leaf-litter frogs” in the dry season (Appendix 3 Fig. D). In Palmarazo in the dry season only, abundance for all anurans was significantly higher in forest than in stream habitat (p=0.049, Randomization test, Table 2). Species richness at this site was generally higher in stream than in forest habitat (Appendix 3 Fig. F). In Cuatro Callitas (45 m elevation), anuran abundance was seen to be higher in forest than in stream habitat, and this trend was significant for “all frogs” in the wet season (p=0.008, Randomization test, Table 2). However, for the majority of anuran guilds at this site, species richness was higher in stream than in forest habitat (Appendix 3 Fig. H). In Altos de Cañada (860 m elevation, pre-epidemic) there was no obvious trend with respect to anuran abundance, but abundance of the “diurnal stream frogs” was significantly higher in stream habitat than in forest habitat (p=0.041, Randomization test, Table 2). A general trend of higher species richness in stream habitat can be seen at this site (Appendix 3 Fig. J). In Cerro Trinidad (540 m), we noted a general trend of higher species richness in stream habitat for many anuran guilds (Appendix 3 Fig. L), but there were no detectable trends with respect to abundance. In Altos del María, a general trend of higher anuran abundance in forest than in stream habitat was found in the dry season, and was significant for “all frogs” (p=0.005, Randomization test) and specifically for the “leaf-litter frogs” (p=0.0004, Randomization test, Table 2). In addition, a general trend of higher species richness in stream habitat can be seen for many anuran guilds at this site (Appendix 3 Fig. N). In Fortuna, a general trend of higher anuran abundance in forest than in stream habitat was found in both seasons for “all frogs”, but this trend was not found to be significant (Appendix 3 Fig. O).
Chytrid prevalence and intensity

Seasonal differences

In Altos del María, chytrid prevalence was significantly higher in the wet (78.3%, CI: 69.6% to 85.4%, n=115) than in the dry season (61.2%, CI: 50.8% to 70.9%, n=98) in stream habitat (p=0.010, Fisher’s exact test, Table 2). We also noted a significant difference in chytrid intensity at this same site, finding that intensity was also statistically higher in the wet (2287.0, CI: 674.9 to 4687.3, mean zoospores, n=90) than in the dry season (17.7, CI: 7.8 to 34.9, mean zoospores, n=69) in stream habitat (p=0.008, Randomization test, Table 2). At Fortuna, prevalence was significantly greater for all frogs in the dry season (76.9%, 46.2% to 95.0%, n=13) than in the wet season (28.6%, CI: 8.4% to 58.1%, n=14) in stream habitat (p=0.021, Fisher’s exact test, Table 2), but this was not detected in forest habitat.

Habitat-specific differences

In Altos de Campana in the wet season, chytrid intensity was seen to be significantly higher in forest habitat (5.7, CI: 3.8 to 8.1 mean zoospores, n=18) than in stream habitat (1.6, CI: 1.4 to 1.9 mean zoospores, n=17, p<0.001, Randomization test, Table 2), although prevalence did not differ between habitats. In Altos del María, intensity was statistically higher in stream (17.7, CI: 7.8 to 34.9, n=69) than in forest habitat (6.4, CI: 3.9 to 9.4, n=21) in the dry season (p=0.05, Randomization test, Table 2), although no habitat-specific differences in prevalence were noted. At Fortuna, chytrid intensity was statistically higher in the forest (2149.3, CI: 5.0 to 6431.3, mean zoospores, n=6) than in the stream (1.9, CI: 1.5 to 2.6, mean zoospores, n=4) in the wet season (p=0.037, Randomization test, Table 2), but not in the dry season.

ANURAN BODY SIZE

Anuran Snout Vent Length (SVL) was a predictor of \emph{B. dendrobatidis} prevalence for two observed species, \emph{Eleutherodactylus crassidigitus} and \emph{Colostethus flotator}, a relationship which was nearly significant for both (Binary Logistic Regression, P=0.057 for \emph{E. crassidigitus} and P=0.076 for \emph{C. flotator}). For both species, there was a general trend of a smaller anuran body size showing a higher propensity towards infection (Fig.
12). Within these two species, no relationship existed between chytrid intensity and anuran body size. Size was not a predictor of chytrid prevalence and/or intensity for the majority of observed species, including *Colostethus panamensis, Colostethus talamancae, Eleutherodactylus cruentus* and *Eleutherodactylus cerasinus*.

**DISCUSSION**

Our results support the idea that the chytrid fungus is a generalist pathogen (Woodhams et al. 2006a) since *B. dendrobatidis* occurred in amphibians of every study site regardless of elevation or stage of epidemic amphibian decline, and was present to some extent in all anuran guilds, in both stream and forest habitats, during both wet and dry seasons, and at all body sizes. There is evidence, however, that anurans show differential susceptibility to the disease chytridiomycosis (eg. Amiche et al. 1999, Lips et al. 2003, Stuart et al. 2004, Woodhams and Alford 2005). Elevation, longitude/latitude, season, host body size, habitat requirements of host and pathogen, host behaviour, host population density, and immunosuppression in response to changing environmental conditions are all ecological factors that may alter disease effects of the chytrid fungus (Lips et al. 2003, Retallick et al. 2004, Woodhams and Alford 2005, Daszak et al. 2004). The concept of differential disease susceptibility should be readily observable through changes in population abundance and composition. Indeed, we found that mean abundance for many anuran guilds in our study is higher along the combined elevational gradients than the epidemics gradient, likely because infection prevalence and intensity is high in at least one site along the epidemics gradient. As well, the elevational transects were composed of fewer post-decline sites than was the epidemics gradient, which could also account for the higher overall abundance along the elevational transects.

**ANURAN ABUNDANCE AND PATHOGEN IMPACTS**

A few detectable trends were found in our study with respect to abundance of some anuran guilds that may have been a result of differential pathogen susceptibility. For example, overall mean abundance of the “diurnal stream frogs” guild was high along both study transects. However, the high variance in abundance of this genus indicates that abundance was highly site-specific. Other researchers have found that “diurnal stream
frogs’ seem to have a high propensity towards chytrid-induced decline, and this is likely due to their reliance on the aquatic habitats that sustain the waterborne pathogen (eg. Lips et al. 2003). Our study supports this finding, as we noted that abundance of this guild was very high in sites where chytrid-induced decline had not yet occurred, but in post-decline sites most species of this guild were absent, except for Colostethus flotator, which was still found in the forest in some post-decline sites and is thought to feed farther away from the riparian zone (Whiles et al. 2006).

We observed an absence of most anuran species at post-decline sites in our study, with the exception of the “leaf frogs”, which were abundant at all sites regardless of stage of epidemic decline and despite many individuals bearing infections at relatively high intensities. We also found a high site-specific variance in abundance of “treefrogs” in our study, noting that overall mean abundance was low along sites of the combined elevational transects but high along some sites of the epidemics transect. Interestingly, the Hyla rufitela (according to Savage 2002) population at the site undergoing decline during our study (Altos del María) appeared to be thriving. Some species of anurans possess highly potent antimicrobial peptides which confer resistance to chytridiomycosis (Amiche et al. 1999). Woodhams et al. (2007) found that the possession of highly potent skin peptides varies greatly at the species, and even individual, level, and immune responses may be both innate and adaptive. There may also be a species-specific threshold of disease intensity that leads to mortality, thus determining the length of time that species survive post-infection (Carey et al. 2006). There is evidence that at least some members of the “treefrogs” and “leaf frogs” guilds in Panama are capable of shedding strong infections (Woodhams et al. 2006a); likely antimicrobial peptide defences conferred greater resistance to “leaf frogs” and possibly Hyla rufitela in our study. In addition, Hyla rufitela populations have been known to undergo apparent explosions at other sites in Panama during the breeding season (Rand et al. 1983), and possibly a similar phenomenon was taking place at this site regardless of the pathogenic outbreak. More work is needed to determine the extent to which chytrid prevalence and intensity differs amongst anuran guilds and whether this is related to ecological factors, phylogenetic signals, or both.
EFFECTS OF HABITAT

Highly stream-associated frog species are considered more likely to be infected with *B. dendrobatidis* and experience chytrid-induced decline than species having a more terrestrial existence (Lips et al. 2003, Woodhams and Alford 2005, Kriger and Hero 2007). In our study, the stream-associated anuran guilds showed either lower or more highly variable abundance across the elevational and epidemics gradients, which may be directly related to the site-specific status of chytrid infection. In addition, species richness was lower for stream-associated groups and high across sites only for the “leaf-litter frogs”, a highly terrestrial guild. However, if stream-associated species are showing greater decline in chytrid-infected zones, then we would expect to see higher *B. dendrobatidis* prevalence and/or intensity for stream-associated anurans, but our results do not support this hypothesis. In particular, when we compared prevalence and intensity between forest and stream habitats in each site for all frogs, we found that chytrid prevalence was not significantly different between habitats in any site, and chytrid intensity was actually higher in the forest than in the stream at two sites, Altos de Campana and Fortuna. This confounding result suggests that the link between infection prevalence/intensity, habitat, and corresponding decline is much more complex than imagined. Certainly it appears that infection prevalence and intensity is not a faithful predictor of disease effects in relation to habitat.

Infection should occur at higher prevalence in (resistant) species with stable populations than in (susceptible) species in which infection leads to mortality and rapid removal from the population (Woodhams and Alford 2005). If there are a large number of terrestrial species with pathogenic resistance then this could account for the observed high prevalence and intensity in the forest. Since the majority of amphibian decline has been noted amongst aquatic species, most research on disease resistance has focused on these amphibians; indeed the majority of species identified to date with potent antimicrobial peptides capable of combating chytrid infection have a high aquatic life history (Woodhams et al. 2006a and 2006b). More work is needed to determine the extent to which terrestrial species exhibit resistance to chytridiomycosis. Resistant species remaining in sites post-decline may act as carriers of the pathogen, passing it on to less resistant re-introduced species through direct contact or by re-introduction into
aquatic environments (Berger et al. 1999; Daszak et al. 2004; Weldon et al. 2004; Beard and O’Neill 2005).

The possibility that observed “chytrid-induced” anuran population declines are not primarily due to *B. dendrobatidis* should not be ruled out. Some authors argue that additional decline factors must be present to cause amphibians to succumb to the disease, and often changing environmental factors and other pathogens are blamed (McCallum et al. 2005, Burgin et al. 2005, Pounds et al. 2006). Both intrinsic and changing environmental factors could cause amphibians in stream habitat to have lower immunities to the disease than amphibians in forest habitat, resulting in more pathogenic effects of the fungus in streams. Water pollution via chemical contamination has been known to decrease immune response in amphibians as well as increase the epidemiology of chytrid blooms through reduction in water pH (Sparrows 1968; Berger et al. 1999; Bosch et al. 2001). During preliminary site analyses we measured both water and soil pH at a number of sites and found that water pH was generally 10-fold lower than soil pH, a fact which could explain why a ubiquitous pathogen could show greater pathogenicity in stream habitat. This condition may have been natural in these streams, as we have no reason to suspect contamination.

**SEASONALITY**

Variable seasonal differences in anuran abundance and species richness were observed throughout sites in our study, but we did note a general trend of higher frog abundance in the wet season and higher species richness in the dry season. Likely the fact that most species breed during the wet season (Savage 2002) and are less cryptic and easier to find at this time led to the seemingly higher abundance. We noted seasonal differences in pathogen prevalence for two of the eight sites sampled in this study, but we found opposing trends for these two sites. Chytrid prevalence and intensity was higher in the wet season in Altos del María, a result that is in accordance with work on pathogen seasonality by both Bosch et al. (2007) and Kriger et al. (2007b). In fact, the disease outbreak noted at Altos del Maria coincided with the onset of the wet season, a trend noted by other researchers (eg. Aplin and Kirkpatrick 2000). However, any seasonal effects observed at this site were likely greatly pronounced due to the disease outbreak
which caused a rapid increase in *B. dendrobatidis* prevalence and intensity. In Fortuna, conversely, we found that pathogen prevalence was significantly higher in the dry season. This might alternatively be explained by the fact that anurans tend to congregate in wet areas in the dry season, which could facilitate pathogen spread (Ibáñez et al. 2002).

Indeed, seasonality has been shown to affect not only the prevalence of *B. dendrobatidis* but also the pathogenicity of chytridiomycosis, with cooler temperatures in the wet season causing increased mortality (Aplin and Kirkpatrick 2000, Berger et al. 2004) and warmer temperatures in the dry season ridding amphibians of infection (Kriger and Hero 2006). The majority of research on seasonality to date however has focused on temperature and not on rainfall, and in fact some authors purport that increased rainfall does not coincide with increased disease prevalence or intensity (Drew et al. 2007). In Panama, temperature does not fluctuate very much by season at high elevation sites (STRI ESP dataset), and so perhaps precipitation has a greater bearing at sites in Panama than in Australia. It is possible that rainfall and relative humidity may have more of a bearing on the prevalence and pathogenicity of the disease than is currently thought, with these effects potentially being overlooked and overridden by temperature correlations.

Because we noted opposing seasonal differences for two sites in our study, and results from one site were confounded by disease outbreak, no conclusive evidence can be drawn from this study regarding effects of season on presence of the chytrid. It is thus possible that season does not play much of a role in pathogen prevalence in Panama, or if it does, the effects may be masked by other factors. Such factors may be intrinsic to anuran populations in Panama, like changes in frog behaviour in response to the changing seasons, or artefacts of our sampling design, since we sampled from sites of varying elevation and stages of decline. All of these factors may have as much or more of an impact on chytrid epidemiology than season. Further research is needed to determine the exact seasonal role on chytrid epidemiology in both montane and lower elevation sites in the Neotropics.

**ANURAN BODY SIZE**

Anuran body size was found to play a role in differential pathogen susceptibility for
at least two observed species in our study, *Eleutherodactylus crassidigitus* and *Colostethus flotator*. For both of these species, there was a general trend of a smaller anuran body size showing a higher propensity towards infection prevalence, but not intensity. Size was not a predictor of chytrid prevalence and/or intensity for the majority of observed species in our study, including *Colostethus panamensis*, *Colostethus talamancae*, *Eleutherodactylus cruentus* and *Eleutherodactylus cerasinus*. The results we found for *E. crassidigitus* and *C. flotator* accord with other authors who found the same correlation of a smaller body size showing higher prevalence and more intense infections than larger body sizes, suggesting that frogs either outgrow infection, have infection-stunted growth, or succumb to pathogen-induced mortality (Kriger et al. 2007).

Aquatic larvae have been implicated as possible disease reservoirs capable of pathogen transfer to juvenile post-metamorphic frogs (Daszak et al. 2003, Blaustein et al. 2005, Woodhams and Alford 2005), and perhaps this could explain the higher infection prevalence in small *C. flotator* individuals. *Eleutherodactylus crassidigitus* has direct-developing reproduction and therefore no aquatic larvae, but perhaps differential behaviours between juvenile and adults may account for the higher infection prevalence in smaller individuals. Subadults of *Eleutherodactylus coqui* are generally found to perch closer to the forest floor than are adults (Beard et al. 2003, Beard and O’Neill 2005), which may make them more susceptible to a water-borne pathogen like *B. dendrobatidis* since the greater moisture content that is found in leaf litter is preferable to subadults (Pough et al. 1983). We cannot ascertain that the other species observed in our study do not follow the same pattern of greater infection susceptibility at a smaller body size since we may not have had a large enough sample size for these other species to detect a trend.

CONCLUSIONS

As suspected, *B. dendrobatidis* does not affect all anuran hosts equally. Moreover, chytrid infection prevalence/intensity is not always a consistent predictor of disease effects in anuran communities. Differential host response to the chytrid was seen as a function of habitat, season and anuran body size, but detected trends were not universal across all frog guilds, elevations, or stages of epidemic decline in Panama. Similar contradictions occur within the literature, and thus it appears that multiple ecological
factors affecting chytrid prevalence and corresponding anuran decline occur simultaneously. Further research is needed to tease apart the ecological factors affecting disease susceptibility in anurans.
**Figure 9.** Overall mean anuran abundance (+/− Standard Deviation) across six sites of varying elevations in the wet season in combined habitat in Panama in 2006 for all anuran guilds. Numbers in bold above each bar represent the sample size n=# species (species richness) for each guild. The “all frogs” category abundance estimate was calculated as the mean abundance of all individual species’ abundances, and was not included in the analysis but is displayed here for comparative purposes. Anuran guilds were not statistically different with respect to abundance.

**Figure 10.** Overall mean anuran abundance (+/− SD) across four sites of varying stages of epidemic decline in the wet season in combined habitat in Panama in 2006 for all anuran guilds. Numbers in bold above each bar represent the sample size n=# species (species richness) for each guild. The “all frogs” category abundance estimate was calculated as the mean abundance of all individual species’ abundances, and was not included in the analysis but is displayed here for comparative purposes. Anuran guilds were not statistically different with respect to abundance.
Table 2: Site-specific differences in anuran abundance, chytrid prevalence and intensity by habitat and season in Panama. Abundance differences and chytrid intensity differences were analyzed using Randomization tests, and Fisher’s exact tests were used to find differences in prevalence.

<table>
<thead>
<tr>
<th>Site</th>
<th>Overall prevalence (%)</th>
<th>Overall intensity (mean zoospores)</th>
<th>Differences in abundance</th>
<th>Differences in prevalence</th>
<th>Differences in intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>El Copé (760 m, recent post-epidemic)</td>
<td>16.2%, CI: 6.2% to 32.0% (n=37)</td>
<td>2.6, CI: 1.7 to 3.8 (n=6)</td>
<td>Wet &gt; Dry for &quot;all frogs&quot; (p=0.024) in forest habitat</td>
<td>None</td>
<td>None</td>
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<tr>
<td>La Rica (250 m)</td>
<td>22.2%, CI: 18.0% to 39.1% (n=76)</td>
<td>69.6, CI: 5.5 to 179.4 (n=21)</td>
<td>Forest &gt; Stream for &quot;all frogs&quot; (p=0.035) and &quot;leaf-litter frogs&quot; (p=0.003) in dry season</td>
<td>None</td>
<td>None</td>
</tr>
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<td>Palmarazo (135 m)</td>
<td>10.6%, CI: 6.1% to 16.9% (n=141)</td>
<td>156.1, CI: 3.2 to 419.8 (n=15)</td>
<td>Wet &gt; Dry for &quot;all frogs&quot; (p&lt;0.001) in stream habitat and Forest &gt; Stream for &quot;all frogs&quot; (p=0.049) in dry season</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cuatro Callitas (45 m)</td>
<td>17.9%, CI: 9.6% to 29.1% (n=67)</td>
<td>694.0, CI: 9.4 to 2019.6 (n=12)</td>
<td>Forest &gt; Stream for &quot;all frogs&quot; (p=0.008) in wet season</td>
<td>None</td>
<td>None</td>
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<td>Altos de Campana (860 m, pre-epidemic)</td>
<td>13.1%, CI: 9.3% to 17.8% (n=267)</td>
<td>3.7 (CI: 2.5 to 5.3, n=35)</td>
<td>Stream &gt; Forest for &quot;diurnal stream frogs&quot; (p=0.041) in wet season</td>
<td>None</td>
<td>None</td>
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<tr>
<td>Cerro Trinidad (540 m)</td>
<td>15.1%, CI: 10.9 to 20.2 (n=245)</td>
<td>40.2 (CI: 13.7 to 75.8, n=37)</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Altos del Maria (during epidemic)</td>
<td>67.3%, CI: 61.2% to 73.1% (n=254)</td>
<td>1210.7, CI: 294.1 to 2399.3 (n=171)</td>
<td>Forest &gt; Stream for &quot;all frogs&quot; (p=0.005) and &quot;leaf-litter frogs&quot; (p&lt;0.001) in dry season</td>
<td>Wet &gt; Dry in stream habitat (78.3%, CI: 69.6% to 85.4%, n=115 vs. 61.2%, CI: 50.8% to 70.9%, n=98) (p=0.010)</td>
<td>Forest &gt; Stream in dry season (2149.3, CI: 5.0 to 6431.3, n=21) vs. 1.9, CI: 1.5 to 2.6, n=4) (p=0.037)</td>
</tr>
<tr>
<td>Fortuna (long post-epidemic)</td>
<td>42.3%, CI: 30.6% to 54.6% (n=71)</td>
<td>441.0, CI: 4.3 to 1306.6 (n=30)</td>
<td>Dry &gt; Wet in stream habitat (76.9%, 46.2% to 95.0%, n=13 vs. 28.6%, CI: 8.4% to 58.1%, n=14) (p=0.021)</td>
<td>Wet &gt; Dry in stream habitat (2287.0, CI: 674.9 to 4687.3, n=90 vs. 17.7, CI: 7.8 to 34.9, mean zoospores, n=69) (p=0.008); Stream &gt; Forest in dry season (17.7, CI: 7.8 to 34.9, n=69 vs. 6.4, CI: 3.9 to 9.4, n=21) (p=0.050)</td>
<td>Forest &gt; Stream in wet season (2149.3, CI: 5.0 to 6431.3, n=6 vs. 1.9, CI: 1.5 to 2.6, n=4) (p=0.037)</td>
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</table>
Figure 11. Cumulative number of species and percent found per proportion of Search Effort Hours in the two sites that were included in both the elevational transects and the epidemics transect in all applicable seasons in Panama in 2006. Sufficient search effort was seen in sites not having experienced chytrid-induced decline (eg. Altos de Campana), but post-decline sites (eg. El Copé) did not show the same trend.
Figure 12. The relationship between anuran body size and chytrid prevalence for two species, *Colostethus flotator* (12A) and *Eleutherodactylus crassidigitus* (12B). Although the relationships were only weakly significant for both species (P=0.057 for *E. crassidigitus* and P=0.076 for *C. flotator*), a trend was detected of smaller individuals showing a higher propensity towards chytrid infection.
MANUSCRIPT THREE EXPLANATIONS

In my final manuscript, appearing as Chapter 5 in this thesis, I discuss the role of non-amphibian disease reservoir and transmission agents. In my first manuscript, I find and discuss results that provide evidence that the chytrid fungus is present throughout central Panama at all elevations and stages of epidemic infection, including both post-decline and pre-decline sites. Despite massive amphibian declines that had occurred at some sites in this study, some amphibian species still remained, thus showing differential susceptibility to this generalist pathogen. In the second manuscript, I identified numerous ecological factors that may be altering disease susceptibility in a Panamanian amphibian assemblage. In particular, I noted that infection prevalence and intensity is similar between both stream and forest habitat, even though *B. dendrobatidis* is known to be a waterborne pathogen and stream-associated amphibian species are experiencing greater decline.

For the chytrid fungus to be distributed as widely as it is in all areas and habitats of Panama there is a great likelihood that the pathogen is living as a saprobe in the environment, on non-amphibian hosts, or both. The presence of non-amphibian reservoir hosts not susceptible to decline that could move between areas and habitats transmitting the pathogen would have innumerable implications for conservation. In particular, the identification of such disease transmission agents could feasibly aid the scientific community in slowing disease spread. In this manuscript, I discuss evidence that reptiles are reservoir hosts that likely facilitate disease transmission between susceptible amphibian communities.
CHAPTER 5: THE PATHOGENIC CHYTRID FUNGUS, *BATRACHOCYTHRIUM DENDROBATIDIS*, IS NOT UNIQUE TO AMPHIBIANS

Kilburn, V.L., Green, D.M., Ibáñez, R., Sanjur, O. and Bermingham, E.

**Abstract:** Chytridiomycosis, the disease caused by *Batrachochytrium dendrobatidis*, is considered to be a disease of amphibians. However, *B. dendrobatidis* may also be capable of living as a saprobe in the environment and non-amphibian hosts or vectors may contribute to disease transmission. Reptiles living in close proximity to amphibians and sharing similar ecological traits could serve as reservoir hosts for the chytrid, harbouring the organism on their keratinised skin without succumbing to disease. We surveyed for the presence of the chytrid among lizards and snakes at eight sites in Panama that were at varying elevations and stages of epidemic infection among amphibians. Detection of *B. dendrobatidis* was done using RT-qPCR analysis. The pathogen was prevalent at varying intensities in the lizards *Anolis humilis* and *Anolis lionatus* (16.1%, CI: 11.4% to 21.8%, n=211 lizards) and in the snakes *Imantodes cenchoa*, *Nothopsis rugosus*, and *Urotheca euryzona* (37.5%, CI: 8.5% to 75.5%, n=8 snakes). These reptiles, therefore, are likely reservoir hosts for the chytrid and could serve as disease transmission agents. Although there is no evidence of chytrid disease-induced declines in reptiles, cases of coincidence of reptile and amphibian declines suggest this potentiality. Our study is the first to provide evidence of non-amphibian hosts for the chytrid in a natural Neotropical environment.
INTRODUCTION

Many factors have been blamed for the phenomenon of global amphibian population decline (Houlahan et al. 2000, Green 2003, La Marca et al. 2005, Lips et al. 2005b, Pounds et al. 2006), but the one factor currently receiving the most scientific and media attention is the amphibian chytrid fungus, Batrachochytrium dendrobatidis. The fungus is generally held to be a water-borne pathogen with motile, flagellated zoospores, composing the infective stage (Berger et al. 1999). Although many aspects of chytrid ecology and pathology remain unclear, in the Tropics, the chytrid appears to prefer cooler, high-elevation, moist, riparian habitats (Berger et al. 1999, Longcore et al. 1999). The chytrid invades and probably feeds on the keratin of amphibian skin (Daszak et al. 2003).

Chytridiomycosis, the disease caused by B. dendrobatidis, is considered to be a disease exclusively of amphibians (Berger et al. 1998). The disease becomes fatal to amphibians either by interfering with cutaneous respiration and water uptake through the skin, by releasing toxic proteolytic enzymes or other active compounds that are absorbed through the permeable skin of the amphibian, and/or through facilitation of other microbial infections (Berger et al. 1998; Pessier et al. 1999). Chytridiomycosis is highly contagious and can be transmitted through physical contact with infected amphibians or water (Berger et al. 1999). Death occurs within 10 – 47 days following initial infection (Berger et al. 1999).

The chytrid appears to be quite capable of survival away from an amphibian host. In the laboratory, infective zoospores can live for up to 4 weeks in tap water and as long as 7 weeks in lake water, can survive for 3 months in sterile moist river sand with no added nutrients, are capable of attaching to and growing on moist bird feathers, and will survive up to 3 hours of drying and transfer to new media and can be cultivated on boiled snake skin (Johnson and Speare 2005). From the above findings, it appears likely that B. dendrobatidis, like other chytrids, is capable of living as a saprobe in the environment or on other non-amphibian hosts (Lips et al. 2006). If so, the fungus could be transferred to new areas in the absence of an amphibian host (Longcore et al. 1999, Johnson and Speare 2005). Non-amphibian hosts in the field have not, as yet, been identified.
Determining the extent to which reservoir hosts act as disease transmission agents is imperative in order to identify management programs for declining populations of amphibians (Lips et al. 2005a). A reservoir host is defined as: “an animal species which carries a pathogen without detriment to itself and serves as a source of infection” (FAO, 1987). There may be a number of possible reservoir hosts for B. dendrobatidis (Lips et al 2005a), including some amphibian species that harbour high infections of B. dendrobatidis but do not usually succumb to chytridiomycosis, including Bufo marinus, Rana catesbeiana, Xenopus laevis and E. coqui (Berger et al. 1999; Daszak et al. 2004; Weldon et al. 2004; Beard and O’Neill 2005. Davidson et al. (2003) demonstrated that the infection could be transmitted between Ambystomatid salamanders and Ranid frogs. Experimental and genetic evidence show that the transfer of pathogens between fish and amphibians is possible (Mao et al. 1999; Kiesecker et al. 2001a); introduced exotic fish such as rainbow trout (Oncorhynchus mykiss) or goldfish (Carassius auratus) are possible culprits (Gillespie and Hero 1999, Lips et al. 2005a). Freshwater shrimp were thought to maintain B. dendrobatidis on their carapaces (Rowley et al. 2006) but this has been found not to be the case (Rowley et al. 2007).

Considering the survivability of the chytrid outside of aquatic media, it may not be necessary for a reservoir host to be aquatic. Reptiles, particularly small lizards and riparian snakes, could be reservoir hosts of the chytrid as they live in close proximity to amphibians, may share similar habitat and microhabitat requirements and may have comparable foraging behaviours and prey preferences (Whitfield et al. 2007). If such reptiles are reservoir hosts they should harbour B. dendrobatidis infections detectable using sensitive Real-Time Quantitative PCR analysis of skin swabs.

**MATERIALS AND METHODS**

**SAMPLE COLLECTION**

Sampling was conducted in Panama intermittently over a 9-month period, from February 18th to October 28th 2006, at eight sites of varying elevations and stages of epidemic decline among anurans: Fortuna, Cuatro Callitas, Palmarazo, La Rica, El Copé, Altos del María, Cerro Trinidad and Altos de Campana (see Chapter 2, Fig. 1). We collected epidermal skin swabs from 221 lizards of 13 species and eight snakes of eight
different species (Appendix 2a). A thorough discussion of the swabbing protocol used, including procedures followed to avoid contamination between samples, can be found in this thesis under the subheading Sample Collection in Chapter 2.

CHYTRID DIAGNOSIS

All DNA extractions and purifications of samples were done at McGill University according to the protocol outlined in Hyatt et al. (2007). After DNA extraction and purification was complete, samples were sent to the Rnomics Platform of Genome Quebec for RT-qPCR analysis, as developed by Boyle et al. (2004) and adapted for a singlicate assay by Kriger et al. (2006b). A thorough discussion of Chytrid Diagnosis procedures used prior to and for Real-Time Quantitative PCR Analysis of epidermal swab samples can be found in Chapter 2 of this thesis. A pilot study was done to ensure that singlicate analysis would be sufficient for this study (see Chapter 2). Three samples were lost and seven samples deemed inhibited by qPCR analyses and were omitted from all subsequent analyses. Thus 211 lizard samples and 8 snake samples were analyzed in total.

DATA AND STATISTICAL ANALYSES

We tested whether infection prevalence and intensity differed between snakes and lizards with all sites combined and within each individual site. We also tested for differences in chytrid prevalence between sites for all reptiles (lizards and snakes combined). In addition, we tested for differences in pathogen prevalence and intensity between reptiles and co-occurring amphibians, using infection data for amphibians presented elsewhere (Chapters 3 and 4). All results for pathogen prevalence and intensity were discussed in the context of results found for co-occurring amphibians. Fisher’s exact tests were used on raw binary infection status data (positive or negative) to test for differences in pathogen prevalence between lizards and snakes for all sites combined, and between reptiles and anurans for all sites combined and within each site ($\alpha = 0.05$). To test for inter-site differences in reptile chytrid prevalence, we used a multiple contingency Pearson chi-square test. To test for differences in pathogen intensity between lizards and snakes for all sites combined and between reptiles and anurans within each site we used
One-tailed randomization tests on log_{10} transformed arithmetic mean zoospore equivalents. To test for inter-site differences in reptile chytrid intensity, we performed a One-way ANOVA through randomization, again on the log_{10} transformed arithmetic mean zoospore equivalent data. Data permutations were done 10,000 times for all samples, with α = 0.05.

Confidence intervals (95%) based on a binomial distribution were calculated for all prevalence estimates, and 95% bootstrap confidence intervals (BCa) were calculated for all intensity estimates. Prevalence was determined as a function of the number of positive samples/total number of samples. Only positive samples (mean zoospore equivalents of ≥ 1.0) were included in analyses involving chytrid intensity. We did not discriminate between seasons or habitats for any analyses and thus included all samples in order to have the greatest possible sample size. Statistical tests and confidence intervals for pathogen prevalence were done using Minitab 15.0 Statistical Software. Statistical analyses involving pathogen intensity and the bootstrap confidence intervals (BCa) for mean intensity were calculated using the Resampling Stats Add-in for Microsoft Excel.

**RESULTS**

The chytrid fungus was found in lizards at all sites in Panama except Palmarazo, which is at the lowest elevation (Table 3). Chytrid prevalence in lizards (all sites combined) was 16.1% (CI: 11.4% to 21.8%, n=211) and intensity was 10.6 (CI: 3.0 to 21.0 (n=34) mean zoospores per sample. Of the 13 species of lizards sampled, the chytrid was found on only two: *Anolis humilis* and *Anolis lionotus*. The chytrid was also found on three individuals of three different species of snakes: *Imantodes cenchoa*, *Notho psis rugosus*, and *Urotheca euryzona*, which were found at three different sites of varying elevation: Cuatro Callitas (45 m), La Rica (250 m), and Altos del María (890 m) respectively. Overall chytrid prevalence in snakes with all sites combined was 37.5% (CI: 8.5% to 75.5%, n=8) and intensity was 43.4 (CI: 1.1 to 65.0) mean zoospores (N=3).

Lizards and snakes were not found to differ in chytrid prevalence but they did differ in chytrid intensity (p=0.035, Randomization test). When lizards and snakes were combined ("reptiles"), a significant difference in infection prevalence between sites was found ($X^2 = 40.3$, p<0.001, Pearson Chi-Square test), but no difference in intensity was seen. Highest
prevalence of *B. dendrobatidis* was seen in reptiles at Altos del Maria, a highland site experiencing an epidemic of chytriomycosis among amphibians (69.2%, CI: 38.6% to 90.9%, n=13). For all other sites where the pathogen was present, site-specific chytrid prevalence in reptiles ranged from 6.9% at La Rica (CI: 0.8% to 22.8%) to 26.3% at Fortuna (CI: 15.5% to 39.7%) (Table 3). Because Altos del Maria was an outlier site, we re-tested for inter-site differences in chytrid prevalence and intensity between the other 7 sites with this site removed, and found that prevalence did still differ ($X^2 = 16.0$, p=0.014, Pearson Chi-Square test) but intensity did not.

When all sites were combined, chytrid prevalence and intensity in reptiles was 16.9% (CI: 12.2% to 22.5%, n=219) and 13.1 (CI: 4.9 to 22.8, n=37) mean zoospores. These were significantly lower than among co-occurring anurans (prevalence = 28.2%, CI: 25.6% to 30.9%, n=1159, p<0.001, Fisher’s exact test; intensity = 715.7, CI: 230.6 to 1303.0 mean zoospores, n=327, p=0.008, Randomization test, Table 3). When analyses were done within each site, only anurans from La Rica showed a significantly higher chytrid prevalence (but not intensity) than reptiles (p=0.032, Fisher’s exact test, Table 3). No significant differences between anurans and reptiles with respect to prevalence and/or intensity were noted for other sites. Infection prevalence in reptiles reflected infection prevalence in amphibians by site; that is, both amphibians and reptiles showed the lowest propensity for infection at Palmarazo, highest infection propensity at Altos del Maria, and similar variations in-between these extremes (Table 3).

**DISCUSSION**

*Batrachochytrium dendrobatidis* is an infective organism that is not restricted to amphibians, as previously thought. We have discovered that the fungus can exist on at least two species of lizards, both terrestrial (*Anolis humilis*) and aquatic (*Anolis lionotus*) and as well on at least three species of colubrid snakes (*Imantodes cenchoa*, *Nothopsis rugosus*, and *Urotheca euryzona*) at levels of prevalence and intensity comparable to anuran amphibians. However, there is no evidence of chytridiomycosis among reptiles. No individual that tested positive for the chytrid showed any symptoms of disease. The fungus is known to feed on the keratin of amphibian skin (Daszak et al. 2003), likely becoming fatal by interfering with cutaneous respiration and water uptake through the
skin, by releasing toxic proteolytic enzymes or other active compounds that are absorbed, and/or through facilitation of other microbial infections (Berger et al. 1998; Pessier et al. 1999). Although *B. dendrobatidis* is obviously capable of existing on keratinised reptile skin under natural field conditions, none of the above fatal interactions are relevant to reptiles, which have a relatively impermeable, non-respiratory integument. Therefore reptiles may act as important reservoir hosts of chytridiomycosis by maintaining the pathogen in the environment without succumbing to disease. This would be important for susceptible amphibians especially at post-decline sites where reservoir hosts would allow the chytrid to remain even after host amphibian populations have crashed (Lips et al. 2005a).

Whitfield et al. (2007) noted a 35-year declining trend in small terrestrial lizard species at La Selva in Costa Rica, in conjunction with anuran decline in similar habitat. The decline appeared to be attributable to climate-driven reduction in standing leaf litter mass. Although Whitfield et al. (2007) addressed the potentiality that chytridiomycosis was causing these declines for both herpetofaunal guilds, they reasoned that the declines were inconsistent with either a novel pathogen invasion (Lips et al. 2006) or disease emergence by changing environmental conditions (Pounds et al. 2006). Considerable debate exists as to whether the disease chytridiomycosis has emerged from a novel invasive pathogen (Lips et al. 2006, 2008) or from an endemic pathogen (Daszak et al. 2004, Pounds et al. 2006), but agents of disease transmission would be important in either scenario. Dissemination of a novel pathogen or more pathogenic strain of an endemic pathogen might be facilitated by reptiles. In addition, long-term infected reservoir hosts could shed zoospores into the environment until it is saturated and transmission between populations and areas is facilitated (Lips et al. 2006).

To our knowledge, there has been no work done in Panama to assess population trends of small lizard species. Population declines have been noted in some riparian snake species in Panama, and are thought to coincide with the loss of amphibian biomass (Whiles et al. 2006). From stable isotope analyses it is known that many of these species rely on adult frogs and frog egg masses as their primary prey (Verburg et al. 2007). Although we do not suspect that *B. dendrobatidis* is causing disease-induced decline of reptiles, evidence of reptiles declining in conjunction with amphibians in some
Neotropical sites suggests that this possibility should be explored further. Histology should be done to determine whether the chytrid is capable of completing its life cycle on reptilian hosts, and further research into how long the pathogen can persist on reptiles and whether it can do so indefinitely in the absence of amphibians are topics warranting further inspection.
Table 3: Summary of statistical tests done on samples to determine if a difference exists in chytrid prevalence and intensity between anurans and reptiles across all sites combined and within each site surveyed. Tests were accepted or rejected at the alpha 0.05 level. Anurans showed a general trend of both higher prevalence and intensity for chytrid under most circumstances, but the difference was only significant in some cases.

<table>
<thead>
<tr>
<th>Site</th>
<th>Statistical Summary</th>
<th>Anurans</th>
<th>Reptiles</th>
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<tbody>
<tr>
<td></td>
<td>(Fisher’s Exact test for prevalence and randomization test for intensity)</td>
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<tr>
<td><strong>All Sites Combined</strong></td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p&lt;0.001</td>
<td>28.2%, CI: 25.6%-30.9% (n=1159)</td>
<td>16.9%, CI: 12.2%-22.5% (n=219)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.008</td>
<td>715.7 (CI: 230.6-1303.0) mean zoospores (n=327)</td>
<td>13.1 (CI: 4.9- 22.8) mean zoospores (n=37)</td>
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<tr>
<td>Infection prevalence and intensity significantly higher in anurans than in reptiles</td>
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<tr>
<td><strong>Altos del Maria (890 m)</strong></td>
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<td>Dry - Forest &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=1.000</td>
<td>67.3%, CI: 61.2%-73.1% (n=254)</td>
<td>69.2%, CI: 38.6%-90.9% (n=13)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.137</td>
<td>1211.0 (CI: 329.2-2338.0) mean zoospores (n=171)</td>
<td>11.3 (CI: 2.6-24.5) mean zoospores (n=9)</td>
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<tr>
<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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<tr>
<td><strong>Altos de Campana (860 m)</strong></td>
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<td>Wet - Forest &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.628</td>
<td>13.1%, CI: 9.3%-17.8% (n=267)</td>
<td>20.0%, CI: 2.5%-55.6% (n=10)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.644</td>
<td>3.7 (CI: 2.5-5.2) mean zoospores (n=35)</td>
<td>3.4 (CI: 2.6-4.2) mean zoospores (n=2)</td>
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<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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<tr>
<td><strong>Cerro Trinidad (540 m)</strong></td>
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<td>Wet - Forest &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.510</td>
<td>15.1%, CI: 10.8%-20.2% (n=245)</td>
<td>21.1%, CI: 6.1%-45.6% (n=19)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.310</td>
<td>40.2 ± (CI: 14.3-73.8) mean zoospores (n=37)</td>
<td>34.7 (CI: 1.2-101.2) mean zoospores (n=4)</td>
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<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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<td><strong>El Copé (760 m)</strong></td>
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<td>Dry - Forest &amp; Stream &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.452</td>
<td>15.8%, CI: 6.0%-31.3% (n=38)</td>
<td>7.4%, CI: 0.9%-24.3% (n=27)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.104</td>
<td>2.6 (CI: 1.7-3.8) mean zoospores (n=6)</td>
<td>1.4 (CI: 1.1-1.8) mean zoospores (n=2)</td>
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<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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<tr>
<td><strong>Fortuna (1215 m)</strong></td>
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<td>Dry - Forest &amp; Stream &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.066</td>
<td>42.3%, CI: 30.6%-54.6% (n=71)</td>
<td>26.3%, CI: 15.5%-39.7% (n=57)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.127</td>
<td>441.0 (CI: 4.2-1305.2) mean zoospores (n=30)</td>
<td>9.5 (CI: 1.6-23.9) mean zoospores (n=15)</td>
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<tr>
<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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<td><strong>La Rica (250 m)</strong></td>
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<td>Dry - Forest &amp; Stream &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.032</td>
<td>27.6%, CI: 18.0%-39.1% (n=76)</td>
<td>6.9%, CI: 0.8%-22.8% (n=29)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: p=0.334</td>
<td>69.6 (CI: 6.2-175.3) mean zoospores (n=21)</td>
<td>1.8 (CI: 1.1-2.4) mean zoospores (n=2)</td>
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<tr>
<td>Infection prevalence significantly higher in anurans than in reptiles</td>
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<td><strong>Palmarazo (135 m)</strong></td>
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<tr>
<td>Dry - Forest &amp; Stream &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.220</td>
<td>10.6%, CI: 6.1%-16.9% (n=141)</td>
<td>0.0%, CI: 0.0%-13.9% (n=20)</td>
</tr>
<tr>
<td>Intensity</td>
<td>Intensity: N/A</td>
<td>156.0 (CI: 3.0-419.3) mean zoospores (n=15)</td>
<td>N/A</td>
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<td>Infection prevalence not different between anurans and reptiles (Intensity N/A)</td>
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<tr>
<td><strong>Cuatro Callitas (45 m)</strong></td>
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<td>Wet - Forest &amp; Stream</td>
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<tr>
<td>Prevalence</td>
<td>Prevalence: p=0.154</td>
<td>17.9%, CI: 9.6%-29.1% (n=67)</td>
<td>6.8%, CI: 1.4%-18.7% (n=44)</td>
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<tr>
<td>Intensity</td>
<td>Intensity: p=0.568</td>
<td>694.0 (CI: 9.4-2008.8) mean zoospores (n=12)</td>
<td>18.7 (CI: 1.2-65.0) mean zoospores (n=3)</td>
</tr>
<tr>
<td>Infection prevalence and intensity not different between anurans and reptiles</td>
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CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS
DISTRIBUTION AND ORIGIN OF CHYTRIDIOMYCOSIS IN PANAMA

The chytrid fungus was distributed from 45 m to 890 m elevation in Panama, and ranged from as far west as the long post-epidemic highland site Fortuna near the Costa Rican border to the pre-epidemic highland site Altos de Campana, just west of the Panama Canal. With the exception of Altos del María which was a site undergoing epidemic decline at the time of study, *B. dendrobatidis* prevalence was low for all anuran communities, ranging from 9.6% at the lowland site Palmarazo to 27.0% at the long post-decline site Fortuna. By using the highly sensitive detection technique, RT-qPCR, I determined mean infection intensity to be low across all sites not undergoing decline in our study, and the vast majority of anurans showed an infection of less than 10.0 mean zoospore equivalents. If I was to have relied upon conventional PCR instead of RT-qPCR for disease diagnosis, detection of chytrid prevalence would have dropped considerably for all sites in this study, and save for Altos del María, infection prevalence would have been close to 0.0% (between 0.0% and 1.1%, site depending). Although we cannot rule out that the novel pathogen hypothesis may explain chytridiomycosis emergence in Panama, my results do not support this hypothesis, and infection prevalence for these sites is within the range reported for endemic chytridiomycosis in other regions (Alemu et al. 2008). Through this work I have provided evidence that the chytrid fungus may be enzootic at least in central Panama.

The “novel pathogen hypothesis” (Lips et al 2006, Skerratt et al. 2007) is problematic since reliable disease diagnostics cannot be done on the limited past data available to us, especially in Neotropical regions. Histology is the only way of diagnosing *B. dendrobatidis* on previously collected specimens, but with histology only high levels of infection can be detected, interpretable as representing the disease chytridiomycosis (Kriger et al. 2006a). To date, the majority of studies insisting that chytridiomycosis is a novel disease (e.g. Lips et al. 2006, Rachowicz et al. 2005) describe healthy amphibian populations when *B. dendrobatidis* is absent, and acute die-offs and subsequent population declines after detection of *B. dendrobatidis*. Generally the date of arrival of the chytrid into a given area is based on histological findings (e.g. Ron et al. 2003, Puschendorf et al. 2006, Lips et al. 2008). Studies using this method to date the arrival of the chytrid are thus only dating the incidence of the disease, chytridiomycosis, and not the
pathogen *B. dendrobatidis*. By using conventional PCR techniques on swab samples obtained at El Copé prior to decline, Lips et al. (2006) presented better evidence that *B. dendrobatidis* was absent from an amphibian community prior to disease outbreak. However conventional PCR cannot detect less than a baseline of 10 zoospore equivalents per sample (Annis et al. 2004). Low, innocuous infections that do not exceed this baseline will remain undetected with this method. Only Real-Time quantitative PCR is capable of detecting very low levels (1 zoospore) of *B. dendrobatidis* and, according to some authors, should be adopted as the primary tool for pathogen diagnostics (Kriger et al. 2007a).

The continuous persistence and seemingly innocuous effects of the pathogen in Fortuna more than a decade post-decline is substantial evidence that the chytrid persists after major population crashes, and certainly evidence of potential co-evolution between host and pathogen post-decline have been noted in other regions (Retallick et al. 2004, McDonald et al. 2005, Briggs et al. 2005). This result is not necessarily evidence of an enzootic pathogen, and I do not rule out the possibility that the Fortuna amphibian community is maintaining the disease as an enzootic infection post “novel” invasion. Nor from the results can I ascertain that amphibian communities in the “uninfected” sites have not just recently experienced “novel” pathogen invasion and are yet to experience decline. However, I do agree with other authors that the novel pathogen hypothesis is oversimplistic (eg. McCallum 2005), and I speculate from our results that the chytrid is likely present at innocuous levels in amphibian communities long before disease outbreaks occur. Chytridiomycosis may be moving in an observable “wave” of infection in Panama as Lips et al. (2006) propose, even if the pathogen is already present (Pounds et al. 2006). Alternatively, it is possible that a more virulent strain of the chytrid has emerged and has spread invasively as a novel disease into areas where more benign strains have always been present (Morehouse et al. 2003). To determine which scenario is most likely, more intensive sampling must occur in advance of the predicted disease wave in Panama using the highly sensitive RT-qPCR diagnostics on many samples. It is estimated that negative samples from at least 300 individual frogs are needed to be 95% confident that the infection is not present (Ibáñez, Pers. Comm., 2006).
ELEVATIONAL DISTRIBUTION OF PATHOGEN AND DISEASE

My results prove that the chytrid fungus is present at low and middle elevations in central Panama. The impact on higher elevation amphibians in the Tropics has been the fundamental observation behind most research to date on the pathogen and has been explained in terms of the chytrid’s proposed reliance on lower temperatures (~23°C) for survival. It has been thought that warmer climates in middle or lower elevation sites in the Tropics may limit chytrid distribution (Longcore et al. 1999). Regardless, we detected symptoms of chytridiomycosis (lethargy, sloughing skin) from at least 16 of 321 of the sampled frogs at low, middle and high elevations along the El Copé elevational gradient. Anuran abundance was indeed significantly lower overall and for some ecological guilds in 2006 as compared to 2001 in the site Cuatro Callitas at 45 m elevation, and species richness was noticeably lower at both lowland sites in 2006 compared to in 2001. Thus the *B. dendrobatidis* pathogen is not only present at low elevations but appears to be causing chytridiomycosis and may be resulting in subsequent amphibian population decline, at least in one locality. This major finding proves that the pathogen is capable of inflicting damage on hosts at temperatures beyond the thermal optimum under natural field conditions. Thus, the low humidity and higher temperatures at lower elevations may not limit the pathogenicity of the chytrid as some authors contend (La Marca et al. 2005). This is not surprising since even under the most extreme temperature regimes there are always microhabitats available to amphibians that are moist and cool enough for zoospores to survive and re-infect their hosts. Certainly climatic trends at the microhabitat scale can dwarf local ambient climatic trends, and in some places the trends might even be entirely opposing (Pounds et al. 2006).

The importance of an abiotic factor like temperature on pathogenicity means that environmental change will have drastic effects on the host-pathogen relationship at presumeably any elevation. Global warming could feasibly cause a temperature-dependent enzootic pathogen to emerge as an epizootic pathogen (eg. Pounds and Crump 1994, Pounds et al. 1999 and Bosch et al. 2007). Increased cloud cover typical of climate change over mountainous forested tropical regions decreases daytime temperatures and increases night-time temperatures, promoting temperatures that shift the fungus towards maximal survival and reproduction at not only higher elevations but a variety of
elevations (Pounds et al. 2006). There is recent evidence that *B. dendrobatidis* has a more complex ecology than was originally thought and appears to do well at a variety of temperatures; the effects of variable environmental temperatures are likely to be complex (Woodhams et al., 2008a). Pounds et al. (2006) found that patterns of amphibian extinction varied over an elevational range, and extinctions actually peaked at middle elevations. They suggested that both warmer temperatures and colder temperatures may limit the pathogenicity of chytrid fungus. Rapid climate change via anthropogenic global warming is a double-edged sword with respect to the disease chytridiomycosis since it can both promote a more prosperous environment for the pathogen and simultaneously weaken the host.

I did note differential pathogenic effects in the lowlands, and these effects may have been due to a number of factors intrinsic to the sites, including higher pathogen prevalence and intensity at Cuatro Callitas, elevational differences conferring more suitable climatic regimes for the fungus (Pounds et al. 2006), and the presence of additional anthropogenic decline factors at Cuatro Callitas that may have caused increased disease susceptibility. Likely these effects were working synergistically to cause decline, and from my results it is not possible to conclude that chytridiomycosis was the prime agent of decline at this site. Furthermore, it is important to acknowledge that any apparent population decline observed in this study may not be indicative of overall trends given that only two sampling years of data were available with which to make comparisons. This reality clearly signifies the importance of long-term studies on amphibians in areas infected with chytridiomycosis. Indeed Ibáñez et al. (2002) reported that high year-to-year variations in all populations of amphibians in Panama underscore the need for long-term monitoring. Amphibian populations are known to be inherently variable, and evidence from one long-term amphibian population study proves that populations wax and wane significantly on an annual basis; conclusions about a population’s decline status from any one year may not be reflective of the overall population status (Green 2003).

**FACTORS INFLUENCING DIFFERENTIAL DISEASE SUSCEPTIBILITY IN HOSTS**

My results are in support of the conclusion made by Woodhams et al. 2006a that the
chytrid fungus is a generalist pathogen. *Batrachochytrium dendrobatidis* occurred in amphibians of every study site regardless of elevation or stage of epidemic amphibian decline, and was present to some extent in all host guilds, in both stream and forest habitats, during both wet and dry seasons, and at all body sizes. There is evidence, however, that anurans show differential susceptibility to the disease chytridiomycosis (eg., Amiche et al. 1999, Lips et al. 2003, Stuart et al. 2004, Woodhams and Alford 2005), and this concept is readily observable through changes in population abundance and composition. Indeed, mean abundance for many anuran guilds in our study is higher along the combined elevational gradients than the epidemics gradient, likely because the elevational transects were composed of fewer post-decline sites than was the epidemics gradient.

**Host Guild**

A few detectable trends were found in our study with respect to abundance of some anuran guilds that may have been a result of differential pathogen susceptibility. We observed an absence of most stream-associated anuran species at post-decline sites in our study, with the exception of the “leaf frogs”, which were abundant at all sites regardless of stage of epidemic decline and despite many individuals bearing infections at relatively high intensities. We also found a high site-specific variance in abundance of “treefrogs” in our study, noting that overall mean abundance was low along sites of the combined elevational transects but high along some sites of the epidemics transect. Some species of anurans possess highly potent antimicrobial peptides which confer resistance to chytridiomycosis (Amiche et al. 1999). Woodhams et al. (2007) found that the possession of highly potent skin peptides varies greatly at the species, and even individual, level, and immune responses may be both innate and adaptive. There may also be a species-specific threshold of disease intensity that leads to mortality, thus determining the length of time that species survive post-infection (Carey et al. 2006). There is evidence that at least some members of the “treefrogs” and “leaf frogs” guilds in Panama are capable of shedding strong infections (Woodhams et al. 2006a); likely antimicrobial peptide defences conferred greater resistance to “leaf frogs” and possibly *Hyla rufitela* in our study. More work is needed to determine the extent to which chytrid
prevalence and intensity differs amongst anuran guilds and whether this is related to ecological factors, phylogenetic signals, or both.

**Habitat**

Highly stream-associated frog species are considered more likely to be infected with *B. dendrobatidis* and experience chytrid-induced decline than species having a more terrestrial existence (Lips et al. 2003, Woodhams and Alford 2005, Kriger and Hero 2007). In my study, the stream-associated anuran guilds showed either lower or more highly variable abundance across transects with combined sites, which appears to be directly related to the site-specific status of chytrid infection. In addition, species richness is lower for stream-associated groups and only the “leaf-litter frogs” guild shows a high species richness across all sites. However, if stream-associated species are showing greater decline in chytrid-infected zones, then I would have expected to see higher *B. dendrobatidis* prevalence and/or intensity for these stream-associated groups as well, but this is not the case. In particular, when I compared prevalence and intensity between forest and stream habitats in each site for all frogs, I found that chytrid prevalence was not significantly different between habitats in any site, and habitat-specific intensity was similar in all sites except Altos de Campana and Fortuna, where infection intensity was actually higher in the forest. This confounding result suggests that the link between infection prevalence/intensity, habitat and corresponding decline is much more complex than imagined. Certainly it appears that infection prevalence and intensity is not a faithful predictor of disease effects in relation to habitat.

Infection should occur at higher prevalence in (resistant) species with stable populations than in (susceptible) species in which infection leads to mortality and rapid removal from the population (Woodhams and Alford 2005). If there are a large number of terrestrial species with pathogenic resistance then this could account for the observed high prevalence and intensity in the forest. Since the majority of amphibian decline has been noted amongst aquatic species, most research on disease resistance has focused on these amphibians; indeed the majority of species identified to date with potent antimicrobial peptides capable of combating chytrid infection have a high aquatic life history (Woodhams et al. 2006a and 2006b). More work is needed to determine the
extent to which terrestrial species exhibit resistance to chytriidiomycosis. Resistant species remaining in sites post-decline may act as carriers of the pathogen, passing it on to less resistant re-introduced species through direct contact or by re-introduction into aquatic environments (Berger et al. 1999; Daszak et al. 2004; Weldon et al. 2004; Beard and O’Neill 2005).

**Seasonality**

Variable seasonal differences in anuran abundance and species richness were observed throughout sites in my study, but I did note a general trend of higher frog abundance in the wet season and higher species richness in the dry season. Likely the fact that most species breed during the wet season (Savage 2002) and are less cryptic and easier to find at this time led to the seemingly higher abundance. I noted seasonal differences in pathogen prevalence for two of the eight sites sampled in this study, but found opposing trends for these two sites. Chytrid prevalence and intensity was higher in the wet season in Altos del María, in accordance with work on pathogen seasonality by both Bosch et al. (2007) and Kriger et al. (2007b), and the disease outbreak at this site coincided with the onset of the wet season, a trend noted by other researchers (eg. Aplin and Kirkpatrick 2000). In Fortuna, conversely, I found that pathogen prevalence was significantly higher in the dry season. This might alternatively be explained by the fact that anurans tend to congregate in wet areas in the dry season, which could facilitate pathogen spread (Ibáñez et al. 2002).

Indeed, seasonality has been shown to affect not only the prevalence of *B. dendrobatidis* but also the pathogenicity of chytriidiomycosis (Aplin and Kirkpatrick 2000, Berger et al. 2004). Because I noted opposing seasonal differences for two sites in our study, and results from one site were confounded by disease outbreak, no conclusive evidence can be drawn from this study regarding effects of season on presence of the chytrid. It is thus possible that season does not play much of a role in pathogen prevalence in Panama, or if it does, the effects may be masked by other factors. Such factors may be intrinsic to anuran populations in Panama, like changes in frog behaviour in response to the changing seasons, or artefacts of our sampling design, since we sampled from sites of varying elevation and stages of decline. All of these factors may
have as much or more of an impact on chytrid epidemiology than season. Further research is needed to determine the exact seasonal role on chytrid epidemiology in both montane and lower elevation sites in the Neotropics.

**Anuran body size**

Anuran body size was found to play a role in differential infection susceptibility for two observed species in our study, *Eleutherodactylus crassidigitus* and *Colostethus flotator*. For both of these species, there was a general trend of a smaller anuran body size showing a higher propensity towards infection prevalence but not intensity of the chytrid. My results accord with other authors who found the same correlation of a smaller body size showing higher prevalence and more intense infections than larger body sizes, suggesting that frogs either outgrow infection or have infection-stunted growth (Kriger et al. 2007). Aquatic larvae have been implicated as possible disease reservoirs capable of pathogen transfer to juvenile post-metamorphic frogs (Daszak et al. 2003, Blaustein et al. 2005, Woodhams and Alford 2005), and perhaps this could explain the higher infection prevalence in small *C. flotator* individuals. *Eleutherodactylus crassidigitus* has direct-developing reproduction and therefore no aquatic larvae, but perhaps differential behaviours between juvenile and adults may account for the higher infection prevalence in smaller individuals. Subadults of *Eleutherodactylus coqui* are generally found to perch closer to the forest floor than are adults (Beard et al. 2003, Beard and O’Neill 2005), which may make them more susceptible to a water-borne pathogen like *B. dendrobatidis* since the greater moisture content that is found in leaf litter is preferable to subadults (Pough et al. 1983).

**REPTILES AS RESERVOIR HOSTS AND DISEASE TRANSMISSION AGENTS**

*Batrachocheiromyrmex dendrobatidis* is an infective organism that is not restricted to amphibians, as previously thought. I have discovered that the fungus can exist on at least two species of lizards, both terrestrial (*Anolis humilis*) and aquatic (*Anolis lionotus*), as well as on three species of Colubrid snakes (*Imantodes cenchoa, Nothopsis rugosus*, and *Urotheca euryzona*). Infection prevalence and intensity was similar for reptiles and anurans, and site-specific differences followed the same trend; reptiles from the site
undergoing decline showed the highest infection prevalence, and at the site where anurans showed lowest overall infection prevalence, no reptiles were infected with the chytrid. However, I noted no evidence of chytridiomycosis among reptiles, and no individual that tested positive for the chytrid showed any symptoms of disease. The fungus is known to feed on the keratin of amphibian skin (Daszak et al. 2003), likely becoming fatal to amphibians either by interfering with cutaneous respiration and water uptake through the skin, by releasing toxic proteolytic enzymes or other active compounds that are absorbed through their permeable skin, and/or through facilitation of other microbial invasions (Berger et al. 1998, Pessier et al. 1999). Although *B. dendrobatidis* is obviously capable of existing on keratinised reptile skin under natural field conditions, none of the above fatal interactions are relevant to reptiles, which have a relatively impermeable, non-respiratory integument.

I propose that reptiles act as important reservoir hosts of chytridiomycosis by maintaining the pathogen in the environment without succumbing to disease. This would be important especially at post-decline sites where reservoir hosts would ensure that the chytrid remains at a site even after host populations have crashed (Lips et al. 2005a). Long-term infected reservoir hosts could shed zoospores into the environment until it is saturated and transmission between populations and areas is facilitated (Lips et al. 2006). Although reptiles likely do not succumb to chytridiomycosis, evidence of reptiles declining in conjunction with amphibians in some Neotropical sites suggests that this possibility should be explored further (Whitfield et al. 2007). These coinciding declines likely represent cascading effects of amphibian biomass loss on other organisms, rather than direct chytridiomycosis effects (Whiles et al. 2006). It remains to be seen whether *B. dendrobatidis* can complete its life cycle on reptile integument, and whether it can persist on reptiles indefinitely in the absence of amphibian hosts.

**CONSERVATION IMPLICATIONS AND CONCLUSIONS**

Currently the only option believed available for amphibian conservation in the short-term is *ex situ* captive breeding, and experts from the CBSG/WAZA are promoting this worldwide initiative (Zippel et al. 2006). *Ex situ* breeding of amphibians has been criticized because it is unclear whether future reintroduction is possible. Unnatural size
reduction of wild populations through removal for such programs will decrease genetic variability and hence disease resistance in the wild, and animals bred in chytrid-free areas in captivity will be bred without disease resistance (Altizer et al. 2003). From work I have done in Panama, I have shown that the chytrid is extremely ubiquitous. It is distributed from low to high elevations; it can be found in both “pre-epidemic” and in “post-epidemic” highland amphibian communities up to 11 years after decline; it is present to some extent in all anuran guilds, in both stream and forest habitats, during both wet and dry seasons, and at all body sizes; and it can even exist on reptiles. All of the above results suggest that re-introduction of captive-bred amphibians might not be possible unless it can be determined that disease resistance is heritable and amphibians can be selectively bred for disease resistance. The identification of all resistant species is the first step in this vital process.

Captive breeding should be supplementary to increasingly rigorous scientific research into additional conservation programs for amphibians in infected areas. To do this, it is imperative that we gain a greater understanding of the ecology of the fungus and its effects of host amphibians under natural conditions. From my study it appears that Batrachochytrium dendrobatidis does not affect all anuran hosts equally. Moreover, B. dendrobatidis prevalence/intensity is not always a consistent predictor of disease effects in anuran communities. Of all the ecological trends detected in this study with respect to host-pathogen relationships, none were universal across all frog guilds, elevations, or stages of epidemic decline in Panama. Similar contradictions occur within the literature, and thus it appears that multiple ecological factors affecting chytrid prevalence and corresponding anuran decline occur simultaneously. Further research is needed to tease apart these ecological factors affecting disease susceptibility in anurans.

A lack of complete long-term biological surveys for anurans in the Neotropics hampers our ability to comment on population fluctuations for both post-infection and pre-infection areas. Very few long-term population studies of any species have been done, thus compromising our ability to accurately detect declines since a confident detection of a “decline” depends on the intensity and duration of investigation (Wright et al. 2001). Batrachochytrium dendrobatidis may appear to be the primary culprit of widespread amphibian decline in Panama and elsewhere, but even so we should not
discourage any research that attempts to elucidate other decline factors or ideas for conservation. We must still do our best to mitigate habitat destruction and promote threat management, especially if *ex situ* amphibian captive breeding programs and subsequent reintroduction continue to be the primary form of amphibian conservation.
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Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin 

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APPENDIX 0 – Species membership in eight different anuran guilds. Site presence (by sampling year) at all eight sites across central Panama is denoted for every species with a check-mark. Site names not accompanied by a sampling year were surveyed only in 2006.

<table>
<thead>
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<th>Guild</th>
<th>Species (according to Savage 2002)</th>
<th>El Copé</th>
<th>La Rica</th>
<th>Palmarazo '06</th>
<th>Palmarazo '01</th>
<th>Cuatro Callitas '06</th>
<th>Cuatro Callitas '01</th>
<th>Altos de Campana '06</th>
<th>Altos de Campana '95</th>
<th>Cerro Trinidad '06</th>
<th>Cerro Trinidad '96</th>
<th>Altos del María</th>
<th>Fortuna</th>
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<tr>
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<td>✓</td>
<td>✓</td>
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<td>✓</td>
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</tbody>
</table>
APPENDIX 1 - Conventional PCR test for *Batrachochytrium dendrobatidis* from skin swabs

This protocol is successful in detecting chytrid from skin swabs collected in Panama and is sensitive enough to detect as little as 10 pg of DNA per microliter, but I do not know how many zoospores it is capable of detecting. This protocol was originally developed by Corinne Richards at the University of Michigan. This version is modified and optimized from her protocol to increase detection sensitivity under laboratory conditions at the Smithsonian Naos Laboratory. To collect the skin swabs, I used the technique developed by Lauren Livo at the University of Colorado.

Protocol - DNA extraction

1. Vortex collection tube containing swab for 30 seconds
2. Remove swab from tube, rubbing it against the side of the tube to get as much liquid off of the swab as possible. I use a pair of tweezers to remove the swab, which I sterilize between each sample.
3. Remove 300 µL of solution from the tube and place it into a 0.5 ml PCR tube and centrifuge until a pellet forms (about 5 minutes should be enough. Sometimes you can’t see a pellet but it still may test positive for Bd). Place the swab back in its original collection tube (remaining solution can be saved for additional tests if desired).
4. After centrifuging the PCR tube containing your sample, remove the supernatant (all the liquid above the pellet) and discard it (save the PCR tube with the pellet). I remove the supernatant by decanting directly into a beaker and then tipping the PCR tubes upside down on a paper towel to allow additional ethanol to drain. Since the pellet is either very tiny or invisible I find decanting through pipetting to be difficult and the likelihood of sucking out the pellet seems pretty high. A small amount of ethanol at the bottom of the tube does not seem to affect the extractions.
5. Add 10 µL of 10x PCR buffer (this should come with your Taq) and 1µL of proteinase K (1mg/ml) to the PCR tube containing the pellet. Vortex for a few seconds to resuspend pellet.
6. Incubate at 55 degrees Celsius for 3 hours. Any kind of incubator works well (thermocycler, water bath, oven), the samples do not need to rotate during incubation.
7. Centrifuge at 12,000 rpm for a few seconds
8. Incubate at 100 degrees Celsius for 5 minutes to inactivate proteinase K. I find this easiest in a thermocycler.
9. Add 20 µL GeneReleaser® to the sample tubes. The GeneReleaser Tips suggest that it is best not to mix the GeneReleaser in with the samples, so I generally just assure that the GeneReleaser has fallen to the bottom of the PCR tube but do not mix. Be sure to store the GeneReleaser at 4 C and not in a freezer. (http://www.bioventures.com/products/gener releaser/index.php)
10. Thermocycle according to GeneReleaser® General Protocol:
    1) 65°C for 30 seconds
    2) 8°C for 60 seconds
    3) 65°C for 90 seconds
    4) 97°C for 180 seconds
5) 8°C for 60 seconds  
6) 65°C for 180 seconds  
7) 97°C for 60 seconds  
8) 65°C for 60 seconds  
9) hold at 80°C until ready to proceed to next step in protocol.

11. Centrifuge at 12,000 rpm for 1 minute 
12. Transfer supernatant to new 0.5mL tube. This will be the sample you will use for the PCR step.

_Polymerase Chain Reaction_

13. Add the following to each PCR tube to make 12 µL samples:  
   1) 1.25 µL dNTPs (10uM)  
   2) 1.25 µL 10xPCR buffer  
   3) 1.0 µL MgCl₂  
   4) 0.05 µL Taq (I use Amplitaq Gold and this protocol has been optimized for this particular Taq. During a Taq Assay I discovered that Amplitaq Gold was superior in detection sensitivity to 4 other kinds of Taq.)  
   5) 0.5 µL of each of the two primers (Bd1a and Bd2a, see below, 10uM)  
   6) 5.45 µL double deionized water. Be particularly careful of contamination in your ddH₂O.  
   7) 2 µL of chytrid sample from step 12 above.  
14. Vortex briefly, then centrifuge briefly all samples prior to putting in the thermocycler for PCR Amplification.

(Note: it is wise to run both a positive and a negative control for each PCR reaction)

_Primers:_

These primers and many steps in this protocol were from Annis _et al._ 2004. A DNA-based assay identifies _Batrachochytrium dendrobatidis_ in amphibians. Journal of Wildlife Diseases. 40(3) 420-428.

Bd1a: (5’– 3’) CAGTGTGCCATATGTCACG  
Bd2a: (5’– 3’) CATGGTTCATATCTGTCCAG

_PCX step thermocycler conditions:_  
   1) 94°C for 5 minutes  
   2) 93°C for 45 seconds  
   3) 60°C for 45 seconds  
   4) 72°C for 60 seconds  
   5) repeat steps 2 through 4, 34 more times  
   6) 72°C for 10 minutes

15. Run PCR products on a 3% agarose gel as normal with TAE Buffer and regular agarose. I load my gels with the whole PCR sample as it allows for more distinct bands in weak chytrid samples. You should expect a band about 300 base pairs in length if _B. dendrobatidis_ is present.
APPENDIX 2a – Summary of herpetofauna sampling by site in both seasons and habitats in Panama in 2006. The number of species recorded is the combined number of species found for both seasons and habitats.

<table>
<thead>
<tr>
<th>Site</th>
<th>Dry Season</th>
<th>Wet Season</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forest</td>
<td>Stream</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>Night</td>
<td>Day</td>
</tr>
<tr>
<td>Cuatro Calitias (45 m)</td>
<td>SHE (61.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (21 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (6 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Palmarazo (135 m)</td>
<td>SHE (82.25)</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (23 sp.)</td>
<td>32</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (3 sp.)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Snakes (1 sp.)</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>La Rica (250 m)</td>
<td>SHE (75.75)</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (19 sp.)</td>
<td>17</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (3 sp.)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Snakes (2 sp.)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>El Copé (760 m) Recent post-epidemic</td>
<td>SHE (68.5)</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td># Frogs (14 sp.)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td># Lizards (4 sp.)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Snakes (2 sp.)</td>
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<td>0</td>
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<tr>
<td>Cerro Trinidad (540 m)</td>
<td>SHE (47)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (15 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (2 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Snakes (1 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Altos de Campana (860 m) Pre-epidemic</td>
<td>SHE (68.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (25 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (4 sp.)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Snakes (1 sp.)</td>
<td>N/A</td>
<td>N/A</td>
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<td>Altos del Mar (890 m) During epidemic</td>
<td>SHE (74.75)</td>
<td>9</td>
<td>8</td>
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<td></td>
<td># Frogs (31 sp.)</td>
<td>11</td>
<td>35</td>
</tr>
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<td></td>
<td># Lizards (4 sp.)</td>
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<td>7</td>
</tr>
<tr>
<td></td>
<td># Snakes (1 sp.)</td>
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<td>0</td>
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<tr>
<td>Fortuna (1215 m) Long post-epidemic</td>
<td>SHE (52)</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td># Frogs (8 sp.)</td>
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<td>N/A</td>
</tr>
<tr>
<td></td>
<td># Lizards (4 sp.)</td>
<td>25</td>
<td>N/A</td>
</tr>
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**APPENDIX 2b** – Summary of chytrid prevalence and intensity by site in both seasons and habitats in Panama in 2006. Only anuran epidermal swabs are included in these analyses.

<table>
<thead>
<tr>
<th></th>
<th>El Copé 760m</th>
<th>La Rica 250m</th>
<th>Palmarazo 135m</th>
<th>Cuatro Callitas 45m</th>
<th>Altos de Campana 850m</th>
<th>Cerro Trinidad 540m</th>
<th>Altos del Marías 950m</th>
<th>Fortuna 1220m</th>
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<tbody>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td>16.2%</td>
<td>27.6%</td>
<td>10.6%</td>
<td>17.9%</td>
<td>13.1%</td>
<td>15.1%</td>
<td>67.3%</td>
<td>45.5%</td>
</tr>
<tr>
<td>(# Pos / Total)</td>
<td>(N=37)</td>
<td>(N=76)</td>
<td>(N=141)</td>
<td>(N=67)</td>
<td>(N=267)</td>
<td>(N=245)</td>
<td>(N=254)</td>
<td>(N=66)</td>
</tr>
<tr>
<td><strong>Intensity</strong></td>
<td>2.6 ± 1.5</td>
<td>69.6 ± 222.9</td>
<td>156.1 ± 481.9</td>
<td>694 ± 2255.0</td>
<td>3.7 ± 4.1</td>
<td>40.2 ± 98.0</td>
<td>1210 ± 6998</td>
<td>441 ± 2344</td>
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<tr>
<td>(Mean zoospores ± StDev)</td>
<td>(N=6)</td>
<td>(N=21)</td>
<td>(N=15)</td>
<td>(N=12)</td>
<td>(N=35)</td>
<td>(N=37)</td>
<td>(N=171)</td>
<td>(N=30)</td>
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<tr>
<td><strong>Forest Wet</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td>13.3%</td>
<td>0%</td>
<td>14.7%</td>
<td>11.1%</td>
<td>16.2%</td>
<td>3.6%</td>
<td>26.1%</td>
<td>N/A</td>
</tr>
<tr>
<td>(# Pos / Total)</td>
<td>(N=15)</td>
<td>(N=1)</td>
<td>(N=34)</td>
<td>(N=18)</td>
<td>(N=111)</td>
<td>(N=28)</td>
<td>(N=23)</td>
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<tr>
<td><strong>Intensity</strong></td>
<td>2.3 ± 0.1</td>
<td>N/A</td>
<td>88.5 ± 142.0</td>
<td>2.3 ± 1.2</td>
<td>5.7 ± 5.0</td>
<td>4.7 ± 0</td>
<td>2149 ± 5242</td>
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</tr>
<tr>
<td>(Mean zoospores ± StDev)</td>
<td>(N=2)</td>
<td></td>
<td>(N=5)</td>
<td>(N=2)</td>
<td>(N=18)</td>
<td>(N=1)</td>
<td>(N=6)</td>
<td></td>
</tr>
<tr>
<td><strong>Stream Wet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td>16.7%</td>
<td>22.2%</td>
<td>6.7%</td>
<td>20.4%</td>
<td>10.9%</td>
<td>16.6%</td>
<td>78.3%</td>
<td>28.6%</td>
</tr>
<tr>
<td>(# Pos / Total)</td>
<td>(N=12)</td>
<td>(N=26)</td>
<td>(N=60)</td>
<td>(N=49)</td>
<td>(N=156)</td>
<td>(N=217)</td>
<td>(N=115)</td>
<td>(N=14)</td>
</tr>
<tr>
<td><strong>Intensity</strong></td>
<td>3.9 ± 2.3</td>
<td>197.0 ± 404.0</td>
<td>2.3 ± 1.2</td>
<td>832.0 ± 2468</td>
<td>1.6 ± 0.6</td>
<td>41.2 ± 99.2</td>
<td>2287 ± 9543</td>
<td>1.9 ± 0.7</td>
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<tr>
<td>(Mean zoospores ± StDev)</td>
<td>(N=2)</td>
<td>(N=6)</td>
<td>(N=4)</td>
<td>(N=10)</td>
<td>(N=17)</td>
<td>(N=36)</td>
<td>(N=90)</td>
<td>(N=4)</td>
</tr>
<tr>
<td><strong>Forest Dry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td>0%</td>
<td>23.5%</td>
<td>12.5%</td>
<td></td>
<td>51.2%</td>
<td></td>
<td></td>
<td>58.8%</td>
</tr>
<tr>
<td>(# Pos / Total)</td>
<td>(N=1)</td>
<td>(N=17)</td>
<td>(N=32)</td>
<td></td>
<td>(N=41)</td>
<td></td>
<td></td>
<td>(N=17)</td>
</tr>
<tr>
<td><strong>Intensity</strong></td>
<td>N/A</td>
<td>6.0 ± 8.7</td>
<td>3.7 ± 4.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.3 ± 7.7</td>
</tr>
<tr>
<td>(Mean zoospores ± StDev)</td>
<td></td>
<td>(N=4)</td>
<td>(N=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(N=10)</td>
</tr>
<tr>
<td><strong>Stream Dry</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td>34.4%</td>
<td>7.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61.2%</td>
</tr>
<tr>
<td>(# Pos / Total)</td>
<td>(N=36)</td>
<td>(N=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(N=98)</td>
</tr>
<tr>
<td><strong>Intensity</strong></td>
<td>1.5 ± 0.2</td>
<td>22.9 ± 60.9</td>
<td>937.3 ± 1319.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.7 ± 54.5</td>
</tr>
<tr>
<td>(Mean zoospores ± StDev)</td>
<td>(N=2)</td>
<td>(N=11)</td>
<td>(N=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(N=60)</td>
</tr>
</tbody>
</table>

Overall Prevalence: 16.2% (N=37) to 45.5% (N=66).
Overall Intensity: 2.6 ± 1.5 zoospores (N=6) to 694 ± 2255.0 zoospores (N=12).

Forest Wet Prevalence: 13.3% (N=15) to 26.1% (N=23).
Forest Wet Intensity: 2.3 ± 0.1 zoospores (N=2) to N/A.

Stream Wet Prevalence: 16.7% (N=12) to 28.6% (N=14).
Stream Wet Intensity: 3.9 ± 2.3 zoospores (N=2) to 1.9 ± 0.7 zoospores (N=4).

Forest Dry Prevalence: 0% (N=1) to 58.8% (N=17).
Forest Dry Intensity: N/A to 6.3 ± 7.7 zoospores (N=10).

Stream Dry Prevalence: 34.4% (N=36) to 83.3% (N=12).
Stream Dry Intensity: 1.5 ± 0.2 zoospores (N=2) to 17.7 ± 54.5 zoospores (N=60).
Appendix 3 - Mean anuran abundance (+/- standard deviation) and species richness in forest and stream habitat during the wet and dry seasons in all eight sites surveyed in Panama in 2006. Abundance of “All Frogs” is the mean abundance of all the guilds.
**Altos Del María abundance**

![Graph showing abundance data for different species at Altos Del María.](image)

**Altos Del María richness**

![Graph showing richness data for different species at Altos Del María.](image)

**Fortuna abundance**

![Graph showing abundance data for different species at Fortuna.](image)

**Fortuna richness**

![Graph showing richness data for different species at Fortuna.](image)
March 12, 2007

The McGill University Animal Care Committee certifies that

**Vanessa Kilburn** has successfully completed the

**Advanced Level**

of the

**Theory Training Course on Animal Use for Research and Teaching**

on

**March 8, 2007.**

The training includes the following topics:

- **Basic Level:** Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety
- **Advanced Level:** Anesthesia, Analgesia, Euthanasia, Categories, Influencing Factors, and Environmental Enrichment
- **Wildlife:** Basic principles for working with wildlife in the laboratory and in the field.

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.


Deanna Collin  
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

*Note: Trainee must keep this certificate as other institutions may request it as evidence of training*