FATE AND DISSEMINATION OF PATHOGENS IN AQUATIC WILDLIFE AND THEIR ECOSYSTEMS

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FATE AND DISSEMINATION OF PATHOGENS IN AQUATIC WILDLIFE AND THEIR ECOSYSTEMS

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ABSTRACT

FATE AND DISSEMINATION OF PATHOGENS IN AQUATIC WILDLIFE AND THEIR ECOSYSTEMS

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The world is experiencing an increase in emergent infectious diseases as a result of expanding world markets, urbanization and intensified animal husbandry. It is not just humans that are affected, but also domestic and wild animals. Many diseases affecting humans are of animal origin and because of population increases and globalization of society we will continue to see increased interactions between humans, domestic animals, and wildlife. The wildlife component of this triad has received inadequate focus in the past to effectively protect not only human health, but also biodiversity of wildlife, agriculture and trade.

The objective of this thesis work is to assess the presence of microbial pathogens such as the bacterium *Salmonella enterica* and the fungus *Batrachochytrium dendrobatidis* (*Bd*) in aquatic environments and elucidate the effects of specific environmental conditions on the fate of these pathogens in the non-host environment and the resulting impact of these conditions to persistence and prevalence of infections.

The first studies provided evidence that there are many species of aquatic animals that can serve as potential reservoirs for the long term persistence of *Salmonella*. Additional studies confirmed the notion that the terrestrial environment can act as a significant source of contamination of the aquatic environment by *Salmonella*. Finally, studies performed demonstrated that naturally occurring biofilms can provide a mechanism for long term persistence and contamination of the aquatic ecosystem by *Salmonella*.

An inexpensive and sensitive nested PCR protocol was developed for studies involving *Bd*. Using this technique, in addition to a qPCR method, the presence of *Bd* in populations including endangered and endemic amphibians in Central Texas was demonstrated and later affirmed. In addition to its presence, spatial and temporal differences in prevalence and intensity of infection were detected. Studies suggest that seasonal temperatures, water quality and permanency of water can contribute to increased infections by the pathogen.

Understanding interactions of pathogens such as these with their host and non-host environments could provide a greater understanding of the organism and be the foundation for preemptive actions and responses that could potentially help limit future impacts to the health of both humans and wildlife populations.

CHAPTER 1

GENERAL INTRODUCTION

Microbes are by far the most abundant life form on the planet. They drive biogeochemical cycles and can act as both essential symbionts and important pathogens of other organisms. Despite their importance, our knowledge of microbial diversity and ecological function is very limited. Recently, powerful, culture-independent, molecular techniques have been developed to examine microbial communities in a variety of environments. Information about the structure and function of microbes in the environment is essential to understanding the underlying processes.

Until recently, advances in molecular microbial ecology have been primarily used to study traditional microbial ecosystems. These same techniques, however, can also be used to study the fate of pathogens in the environment. Pathogenic microorganisms are often passed into the aquatic environment and must survive until infection of a new host. Interactions of pathogens with the non-host environment may therefore play an important role in a pathogen's life history. Understanding the interactions of pathogens with the environment is therefore essential to understanding the organism and limiting potential future impacts.

The world is experiencing an increase in emergent infections as a result of a world market, urbanization and intensified animal husbandry. It is not just humans affected, but

also domestic and wild animals. Many diseases affecting humans are of animal origin and because of population increases and globalization of society we will continue to see increased interactions between humans, domestic animals, and wildlife. With rising population, intensified agriculture and serious threats to biodiversity, this problem will only get worse. There is now a strong need to have sound information about diseases of humans, livestock and wildlife. The wildlife component of this triad has received inadequate focus in the past to effectively protect not only human health, but biodiversity, agriculture and trade.

My research focuses on the interactions of microbial pathogens such as *Salmonella* and the fungus *Batrachochytrium dendrobatidis* with animals and aquatic ecosystems. Understanding interactions of pathogens such as these with their host and non-host environments could provide the foundation for preemptive actions and responses that could potentially help minimize disease impacts on human and animal health.

Salmonella

One area of interest is in the interactions of *Salmonella* with aquatic wildlife and the environment. Although not all strains cause disease, *Salmonella* is recognized worldwide as an important public health concern (Humphrey 2000). Annually, infections with *Salmonella* account for 1.3 billion cases of gastroenteritis worldwide (Pang et al. 1995) and as many as 1.4 million cases in the United States alone (Mead et al. 1999). The majority of illnesses result from exposure to undercooked animal products or cross-contamination with foods consumed raw (Tauxe 1997, World Health Organization 2002)

although salmonellosis can also result from direct contact with contaminated water (Foltz 1969, Harvey et al. 1969) or infected animals (Sanyal et al. 1997, Wells et al. 2004, Nakadai et al. 2005).

The native habitat of *Salmonella* is widely considered to be the intestinal tract of taxonomically diverse group of vertebrates including birds, fish, mammals, invertebrates and reptiles (Janssen & Meyers 1968, Foster & Spector 1995, Gray 1995, Refsum et al. 2002, Briones et al. 2004). The general pattern of human infection by *Salmonella* is primarily related to animal reservoirs (Turnbull 1979, Anonymous 1980) even though many animals are mostly considered to be asymptomatic carriers of *Salmonella* (Chiodini & Sundberg 1981, Anonymous 1995). The release of feces from these animals can be a source of contamination of fresh- or marine waters, estuarine environments, vegetables, compost, or soils and sediments (Thomason et al. 1975, Polo et al. 1998, Refsum et al. 2002 Tavechio et al. 2002, Martinez-Urtaza et al. 2004). The occurrence of *Salmonella* in these environments is therefore frequently linked to environmental contamination through manure or wastewater discharges (Polo et al. 1998, Martinez-Urtaza et al. 2004). The general assumption is that once *Salmonella* enters the environment through fecal contamination it persists only for a short time (Morinigo et al. 1990, Monfort & Baleux 1994) before finding a new host or dying. Despite this assumption, however, *Salmonella* have been detected in pristine aquatic systems (Fair & Morrison 1967, Hendricks & Morrison 1967) and shown to persist in the environment for extended periods of time without significant impact from terrestrial animals (Hendricks 1971, Chao et al. 1987). *Salmonella* have even been suggested to be ubiquitously distributed and part of the natural flora present in some aquatic ecosystems (Jimenez et al. 1989, Marsh et al. 1998).

Associations with biofilms (Armon et al. 1997, Barker & Bloomfield 2000) or sediments (Hendricks 1971, Marsh et al. 1998) are assumed to help *Salmonella* survive in the environment. Although several studies have established the potential for *Salmonella* to form biofilms on surfaces under laboratory conditions (Austin et al. 1998, Joseph et al. 2001, Prouty & Gunn 2003), little data exists to show this is possible in a natural system. Despite the evidence that *Salmonella* have complex interactions with the non-host environment little is known about the organism's fate outside of host organisms. Understanding the ecology of *Salmonella* and its interaction with hosts and the environment is therefore imperative to limiting potential future infections.

Batrachochytrium dendrobatidis

The second area of study in my dissertation is the interaction of the fungus *Batrachochytrium dendrobatidis* with amphibians and the environment. Amphibians are an important group of animals and their losses could have profound effects on humans and ecosystems not only through direct means but also by indirect effects such as destabilizing ecosystems. Amphibians can be important as both predators and prey (Pounds et al. 1999, Poulin et al. 2001) as adults as well as important herbivores in the larval stage (Flecker et al. 1999, Ranvestel et al. 2004). Furthermore because they can be the most abundant land vertebrate in some tropical systems (Stebbins & Cohen 1995), the loss of amphibians would have profound effects on ecological function (Flecker et al. 1999, Ranvestel et al. 2004). Amphibians, however, are not just important to natural systems. They are also important to humans in many ways. Amphibians constitute a major source of protein for humans in some developing countries, are important

consumers of insects which may act as pests or vectors of disease (Stewart & Woolbright 1996, Beard et al. 2002, Beard et al. 2003), and also contain an incredibly diverse array of toxins that protect them from predation and invasion by microorganisms which may potentially be useful in the medical and industrial fields.

Amphibian populations have been declining worldwide at an alarming rate (Stuart et al. 2004). Widespread and severe amphibian declines were first noticed starting in the 1970s concerns continued to grow. But it was not until 1989 at the First World Congress of Herpetology that the true scope of the problem was realized. Evidence for declines included studies indicating losses were more widespread and severe than would be expected by random chance (Blaustein & Olson 1991). Montane and riverine species were suffering disproportionately high declines (Pounds et al. 1997, Lips et al. 2003), and several species were driven to extinction (Pounds & Crump 1994, Schloegel et al. 2006). Losses were beginning to appear non-random and unidirectional. What was more disconcerting is the fact that even species in relatively pristine habitats, protected from human impacts, were also suffering losses (Ron et al. 2003, Burrowes et al. 2004). A status report of amphibians summarized the extent of the problem (Stuart et al. 2004). Stuart and his colleagues examined the International Union for Conservation of Nature's (IUCN) Red List for amphibians. They discovered that, as of 2004, 43% of all amphibians were currently experiencing declines with 32% listed as threatened. Furthermore, another 22% of amphibians were at the time data deficient and probably also declining. They concluded that as much as 65% of all known amphibians were declining and in some danger of extinction. It was also noted that the number of critically endangered amphibians on the Red List has doubled since 1985, suggesting that

the rate of listing is increasing. Finally, Stuart and his colleagues highlighted exactly how dire the situation is for amphibians by noting that 85% of all vertebrate species added to the Red List in the 20 years prior to 2004 have been amphibians even though they represent only 10% of the vertebrate diversity.

Amphibians have several unique characteristics that make them especially prone to population losses such as highly permeable skin, an egg lacking a protective shell, as well as a complex lifestyle that exposes them to both the terrestrial and aquatic ecosystems. In addition, amphibians are equally prone to the major factors driving declines of all groups of animals such as overexploitation, habitat loss, environmental degradation, and impacts from novel diseases. One such novel disease (chytridiomycosis) is caused by the fungal pathogen *Batrachochytrium dendrobatidis* and has been implicated as a major contributing factor in amphibian declines (Berger et al. 1998, Daszak et al. 1999, Daszak et al. 2000, Lips et al. 2006).

Batrachochytrium dendrobatidis (*Bd*) was first identified in 1997 (Berger et al. 1998) and fully described in 1999 (Longcore et al. 1999). *Bd* is located in the division Chytridiomycota of the kingdom Fungi and are among the most primitive groups of fungi. The chytrid fungi are unique among fungi in that they have a motile flagellate zoospore (James et al. 2006). Most chytrid fungi live in aquatic environments and soils as saprobic decomposers of organic matter and are typically free-living or commensal organisms. Some species of chytrid fungi can however parasitize algae, invertebrates, plants and even other species of fungus (Gleason et al. 2008). *Bd* is unusual among the chytrid fungi in that it is one of the only two chytrid fungi that are known to parasitize vertebrates. *Bd* is a keratinaecous fungus that parasitizes the skin of adult amphibians as

well as the mouthparts of larvae (Berger et al. 1998, Longcore et al. 1999, Marantelli et al. 2004). The direct cause for mortality has been determined to be a disruption of the osmoregulatory function of amphibian skin causing electrolyte imbalance followed in many cases by death from asystolic cardiac arrest (Voyles et al. 2009). *Bd* is now known to infect over 350 different species of amphibians and although infection does not always lead to death, it has been implicated in the decline of over 200 species (Stuart et al. 2004, Skerratt et al. 2007). Although traditional disease models do not predict extinctions (McCallum & Dobson 1995), there is good evidence that *Bd* has been the proximal cause of extinction in at least three frog species (Retallick et al. 2004, Schloegel et al. 2006, Gewin 2008).

Infection by *Bd* can however have variable effects on amphibian populations with potential impact ranging from persistence with no mortalities (Daszak et al. 2004, Retallick et al. 2004) to mass die-offs without recovery (Daszak et al. 2003, Schloegel et al. 2006). The prevalence and pathogenicity of *Bd* on host organisms can vary across different species (Carey 2000, Retallick et al. 2004), and developmental stages (Lamirande & Nichols 2002), and is affected by environmental factors such as temperature (Woodhams & Alford 2005, Kriger & Hero 2007), precipitation (Ron 2005), and pollutants (Bosch et al. 2001, Davidson et al. 2001).

Although the presence of *Bd* has been confirmed on all continents containing amphibians, and in many states of the USA (Ouellet et al. 2005, Longcore et al. 2007, Rothermel et al. 2008) its presence in Texas is not well documented. Central Texas is home to many endemic and threatened species of amphibians including the Houston toad

(*Bufo houstonensis*) and several species of *Eurycea* salamanders. The potential effects to amphibians of central Texas from this pathogen has yet to be assessed.

Objectives

The objective of this thesis work is to assess the presence of *Salmonella* and *Bd* in local aquatic ecosystems and elucidate the effects of specific environmental conditions on the fate of these pathogens in the non-host environment and the resulting impact of these conditions to prevalence and pathogenicity. Most pathogens are thought to be affected primarily by host defenses, but environmental conditions may be an important factor in a pathogen's life history. Interaction with the environment can be intense on pathogens that affect the skin or while in the non-host environment searching for a new host or during re-infection. Previous studies on *Salmonella* have had conflicting results ranging from a low prevalence in the environment to suggestions of *Salmonella* being part of the natural flora (Jimenez et al. 1989) and those on *Bd* have demonstrated effects of environmental factors such as temperature, precipitation and naturally occurring antimicrobial peptides on the fungus (Carey et al. 1999, Davidson et al. 2001, Hopkins & Channing 2002). The objective of this dissertation was to perform a series of studies to study the fate of these two pathogens in aquatic environments specifically addressing these points:

Salmonella

1. Increase understanding of *Salmonella* in the non-host environment.
2. Test potential reservoirs.

3. Test factors contributing to persistence and dissemination of *Salmonella* in the environment.
4. Determine the potential of biofilms for the persistence of *Salmonella* in the non-host environment.

Batrachochytrium dendrobatidis

1. Determine if the pathogen is found in central Texas.
2. Determine if *Bd* is present in central Texas.
3. Determine environmental factors contributing to the prevalence of *Bd* in local populations of amphibians.

Expected Results

The major outcome of these studies will be a better understanding of the habits of *Batrachochytrium dendrobatidis* and *Salmonella* outside their host environment. Despite the general assumption that the natural environment of *Salmonella* is in the intestinal tract of animals, there is evidence of its persistence outside of the host. This study should help to explain the conditions necessary for *Salmonella* to persist, as well as help identify potential survival strategies such as associations with sediments or biofilms. Similarly, with *B. dendrobatidis*, this study should help elucidate the effects of specific environmental parameters on the prevalence and pathogenicity of the fungus. Understanding the interactions of pathogens with the non-host environment can help limit future infections.

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CHAPTER 2

DETECTION OF *SALMONELLA* IN DIFFERENT TURTLE SPECIES WITHIN A HEADWATER SPRING ECOSYSTEM[1]

Abstract

Sediments and water from the slough arm of Spring Lake, the headwaters of the San Marcos River, Texas, USA, as well as swabs from biofilms on carapace and from the cloacae of 18 common musk turtles (*Sternotherus odoratus*), 21 red-eared sliders (*Trachemys scripta elegans*), nine Texas river cooters (*Pseudemys texana*), one snapping turtle (*Chelydra serpentina serpentine*) and three Guadalupe spiny soft-shell turtles (*Apalone spinifera guadalupensis*) caught at the same site, were analyzed for *Salmonella* by culture and molecular techniques. Although enrichment cultures from sediment and water samples were negative for *Salmonella* in polymerase chain reaction (PCR)-based analyses, this technique detected *Salmonella* in the enrichments from both carapace and cloacae of 11 musk turtles (61%), eight red-eared sliders (38%) and the snapping turtle. *Salmonella* could also be detected in enrichments from the carapace of two additional red-eared sliders and two Texas river cooters; the remaining samples were negative. Further characterization of isolates obtained from the enrichment cultures of seven

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selected individuals that represented all turtle species with *Salmonella* confirmed the presence of *Salmonella. enterica* subspecies *enterica*, with serovars Rubislaw, Newport, Gaminara and Thompson identified. These results demonstrate the presence of differentstrains of potentially human pathogenic *Salmonella* naturally occurring on several turtle species with different life histories even within supposedly pristine environments.

Temporal analysis of *Salmonella* in a headwater spring ecosystem

Salmonella have been detected in the gastrointestinal tract of a large taxonomic variety of free-living and captive animals including mammals, birds, and reptiles throughout the world (Gray 1995, Refsum et al. 2002, Briones et al. 2004). Although both free-living and captive animals have been identified as reservoirs for human-associated salmonellosis (Guard-Petter 2001, Santos et al. 2001), the significance of free-living animals as potential carriers and vectors for *Salmonella* in human-associated salmonellosis is often less well established than for captive animals. Pet turtles, for example, are well-known reservoirs for *Salmonella* (Johnson-Delaney 1996), and many studies have demonstrated their significance in human-associated salmonellosis (Shane et al. 1990, Anonymous 1995, Sanyal et al. 1997b, Anonymous 1999, Geue & Löschner 2002, Mermin et al. 2004, Nakadai et al. 2005). However, studies on the occurrence of *Salmonella* in free-living turtles are scarce and the results are contradictory (Brenner et al. 2002, Briones et al. 2004, Richards et al. 2004, Chambers & Hulse 2006, Saelinger et al. 2006). While some studies failed to find any *Salmonella* in cloacal, fecal or gastrointestinal mucosal samples of free-living turtles, and thus questioned the

importance of free-living turtles as potential vectors for human-associated salmonellosis (Brenner et al. 2002, Richards et al. 2004, Saelinger et al. 2006), other investigations emphasized the role of reptiles including turtles as reservoirs for *Salmonella*, with *Salmonella* present in 41% of lizards, 54% of snakes and 32% of turtles analyzed (Briones et al. 2004) or even in 100% of all turtles tested (Chambers & Hulse 2006).

In a recent study, we demonstrated the presence of *Salmonella* in common musk turtles (*Sternotherus odoratus*) living in the headwaters of the San Marcos River, Texas, USA (Hahn et al. 2007). Although no *Salmonella* could be detected in sediment and water samples, about 50% of the musk turtles harbored *Salmonella* in both cloacal samples as well as in biofilms on the carapace, supporting the idea that free-living turtles can serve as reservoir for *Salmonella*. A generalization of this statement, however, requires additional data on the distribution of *Salmonella* in different taxa of free-living turtles in order to examine potential differences resulting from different life histories such as basking or foraging behavior.

The aim of this study was therefore to expand our previous survey and analyze additional turtle species for *Salmonella* in other areas of Spring Lake, the headwaters of the San Marcos River, Texas, USA (79°53'N, 97°55'W). In our previous study (Hahn et al. 2007) we examined the spring arm that is characterized by relatively constant environmental conditions in depth and throughout the year with high mineral nutrient availability regulated by the permanent supply of aquifer water through numerous springs and the resulting fast flow and exchange of the surface water (Groeger et al. 1997). Spring Lake also has a distinct slough arm, representing a more lentic environment with slow flow, large seasonal changes in temperature and redox conditions, and large

deposition of organic material (Groeger, pers. communication). We collected sediment and water samples as well as biofilms from the turtle carapace and direct cloacal samples from turtles living in this habitat.

Water and sediment samples were retrieved from several sites at the upper slough arm of Spring Lake with a vertical point water sampler and a bottom dredge, respectively, at a distance less than half a kilometer from our previous sampling sites in the spring arm (Fig. 2.1). Water samples were taken just below the surface and at a depth of about50 cm, just above the sediment. Turtles were collected with the use of a dip net or were caught in baited hoop nets. Fifty-two turtles were retrieved, i.e., 18 common musk turtles (*Sternotherus odoratus*, nine females, nine males), 21 red-eared sliders (*Trachemys scripta elegans*, nine females, 12 males), nine Texas river cooters (*Pseudemys texana*, all female), one snapping turtle (*Chelydra serpentina serpentine*, male) and three Guadalupe spiny softshell turtles (*Apalone spinifera guadalupensis*, two females, one male). Thus, we replicate our efforts from the spring arm (Hahn et al. 2007) by including a matching number of *S. odoratus* and *C. serpentina*, but significantly expand the overall taxonomic coverage and total number of turtles sampled.

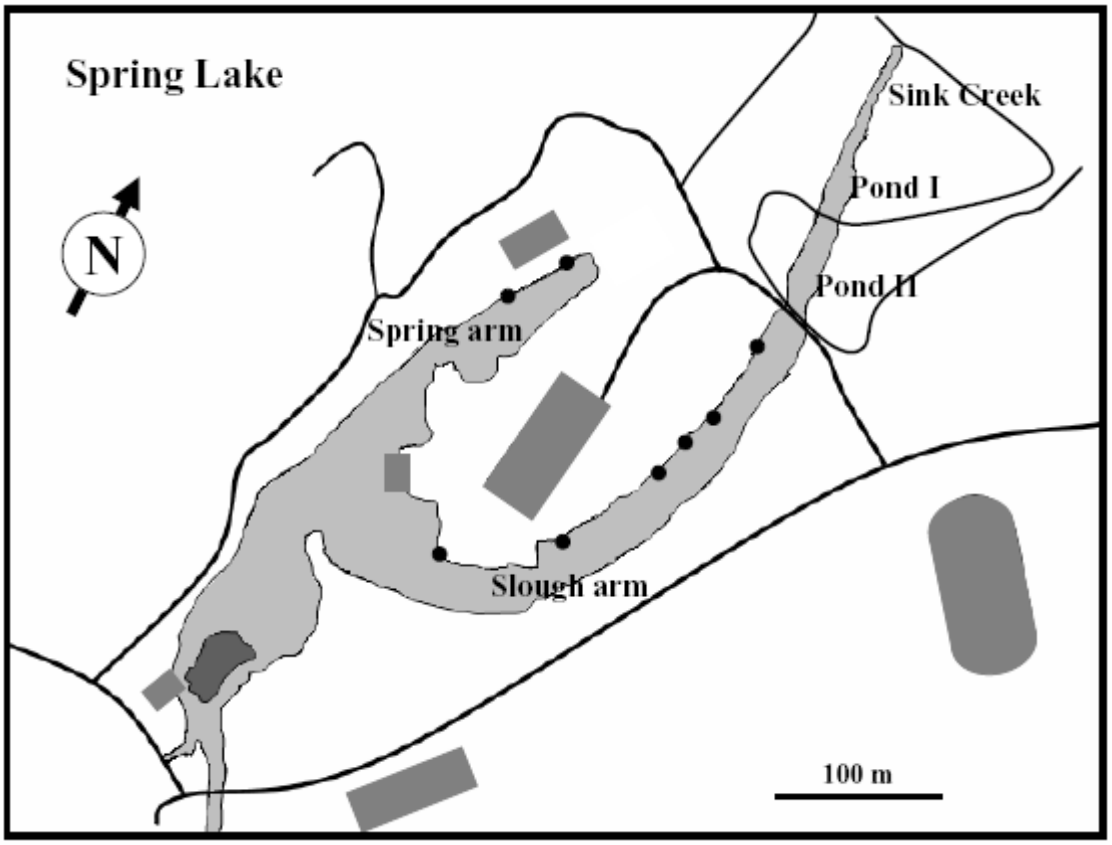

Figure 2.1: Schematic presentation of sampling sites. Sampling sites appear as dots in both spring and slough arms of Spring Lake, the head waters of the San Marcos River, Texas, USA (79o53'N, 97o55'W).

Samples were taken with sterile cotton wool swabs from the carapace and the cloacae of all turtle species (Hahn et al. 2007), except for the Guadalupe spiny softshell turtles, from which only cloacal samples were retrieved. Swabs, sub-samples of sediment (about 100 mg wet weight), and pellets obtained from 1 ml of the water samples centrifuged at 14,000 rpm for 2 min, were incubated in 1 ml of Buffered Peptone Water in 2-ml cryotubes at 37°C for 16-20 hours (International Standard Organization 1993). Sub-samples (100-µl) of these pre-enrichment cultures were transferred to 2-ml cryotubes

that contained 1 ml of Rappaport-Vassiliadis (RVS) Broth (Vassiliadis et al. 1981b) and incubated at 43°C for 24 hours in order to enrich for *Salmonella* (Vassiliadis et al. 1981).

Pre-enrichment and enrichment cultures of all samples, i.e., all sediment and water samples from the slough arm of Spring Lake as well as of all samples from carapace and cloacae of turtles, showed significant increment in turbidity during incubation suggesting microbial growth. Since enrichment conditions in RVS broth were only semi-selective, the increase in turbidity in enrichment cultures did not presuppose enrichment of *Salmonella*, and thus their potential presence in enrichment cultures required confirmation (Hahn et al. 2007). This confirmation was based on PCR-assisted detection of a 284-bp-fragment of the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by *Salmonella* (Suárez & Rüssmann 1998, Khan et al. 2000). This assay detects all *Salmonella enterica* subspecies as well as *S. bongori* and was recently validated and proposed as international standard diagnostic method for quality assurance laboratories in epidemiological studies on *Salmonella* spp. (Malorny et al. 2003).

For the detection of *Salmonella* by PCR, 100-µl-samples of pre-enrichment and semi-selective enrichment cultures as well as of cultures of *Salmonella typhimurium* ATCC 14028 that was always used as positive control, were centrifuged at 14,000 rpm for 1 minute. The bacterial pellets were washed once in sterile distilled water, and bacteria lysed in 100 µl of 50 mM NaOH by incubation at 65°C for 15 minutes (Hahn et al. 2007). One µl of this lysate was used as template for PCR amplification with primers 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC C) (Rahn et al. 1992). PCR was carried out in a total volume of 50

µl containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (5 U μl^{-1}), and 1 µl of each primer (100 ng μl^{-1}). After an initial 10-min-denaturation at 96°C, and subsequent addition of *Taq* polymerase (hot-start PCR), thirty-five rounds of temperature cycling were performed in a PTC-200 Thermocycler (BioRad, Hercules, CA) with denaturation at 96°C, primer annealing at 54°C, and elongation at 72°C, each for 30 seconds (Malorny et al. 2003). This was followed by incubation at 72°C for 7 min (Hahn et al. 2007).

Agarose gel electrophoresis (2% agarose in TAE [Tris-acetate-ethylenediaminetetraacetic acid] buffer) (Sambrook et al. 1989) of 10-µl subsamples of the PCR did not display amplicons of *inv*A gene fragments in any of the environmental samples, i.e., water and sediment samples. These samples remained negative even when inoculum sizes into pre-enrichment medium were increased, i.e., from 100 to 500 µl of sediment, and from cells in 1 ml to cells in 100 ml water samples, and two sequential enrichment steps in RVS broth were used. These results corroborate with those of our previous study on the spring arm of Spring Lake where none of the environmental samples were positive for *Salmonella* (Hahn et al. 2007). Although *Salmonella* had been recovered from rivers and streams in remote areas without any significant human impacts (Fair & Morrison 1967, Hendricks & Morrison 1967, Thomason et al. 1975), our results indicate that neither water nor sediments in both the lotic spring and the lentic slough arm of Spring Lake provide conditions that allow *Salmonella* to persist in densities that are detectable by our methodology.

In contrast to environmental samples, *inv*A gene fragments were detected in carapace and cloacal enrichment samples of 11 out of 18 musk turtles (i.e., in 61%, five females, six males) with generally strong signals (Fig. 2.2). Carapace and cloacal samples were also positive for eight red-eared sliders (38%, three females, five males) and the snapping turtle (male). *Salmonella* could also be detected in enrichments from the carapace of two additional red-eared sliders (two females) and of two Texas river cooters (two females); analyses of the cloacal samples of these animals remained negative (Fig. 2.2), as did analyses of samples from the three Guadalupe spiny soft-shell turtles. These results again corroborate with our previous analysis on musk turtles of the spring arm of Spring Lake, Texas, USA, that demonstrated the presence of *Salmonella* in about 50% of the musk turtles analyzed (Hahn et al. 2007). These results are also comparable to percentages of detection, i.e., 32% of the turtles analyzed, obtained in other studies (Briones et al. 2004).

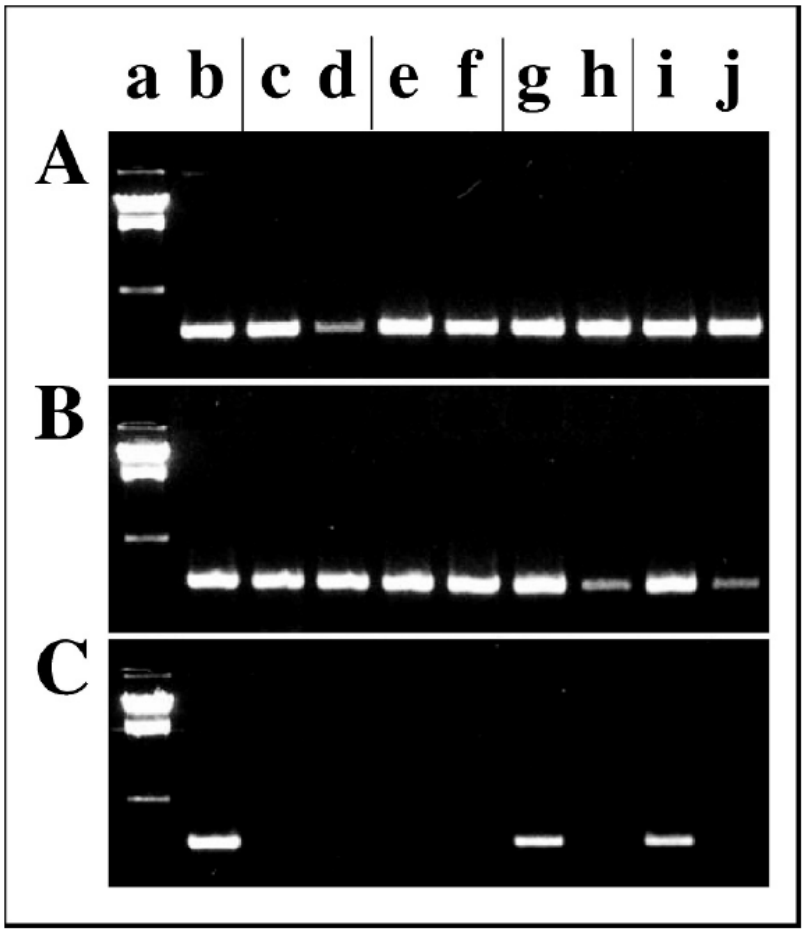

Figure 2.2: PCR amplification products of selected samples. PCR amplification products showing the presence of *inv*A gene fragments in enrichment cultures for *Salmonella* from swabs obtained from carapace (lanes c, e, g, i) or cloacae (lanes d, f, h, j) of 4 individuals of either musk turtles (*Sternotherus odoratus*) (a), red-eared sliders (*Trachemys scripta elegans*) (b), or Texas river cooters (*Pseudemys texana*) (c). Lanes marked a show a Lambda HindIII size marker; and lanes b the *inv*A gene fragment from *Salmonella typhimurum* ATCC 14028.

The presence of *Salmonella* varied among the turtle taxa, but did not follow a trend affected by foraging or basking behavior. Musk and snapping turtles that are routinely found foraging along the bottom of the spring and slough arm, are primarily opportunistic feeders, and rarely bask in air (as opposed to the water surface). Softshell turtles bask infrequently and are piscivorous. Red-eared turtles are opportunistic feeders that take vegetative and animal matter, whereas the Texas river cooter is strictly

herbivorous. The latter two species frequently bask in air for long periods (Ernst et al. 1994). Softshell turtles, because of the nature of their carapacial integument, do not support algal mats, which are frequently found on the other species. In the Spring Lake system, red-eared turtles and Texas river cooters shed the outer keratin layer (scutes) annually; snapping and musk turtles do so less frequently. Basking, thus, does not seem to eliminate *Salmonella* from the carapace.

Compared to samples from the carapace, cloacal samples often produced less amounts of amplicons than carapace samples or no amplicons at all (Fig. 2.2) suggesting lower amounts of template and thus smaller numbers of *Salmonella* in cloacal samples. Since cloacal swabs generally contained less material used as inoculum than carapace swabs, this assumption, however, might more reflect a sampling bias caused by different amounts of feces and thus numbers of *Salmonella* present before or after shedding than an accurate result (Kaufmann et al. 1967). Because detection of amplicons in carapace samples was generally confirming that in cloacal samples, except for two cases where no amplicons were detected in cloacal samples (Fig. 2.2), carapace samples could probably serve as sole resource for the analyses of *Salmonella* on turtles and circumvent potential sampling bias of cloacal samples. These results also demonstrate the ability of *Salmonella* to at least survive outside of the animals, which is in contrast to the general assumption that *Salmonella* live primarily in the intestinal tracts of animals (Foster & Spector 1995).

In order to confirm the molecular identification of *Salmonella* in the cloacae and on the carapace of the different turtle species, isolation attempts were undertaken from positive enrichment cultures of individuals of each species (i.e., 2 musk turtles, 2 red-

eared sliders, 2 Texas river cooters, and the snapping turtle). From the semi-selective enrichment in RVS Broth, bacteria were plated onto RVS Agar (i.e., RVS Broth solidified with 15 g agar l^{-1}) and incubated at 43°C for 24 hours. Selected colonies (n=10 per sample) were sub-cultured in LB medium (l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and identified as *Salmonella* by PCR detection of the *inv*A gene as described above. This screening resulted in the detection of isolates of *Salmonella* in samples of all individual turtles analyzed, with usually all 10 isolates being positive except for the snapping turtle where only 5 isolates were producing amplicons, and thus resembled *Salmonella*.

All PCR-positive isolates were further analyzed by rep-PCR, a PCR-assisted fingerprinting technique targeting consensus motifs of repetitive elements common to prokaryotic genomes (Bennasar et al. 2000, Woo & Lee 2006), with primer BoxA1R (5'CTA CGG CAA GGC GAC GCT GAC G) (Versalovic et al. 1998), in order to reduce redundancy of isolates as outlined in our previous study (Hahn et al. 2007). Profiles were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 μg ml^{-1}) (Sambrook et al. 1989). Profiles were found to be identical for all isolates from carapace and cloacal samples for each individual, similar to results in our previous study (Hahn et al. 2007). Except for isolates from samples of the two red-eared sliders that produced identical profiles, all profiles between individual turtles differed from each other (Fig. 2.3). However, profiles of *Salmonella* from both musk turtles were identical to profiles from the Texas river cooters (M_1 to P_1, and M_2 to P_2) (Fig. 2.3), with one profile (M_1, P_1) being identical to those of *Salmonella* retrieved from musk turtles in the spring arm of Spring Lake in our previous study (Fig. 3, (Hahn

et al. 2007). These results again indicate that the different life histories of musk turtles and Texas river cooters with respect to basking or foraging behavior were not impacting the presence of *Salmonella* overall and of specific *Salmonella* strains in particular. In addition, the presence of the same strain established at both sites, i.e., the spring and the slough arm of Spring Lake analyzed about 6 months apart, and on different turtle species, i.e., musk turtles and Texas river cooters, suggests that *Salmonella* persist in supposedly pristine environments like Spring Lake in and on different turtle species.

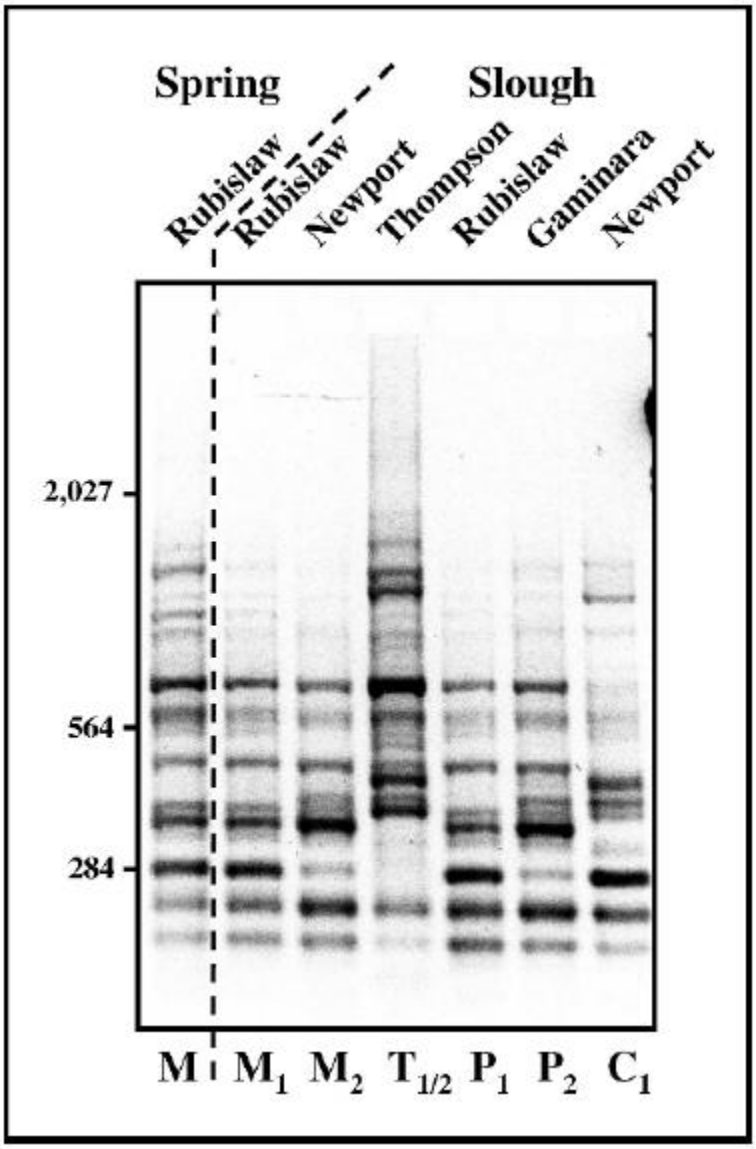

Figure 2.3: Profiles of representative isolates. rep-PCR profiles of representative *Salmonella* isolates from two individual musk turtles (M_1 and M_2), two red-eared sliders ($T_{1/2}$), two Texas river cooters (P_1 and P_2), and the snapping turtle (C_1) caught in the slough arm of Spring Lake. The left lane show a representative rep-PCR profile from a *Salmonella* strain isolated from a musk turtle in the spring arm (M) in our previous study (Hahn et al. 2007). The assignments on top of the profiles (i.e., Rubislaw, Newport, Thompson and Gaminara) represent the corresponding results of the serotyping.

Characterization to the serotype level by slide agglutination using *Salmonella* specific antisera at the Texas Department of State Health Services (Austin, TX) resulted in the identification of serovar Rubislaw for those isolates showing identical rep-PCR

profiles to the *Salmonella* isolate from the spring arm (Fig. 2.3). In our previous study on the spring arm all isolates had been identified as serovar Rubislaw (Hahn et al. 2007), and thus our serotyping results supports the idea that the same strain persisted in different habitats. In addition to serovar Rubislaw, however, other serovars were identified. Different strains of serovar Newport were found on a musk and the snapping turtle, while *Salmonella* of the red-eared sliders were identified as serovar Thompson, and those of the second Texas River cooter as serovar Gaminara (Fig. 2.3). All serovars have been identified in human-associated salmonellosis (Cook et al. 1998, Lyytikainen et al. 2000, Kimura et al. 2005), but were also detected in amphibians (Cook et al. 1998, Chambers & Hulse 2006) and reptiles (Kaufmann et al. 1972, Johnson-Delaney 1996, Chambers & Hulse 2006). Our results support the idea that free-living turtles are a reservoir for *Salmonella* that are involved in human-associated salmonellosis. The failure to detect *Salmonella* in the environmental samples, however, suggests that turtles are not acting as a vector for large scale dispersion of *Salmonella* into the environment, but are more likely reflecting isolated islands where *Salmonella* can persist as normal flora without becoming a human health hazard.

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CHAPTER 3

TEMPORAL ANALYSES OF *SALMONELLA* IN A HEADWATER SPRING ECOSYSTEM[1]

Abstract

Sediments and water from the spring and slough arm of Spring Lake, the pristine headwaters of the San Marcos River, Texas, were analyzed for *Salmonella* by culture and molecular techniques before and after three major precipitation events, each with intermediate dry periods. Polymerase chain reaction (PCR)-assisted analyses of enrichment cultures detected *Salmonella* in samples after all three precipitation events, but failed to detect them immediately prior to the rainfall events. Detection among individual locations differed with respect to the precipitation event analyzed, and strains isolated were highly variable with respect to serovars. These results demonstrate that rainwater associated effects, most likely surface runoff, provides an avenue for short-term pollution of aquatic systems with *Salmonella* that do not, however, appear to establish for the long-term in neither water nor sediments.

[1] Authors: James P. Gaertner, Tiffany Garres, Jesse C. Becker, Maria L. Jimenez, Michael R.J. Forstner, Francis L. Rose, Dittmar Hahn. Publication: Journal of Water and Health, 07(1): 115-121 (2009).

Introduction

Salmonella have been detected in the gastrointestinal tract of a large variety of wild and captive animals including mammals, birds, and reptiles throughout the world (Gray 1995, Refsum et al. 2002b, Briones et al. 2004). The intestinal tract of these warm- and cold-blooded vertebrates is supposed to be the native habitat of *Salmonella* (Woodward et al. 1997), from which the organisms can spread to other environments. Feces released are assumed to act as the source of potential contamination with *Salmonella* for environments such as fresh- or marine waters, estuarine environments, vegetables, compost, or soils and sediments (Thomason et al. 1975, Polo et al. 1998, Refsum et al. 2002b, Tavechio et al. 2002, Martinez-Urtaza et al. 2004). The occurrence of *Salmonella* in these environments is therefore frequently linked to environmental contamination through, e.g., manure or grey- or wastewater discharges (Polo et al. 1998, Martinez-Urtaza et al. 2004). *Salmonella*, however, have also been recovered from rivers and streams in remote areas without any significant human impacts (Fair & Morrison 1967, Hendricks & Morrison 1967, Thomason et al. 1975), nor any detectable impact from potential host animals (Hendricks 1971, Chao et al. 1987).

In recent studies in Spring Lake, the pristine headwaters of the San Marcos River, Texas, USA (79°53’N, 97°55’W), we failed to detect *Salmonella* in water or sediment samples, however, could demonstrate their presence in, and on, different turtle species. About 50% of all turtles that included common musk turtles (*Sternotherus odoratus*) (Hahn et al. 2007), red-eared sliders (*Trachemys scripta elegans*), Texas river cooters (*Pseudemys texana*), and snapping turtles (*Chelydra serpentina serpentine*) (Gaertner et al. 2008) harbored *Salmonella* in the cloacae as well as in biofilms on the carapace,

supporting the idea that wild turtles could serve as a reservoir for *Salmonella*. The absence of *Salmonella* in water and sediments from the turtles' habitat, however, indicated that these *Salmonella* were not disseminated in significantly high numbers allowing detection from the environment, and thus their presence in, and on, turtles did not provide reasons for human health concerns. The concomitant lack of re-inoculation through *Salmonella* released from feces has led us to investigate sources of *Salmonella* in this headwater system. One hypothesis was that the microorganisms could arrive by being deposited in surrounding terrestrial habitats and transferred into the aquatic system by rain associated runoff. This would imply that "pulses" of *Salmonella* arising from non-point sources would be detectable in the system just after natural precipitation events.

To test this hypothesis, water and sediment samples were collected at several sites from Spring Lake before and after significant rainfall events with intermediate dry periods, and analyzed for *Salmonella* using traditional enrichment culture techniques in combination with molecular detection and identification tools (Hahn et al. 2007). Because the lake lies within the urban-rural interface of San Marcos, Texas, several potential non-point source pollution sources for *Salmonella* are provided that include cattle ranching operations as well as wildlife habitat provided by an adjacent golf course.

Material and Methods

Sampling

Water and sediment samples were retrieved from nine sites at Spring Lake (Fig. 3.1) (Hahn et al. 2007) at four times during spring 2007. Six of the nine sites represented permanent waters, while three sites (1, 3 and 6) carried water only after significant

rainfall events. Site 1 was located in a discharge area of Sink Creek into the Slough arm of Spring Lake, while sites 3 and 6 represented flood water puddles on a golf course which surrounds the Slough Arm (Fig. 3.1). Each sampling consisted of two events, the first after a 1-month-period without significant precipitation, and a second sampling following significant precipitation events. The samplings were carried out March 10 (no significant precipitation for about 2 months) and March 14 (approx. 460 mm precipitation the previous 2 days), May 22 (no significant precipitation for about 1 month) and May 24 (approx. 700 mm precipitation the previous 2 days), and then June 16 (no significant precipitation for about 1 month) and June 18, followed by an additional sampling June 21 (approx. 100 mm precipitation the previous 4 days) (Fig. 3.2). Water samples were taken just below the surface with a vertical point water sampler, while sediment samples were obtained from the same sites with a bottom dredge.

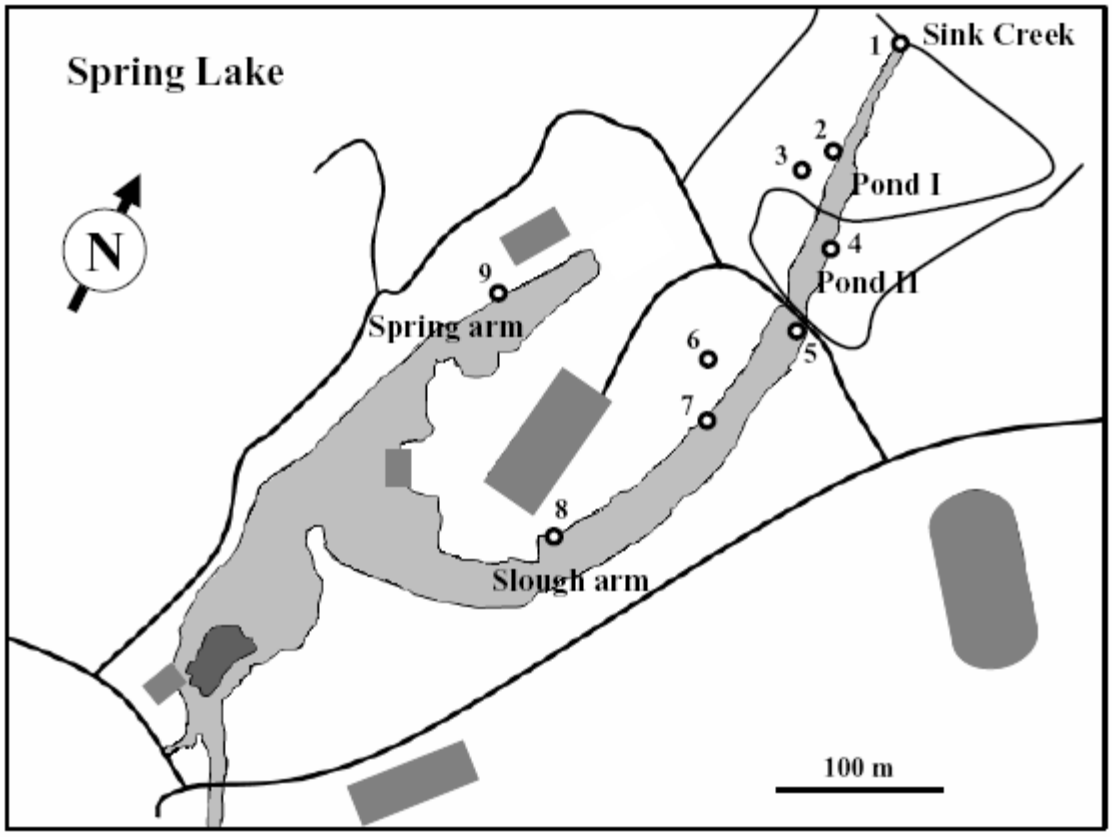

Figure 3.1: Schematic presentation of sampling sites. Sites 1-9 in both spring and slough arms of Spring Lake, the head waters of the San Marcos River, Texas, USA ($79^0$53'N, $97^0$55'W). Dark lines represent roads and squares buildings.

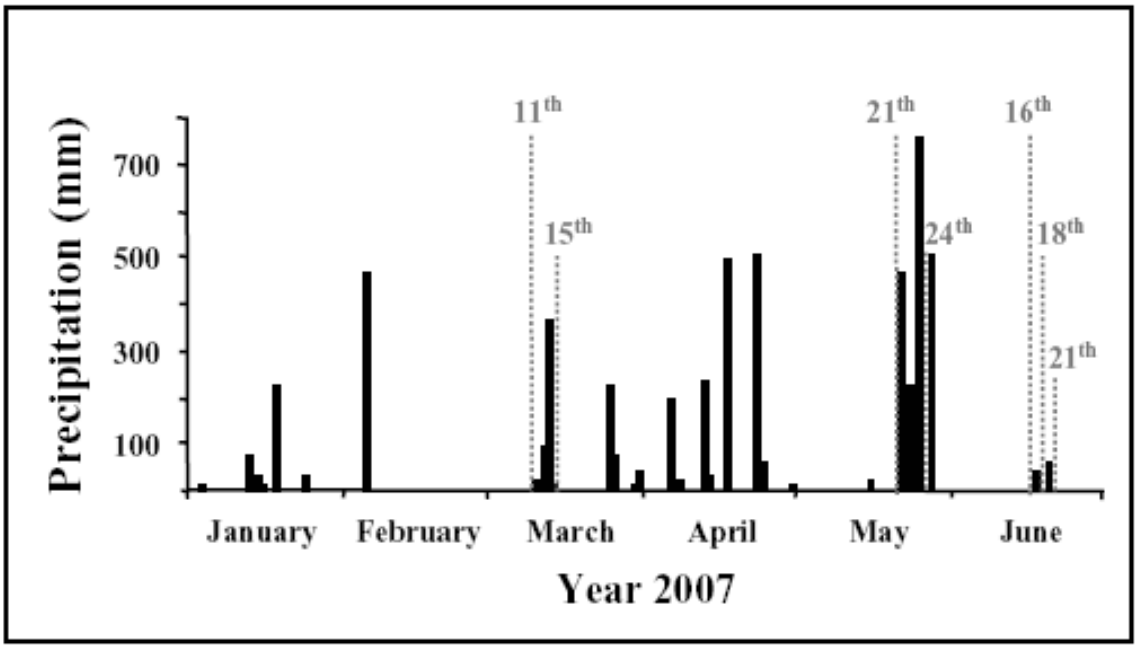

Figure 3.2: Climatic data collected. Precipitation data (dark bars) and sampling dates before and after significant rainfall for the first six months of 2007.

Enrichment

Sub-samples of sediment (about 100 mg wet weight), and pellets obtained from 50 ml of the water samples centrifuged at 16,000 x g for 2 minutes, were incubated in 1 ml of Buffered Peptone Water in 2-ml cryotubes at 37°C for 16-20 hours (International Standard Organization 1993). Sub-samples (100-µl) of these pre-enrichment cultures were transferred to 2-ml cryotubes that contained 1 ml of Rappaport-Vassiliadis (RVS) Broth (Vassiliadis et al. 1981) and incubated at 43°C for 24 hours in order to enrich for *Salmonella* (Vassiliadis et al. 1981). In order to enhance the sensitivity of the detection procedure, 100 µl of these enrichment cultures were subjected to a second enrichment in 1 ml of RVS Broth. Sub-samples (100 µl) of pre-enrichment and first and second semi-selective enrichment cultures were then used for molecular analyses. The remainder of the cultures were mixed with 600 µl of 60% glycerol in water and preserved at -80°C.

Polymerase chain reaction (PCR)-based detection

For the detection of *Salmonella* by PCR, 100-µl-samples of semi-selective enrichment cultures as well as of cultures of *Salmonella typhimurium* ATCC 14028 which were used as a positive control, were centrifuged at 16,000 x g for 1 minute. The bacterial pellets were washed once in sterile distilled water, and bacteria lysed in 100 µl of 50 mM NaOH by incubation at 65°C for 15 minutes (Hahn et al. 2007). One µl of this lysate was used as template for PCR amplification with primers 139 (5'GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 (5'TCA TCG CAC CGT CAA AGG AAC C) (Rahn et al. 1992) targeting the *inv*A gene. The *inv*A gene encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by *Salmonella* (Suárez & Rüssmann 1998, Khan et al. 2000), and is present in all *Salmonella enterica* subspecies as well as in *S. bongori* (Malorny et al. 2003).

PCR was carried out in a total volume of 50 µl containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (5 U μl^{-1}), and 1 µl of each primer (100 ng μl^{-1}). After an initial 10-min-denaturation at 96°C, and subsequent addition of *Taq* polymerase (hot-start PCR), thirty-five rounds of temperature cycling were performed in a PTC-200 Thermocycler (BioRad, Hercules, CA) with denaturation at 96°C, primer annealing at 54°C, and elongation at 72°C, each for 30 seconds (Malorny et al. 2003). This was followed by incubation at 72°C for seven minutes (Hahn et al. 2007). The presence of 284-bp-fragments was examined by agarose gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989).

Isolation and characterization

From the semi-selective enrichment in RVS Broth, bacteria were plated onto RVS Agar (i.e., RVS Broth solidified with 15 g agar l^{-1}) and incubated at 37°C for 24 hours. Selected colonies (n=10 per sample) were sub-cultured in LB medium (l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and identified as *Salmonella* by PCR detection of the *inv*A gene (Malorny et al. 2003) as described above. Lysates of all PCR-positive isolates were further analyzed by rep-PCR, a PCR-assisted fingerprinting technique targeting consensus motifs of repetitive elements common to prokaryotic genomes (Bennasar et al. 2000, Woo & Lee 2006), in order to reduce redundancy of isolates. Rep-PCR was performed in a total volume of 25 µl with primer BoxA1R ($^{5'}$CTA CGG CAA GGC GAC GCT GAC G), and 2 µl of lysate as described in (Hahn et al. 2007). Profiles were screened by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989). Representative isolates were subsequently characterized to the serotype level by slide agglutination using *Salmonella* specific antisera at the Texas Department of State Health Services (Austin, TX).

Results

PCR analysis of water and sediment samples from Spring Lake required semi-selective enrichment culture in order to detect *inv*A gene fragments indicating the presence of *Salmonella*. While enrichment cultures from all sites remained negative for *inv*A fragments at sampling times before rainfall and after extended dry periods (i.e., March 11, May 21, and June 16), *inv*A fragments and thus *Salmonella* were detected in samples after all four precipitation events (Fig. 3.3). Detection at individual sites differed

with respect to precipitation event analyzed, with 5, 1, 2 and 6 sampling sites found positive for *Salmonella* for the March 15, May 24, June 18 and June 21 sampling events, respectively (Fig. 3.3). *Salmonella* were generally only detected in water samples; with one exception (May 24, site 8), sediment samples remained negative for *inv*A gene fragments. None of the water samples from any site carried *Salmonella* at all four sampling events. *Salmonella* could be detected at three sampling events at sites 7 and 8, and at two events at sites 2 and 5. The remaining sites were positve for *Salmonella* only once, or not at all (i.e., site 3, a puddle on the golf course) (Fig. 3.3).

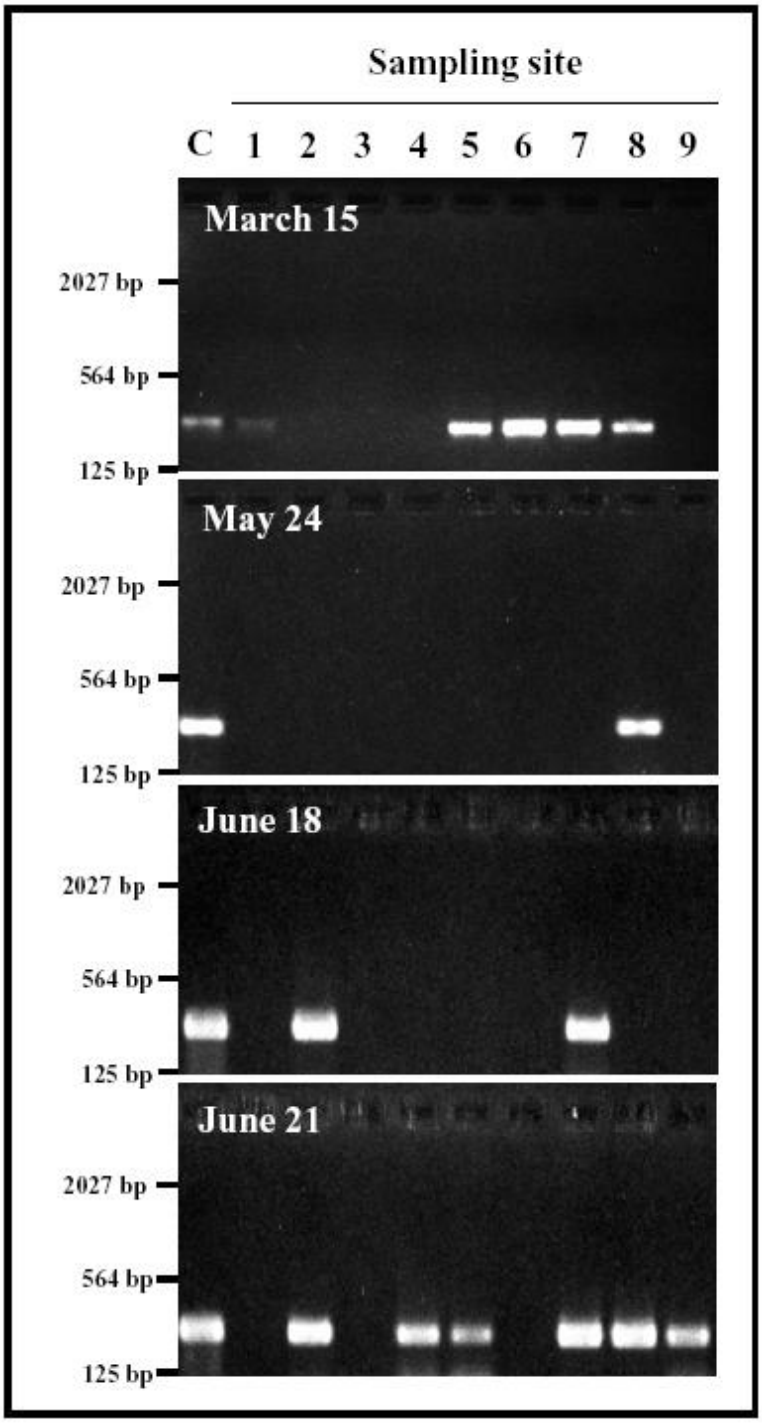

Figure 3.3: Gel picture of selected results. PCR amplification products showing the presence of *inv*A gene fragments in enrichment cultures for *Salmonella* from water samples obtained at different sites (sites 1–9) at Spring Lake (see Fig 3.1) after significant rainfall at sampling dates March 15, May 24, June 18, and June 21, 2007. Lane C shows the *inv*A gene fragment from *Salmonella typhimurum* ATCC 14028. Fragment sizes on the left represent those of a Lambda HindIII size marker.

Except for samples from site 4 on March 24, and site 9 on June 21, isolates were obtained for all samples. Rep-PCR analyses and serotyping revealed the presence of different strains in samples from all sampling dates (Fig. 3.4). On March 15, *Salmonella* obtained from samples from site 1 at Sink Creek belonged to serovar Kentucky, while the remaining isolates from sites 5, 6, and 8 were identified as serovar Montevideo. Isolates from the May 24 sampling were only obtained from site 8, with serovars Muenchen and Give obtained from water, and serovar Newport from sediment samples. Serovars Javiana as well as Give and Javiana were retrieved from sites 2 and 7, respectively, on June 18, and serovars Give and Cubana, Javiana, Kentucky and Gaminara from sites2, 4, 5, 6 and 7, respectively, on June 21 (Fig. 3.4). Serovars Kentucky, Javiana, and Give that were obtained at different sampling times and at different sites displayed identical rep-PCR profiles, as did serovars Montevideo (with one exception) and Javiana obtained at different sites, but at the same sampling time (Fig. 3.4).

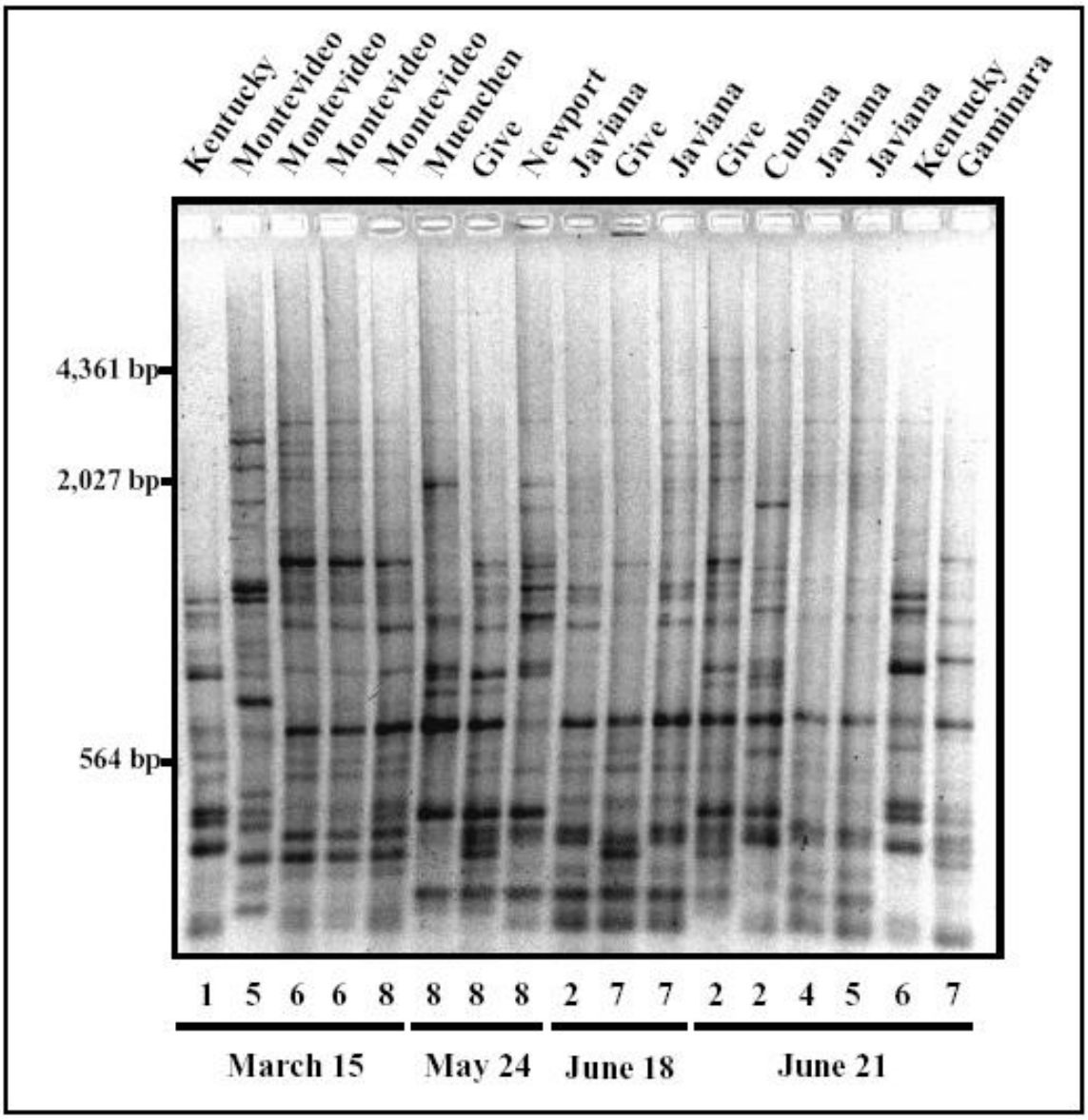

Figure 3.4: Gel picture of rep-PCR profiles of isolates. Rep-PCR profiles and serovar assignments of representative isolates from enrichment cultures for *Salmonella* from water samples obtained at different sites (sites 1–9) at Spring Lake (see Figure 3.1) after significant rainfall at sampling dates March 15, May 24, June 18 and June 21, 2007. Fragment sizes on the left represent those of a Lambda HindIII size marker.

Discussion

The detection of *Salmonella* in water of Spring Lake exclusively after significant rainfall, but not before provides evidence for potential contamination through non-point rather than point sources. These non-point sources most likely include agricultural run-off

from livestock ranching, input from soils of areas surrounding the lake, and from fecal droppings from wildlife (Kinzelman et al. 2004). Cattle (Veling et al. 2002), goats (Woldemariam et al. 2005, Chandra et al. 2006) and sheep (Woldemariam et al. 2005) have been shown to be reservoirs of *Salmonella*, similar to free-ranging animals such as deer (Branham et al. 2005, Renter et al. 2006) and birds (Refsum et al. 2002a, Refsum et al. 2002b). Released from animal reservoirs into the environment, *Salmonella* have been shown to survive, e.g., in manure of cattle (Kearny et al. 1993, Himathongkham et al. 1999) or in soils (Islam et al. 2004, Cote & Quessy 2005, Franz et al. 2005) for time periods that exceed the dry periods of about 1 month experienced in our analyses. Thus, *Salmonella* released by animals in the vicinity of Spring Lake can be considered a potential non-point source of contamination for water and sediments of the lake when transported into the aquatic system by strong rainfall and associated runoff events (Kinzelman et al. 2004).

Support for a scenario as outlined above is provided by the exclusive detection of *Salmonella* after significant rainfall events, by the spotty detection of *Salmonella* along the sequence of 9 sampling sites, with different detection patterns depending on the sampling time, and by the retrieval of different serovars at different sites at the same sampling time (Figs. 3.3, 3.4). Further support for non-point source contamination of Spring Lake with *Salmonella* is provided by the detection of *Salmonella* in puddles on terrestrial areas in the vicinity of the water body of Spring Lake (i.e., Site 6) that remained separated from the water body of the lake even during the rainfall events. Detection of the same strain in the water body further downstream (i.e., Site 8) at the same sampling time suggests transport of this strain from the terrestrial environment into

the water body. This assumption is also supported by the fact that from sites further upstream either no *Salmonella* or different serovars or strains (i.e., serovars Kentucky and Montevideo at Site 1 and 5, respectively) were retrieved.

Although all data retrieved support non-point source contamination of the water body through a source from terrestrial environments, some of these data require additional studies for more explicit explanations that go beyond that of pure speculation. For example, the same strain detected at site 1 at the March 15 sampling (i.e., serovar Kentucky) was retrieved three months later (i.e., June 21) at Site 6, where intermediate sampling events did not succeed to detect *Salmonella*. Since Site 6 represents a terrestrial site without direct contact to the water body, reinoculation of this site with feces from animals such as deer harboring this *Salmonella* strain seems to be the most likely cause for this detection. Other examples include the detection of several *Salmonella* at one site (e.g., serovars Muenchen, Give and Newport at site 8 at the May 24 sampling) or predominantly one strain within the entire system (e.g., serovar Javiana at Sites 2 and 7, and Sites 4 and 5 at samplings June 18 and 21, respectively) which indicates different sources of contamination (e.g., run-off *versus* sewage or storm water) (Geldreich 1996).

An additional source of contamination of the water body could be due to resuspension of bacteria associated with bottom sediments as has been previously documented for coliforms (Sherer et al. 1988, Davis et al. 2005, Jamieson et al. 2005). Sediments were proposed to be potential reservoirs for *Salmonella* since decreases were found to be less pronounced than in the water body (Craig et al. 2003) where physical dilution, sunlight or competition have been identified as factors reducing numbers of *Salmonella* in time (Moriñigo et al. 1989, Chandran & Hatha 2005). In our analyses,

however, none of the sediment samples was found contaminated with *Salmonella*, except for Site 8 at the May 24 sampling. These results are in conflict with the assumption that sediments provide an adequate environment for long-term persistence of *Salmonella*. In order to evaluate potential short-term persistence, however, additional studies are required which evaluate a time span after the detection of non-point source contamination in order to monitor disappearance of *Salmonella* in water or their potential appearance in sediments.

All results obtained in this study could be affected by the transient nature of the contamination in run-off, storm water and water body of the lake (Novotny 2002). It is also possible, however, that the high resolution identification approach of *Salmonella* undertaken in this study could have introduced sampling and analyses biases. The specific identification of *Salmonella* down to the strain level has been used successfully to analyze *Salmonella* in, and on, turtles (Hahn et al. 2007); however, these systems were limited with respect to diversity of *Salmonella* populations. The potentially much higher diversity of *Salmonella* in terrestrial samples and run-off might therefore not have been retrieved accurately in this study due to the very limited sample number per site, the sample size, the potentially highly heterogeneous distribution of *Salmonella* in the environment, and the potential preferential enrichment and detection of a single *Salmonella* strain from a heterogeneous mixture in the original sample. Therefore, subsequent studies that focus on the strain level of identification (i.e., isolation, followed by rep-PCR) and include the analyses of multiple samples from the same site, and of significantly larger numbers of isolates retrieved per sample, are necessary to explain the distribution of specific *Salmonella* strains in Spring Lake as a consequence of non-point

contamination. The results do highlight the complex nature of *Salmonella* in natural systems and the importance of documenting the ecology of these potential human pathogens as they move through such ecosystems.

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CHAPTER 4

DETECTION OF *SALMONELLA* FROM FISH IN A NATURAL RIVER SYSTEM[1]

Abstract

Sediment, water, and fish gut samples taken at three sites near the headwaters of the San Marcos River, Texas, were analyzed for *Salmonella* spp. by culture and molecular techniques. While enrichment cultures from sediment and water samples from the two uppermost sites were negative for *Salmonella* in polymerase chain reaction analyses, both sediment and water samples were positive at the downstream site. At all sites, *Salmonella* were present in the guts of different fishes (e.g., largemouth bass *Micropterus salmoides*, channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, and suckermouth catfish *Hypostomus plecostomus*). The highest percentage of detection (33% of analyzed fish) occurred at the downstream site, whereas detection percentages at the upper two sites were 18% and 17%. Detection of *Salmonella* was usually limited to one segment of the gut (i.e., upper or lower part). Serovars were highly variable among individuals and differed between the upper and lower gut in the only individual (a common carp) that had *Salmonella* in both gut segments. In situ hybridization demonstrated that *Salmonella* were normally associated with particulate material in the gut and occurred in highly variable

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numbers ranging from an occasional organism to a majority of the gut microbe population. These results demonstrate the presence of different serovars of potentially human pathogenic *Salmonella* among four ecologically distinct fishes within natural environments. They also suggest that *Salmonella* are not components of the indigenous microbial community in fish intestines but rather are ingested with particulate material.

Introduction

Infection of humans with *Salmonella* spp. is a serious public health problem presenting up to 1.3 billion cases worldwide (Pang et al. 1995), including 1.4 million cases in the United States alone (Mead et al. 1999). The majority of infections with *Salmonella* result from exposure to undercooked animal products or cross-contamination with fruits and vegetables (Pang et al. 1995, Tauxe 1997); however, salmonellosis can also result from direct contact with contaminated water (Foltz 1969, Harvey et al. 1969). Water is typically contaminated with *Salmonella* via the feces of many types of warm- and cold-blooded animals that might serve as a constant source of environmental contamination (Foltz 1969, Refsum et al. 2002). *Salmonella* can, however, persist in the environment for extended periods of time (Hendricks 1971, Chao et al. 1987) and even occur in apparently pristine aquatic systems (Fair & Morrison 1967, Hendricks & Morrison 1967).

Although *Salmonella* are not considered to be part of the normal intestinal flora of fish (Janssen & Meyers 1968, Pal & Dasgupta 1991), fish exposed to *Salmonella* can become asymptomatic carriers of these pathogens (Brunner 1974, Bocek et al. 1992). *Salmonella* may exist in the digestive tracts of fish in numbers up to one order of

magnitude higher than those occurring in the water (Pal & Dasgupta 1991, Ampofo & Clerk 2003). *Salmonella* have also have been shown to multiply in the intestine and to be shed in the feces (Morse et al. 1978a, Morse et al. 1978b, Lesel & Legac 1983). Consequently, fish constitute a potentially important source for the dissemination and persistence of *Salmonella* in aquatic environments (Lawton & Morse 1980). Studies on the occurrence of *Salmonella* in fish have been mainly limited to aquaculture systems, in which treatment with antibiotics or fertilization with sewage was thought to represent the mechanism of contamination with *Salmonella* and similar pathogens (Balasubramanian et al. 1992, Khalil & Hussein 1997, Pullela et al. 1998). Studies that evaluate the presence of *Salmonella* in wild fish of natural systems are lacking.

The purpose of this study was to investigate the potential for fish to disseminate *Salmonella* in a riverine ecosystem, the San Marcos River, Texas. Our previous study (Hahn et al. 2007) tested for the occurrence of *Salmonella* in the river's headwaters, Spring Lake. Spring Lake is fed by the second-largest spring system in Texas, encompassing more than 200 individual spring heads (Groeger et al. 1997). In the prior study, *Salmonella* were not recovered from water or sediments of Spring Lake; however, they were frequently detected in carapace biofilms and cloacae of common musk turtles Sternotherus odoratus (Hahn et al. 2007). In the present study, sediment, water, and fish gut samples collected at three sites downstream from Spring Lake were analyzed for *Salmonella* by use of traditional enrichment culture techniques in combination with molecular detection and identification tools, such as polymerase chain reaction (PCR) and in situ hybridization. Such tools were used successfully in our previous study. A

more specific analysis of isolates was performed by repetitive sequence-based PCR (rep-PCR), followed by standard serotyping of representative isolates (Hahn et al. 2007).

Materials and Methods

Water samples, benthic sediments, and fish were obtained from three sites on the San Marcos River (Hays County; Fig. 4.1) between April 1 and June 10, 2007. Site 1 (29°51’28.46”N, 97°55’52.19”W) was located about 5 km downstream from the river’s headwaters and upstream from two low-head dams that prevented fish movement upstream during average flow conditions. Site 2 (29°51’59.99”N, 97°55’32.13”W) was located downstream from the point of effluent releases by the City of San Marcos Wastewater Treatment Plant. Site 3 (29°51’14.20”N, 97°51’40.49”W) was located about 10 km downstream from site 2 and was also downstream from the confluence with the Blanco River. At each site, four water column samples were taken with a vertical point water sampler, and four benthic sediment samples were taken with a dredge. Water and benthic sediment samples were placed on ice and processed within 4 h after sampling. Fish were captured by hook and line. Immediately upon capture, each fish was pithed and the abdomen was cut with sterile scissors, exposing the entire viscera. About 200 mg (wet weight) of gut contents were removed from each of two gut sections (upper and lower) by use of scissors and a small spatula. Gut contents were placed in separate 1.5-mL tubes and stored on ice until processing. The scissors and spatula were sterilized with ethanol and flaming between each use.

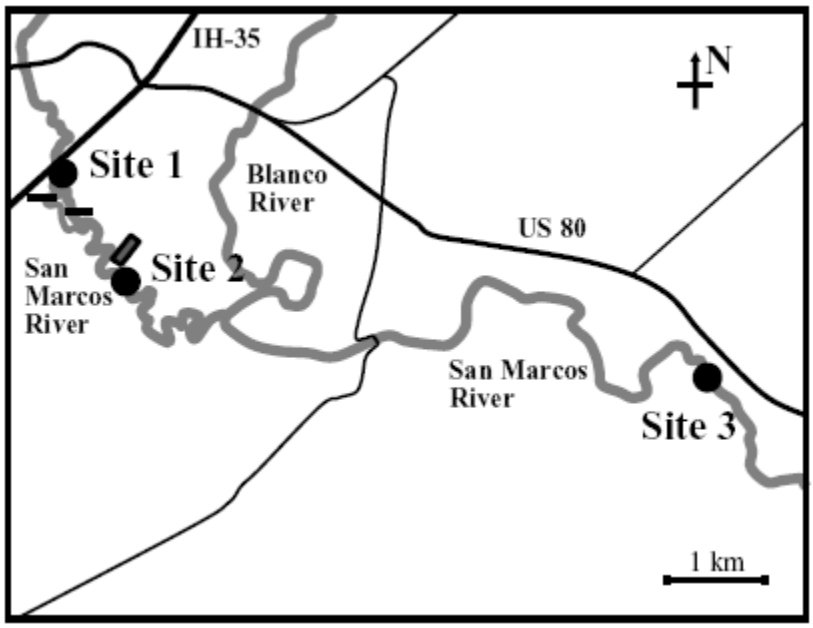

Figure 4.1. Schematic presentation of sampling locations. Sampling locations (sites 1–3) along the San Marcos River, Texas. Site 1 is located about 5 km downstream from the river's headwaters and upstream from two low-head dams that prevent fish movement upstream during mean flow conditions. Site 2 is located downstream from the point of effluent releases by the City of San Marcos Wastewater Treatment Plant. Site 3 is located about 10 km downstream from site 2 and is downstream from the Blanco River confluence. Major roads (e.g., Interstate Highway [IH] 35 and U.S. Route 80) are shown.

Salmonella in pre-enrichment and enrichment cultures obtained from water samples, benthic sediments, and gut contents of fish were analyzed by the procedure outlined in Hahn et al. (Hahn et al. 2007). To obtain preenrichment cultures, approximately 100 mg (wet weight) of gut contents, benthic sediment samples, or pellets from centrifugation (14,000 revolutions/min for 2 min) of 50-mL water samples were transferred into 2- mL cryotubes that contained 1 mL of buffered peptone water (10 g of peptone/L of water, 5-g/L NaCl, 9-g/L Na_2HPO_4, and 1.5-g/L KH_2PO_4 [pH = 7.2]) and were incubated at 37°C for 16–20 h (International Standard Organization 1993). The first semiselective enrichment culture was created by transferring 100 µL of pre-enrichment culture into 2- mL cryotubes that contained 1 mL of Rappaport– Vassiliadis (RVS) broth

(4.5-g/L peptone [soymeal], 29-g/L $MgCl_2$ • 7[H2O], 8-g/L NaCl, 0.4-g/L K_2HPO_4, 0.6-g/L KH_2PO_4, and 0.036-g/L malachite green; pH= 5.2) and incubating at 43°C for 24 h (Vassiliadis et al. 1981). To enhance the sensitivity of the detection procedure, a second semiselective enrichment culture was made, which consisted of subjecting 100 µL of the first semiselective enrichment culture to RVS broth. Subsamples (100 µL) of pre-enrichment and first and second semiselective enrichment cultures were then used for molecular analyses. The remaining cultures were mixed with 600 µL of 60% glycerol in water and preserved at -80°C. For molecular analyses, 100-µL subsamples of pre-enrichment cultures, first and second semiselective enrichment cultures, and cultures of *Salmonella typhimurium* ATCC (American Type Culture Collection) 14028 (control) were centrifuged at 14,000 revolutions/min for 1 min. Bacterial pellets were washed once with 1 mL of sterile distilled water, and bacteria were lysed in 100 µL of 50-mM NaOH by incubation at 65°C for 15 min (Hahn et al. 2007). One microliter of this lysate was used as template for PCR amplification with primers 139 (5'-GTGAAATTATCGCCACGTTCGGGCAA) and 141 (5'-TCATCGCACCGTCAAAGGAACC) (Rahn et al. 1992). The PCR was carried out in a total volume of 50 µL containing 10X PCR buffer (500-mM KCl, 25-mM $MgCl_2$, 200-mM tris HCl [pH = 8.4], and 0.1% Triton 100), 1 µL of each deoxynucleotide triphosphate (dNTP; each 10 mM in 10-mM tris HCl [pH = 7.5]), 0.2 µL of Taq polymerase (5 units/µL; enzyme code 2.7.7.7; IUBMB 1992), and 1 µL of each primer (100 ng/µL). After an initial 10-min denaturation at 96°C and subsequent addition of *Taq* polymerase (hot-start PCR), 35 rounds of temperature cycling were performed in a PTC-200 thermocycler (BioRad, Hercules, California) with denaturation at 96°C, primer

annealing at 64°C, and elongation at 72°C (each for 30 s) (Malorny et al. 2003). This was followed by incubation at 72°C for 7 min (Hahn et al. 2007). The presence of 284-base pair fragments was examined by agarose gel electrophoresis (2% agarose in tris–acetate–EDTA [TAE] buffer) (Sambrook et al. 1989).

As in our previous studies (Hahn et al. 2007, Gaertner et al. 2008), second semiselective enrichments in RVS broth that tested positive for *Salmonella* invasion gene (*inv*A) fragments were used for method verification. This verification was based on the isolation of *Salmonella* from these enrichments and their subsequent characterization. Bacteria were plated onto RVS agar (i.e., RVS broth solidified with agar at 15 g/L) and incubated at 37°C for 24 h. Selected colonies (n = 10 colonies/sample) were subcultured in Luria–Bertani medium (10-g/L tryptone, 5-g/L yeast extract, and 5-g/L NaCl) and identified as *Salmonella* by PCR detection of *inv*A (Malorny et al. 2003) as described above. Lysates of all PCR-positive isolates were further analyzed by rep-PCR to reduce redundancy of isolates. The rep-PCR was performed in a total volume of 25 µL containing 5X Gitschier buffer (83-mM $[NH_4]_2SO_4$, 33.5-mM $MgCl_2$, 335-mM tris HCl [pH = 8.8], 33.5-µM EDTA, and 150-mM β-mercaptoethanol), 1.25 µL of each dNTP (each 100 mM; mixed 1:1:1:1), 2.5 µL of dimethyl sulfoxide, 0.2 µL of bovine serum albumin (20 mg/mL), 1.3 µL of primer BoxA1R (300 ng/µL; 5'-CTACGGCAAGGCGACGCTGACG) (Versalovic et al. 1998), 0.4 µL of *Taq* polymerase (5 units/µL), and 2 µL of lysate (Rademaker & de Bruijn 1997, Dombek et al. 2000). After an initial denaturation at 95°C for 2 min, 30 rounds of temperature cycling were performed in a PTC-200 thermocycler with denaturation at 94°C for 3 s and 92°C for 30 s, primer annealing at 50°C for 1 min, and elongation at 65°C for 8 min.

These were followed by incubation at 65°C for 8 min (Rademaker & de Bruijn 1997, Dombek et al. 2000). Profiles were screened by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989). Representative isolates were subsequently characterized to the serotype level by slide agglutination using *Salmonella*-specific antisera; this work was performed by the Texas Department of State Health Services, Austin.

In contrast to PCR-based analyses, *in situ* hybridization allows visualization of individual cells in samples. For in situ hybridization, gut content samples (~100 mg) were fixed by mixing with 300 µL of 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.13-M NaCl, 7-mM Na_2HPO_4, and 3-mM NaH_2PO_4; pH = 7.2) and subsequent incubation on ice for 16 h (Zarda et al. 1997). Fixed cells were washed with PBS twice, resuspended in 50% ethanol in PBS, and stored at -20°C until further use (Amann et al. 1990). Aliquots (1 µL) of the samples, as well as fixed cells of *S. typhimurium* ATCC 14028 used as positive controls, were spotted onto gelatin-coated slides (0.1% gelatin, 0.01% $KCr[SO_4]_2$ in distilled water), allowed to air dry, and subsequently dehydrated in 50, 80, and 96% ethanol for 3 min each (Amann et al. 1990). Hybridizations were carried out in 9 µL of hybridization buffer (0.9-M NaCl, 20-mM tris HCl, and 5-mM EDTA, and 0.01% sodium dodecyl sulfate [SDS]; pH= 7.2) in the presence of 10% formamide, 1 µL of cyanine-3 (Cy3) labeled oligonucleotide probe Sal3 (25 ng/µL), and 1 µL of 40,6-diamadino-2-phenylindole (DAPI; 200 ng/µL) at 42°C for 2 h (Zarda et al. 1997). Probe Sal3 (5’-AAT CAC TTC ACC TAC GTG; *Escherichia coli* position 1713–1730) (Nordentoft et al. 1997) binds to 23S ribosomal RNA of all *S. enterica* subspecies except IIIa but should not detect *S. bongori* (Fang et al. 2003).

After hybridization, slides were washed in buffer containing 20-mM tris HCl (pH=7.2), 10-mM EDTA, 0.01% SDS, and 440-mM NaCl for 15 min at 48°C; slides were subsequently rinsed with distilled water and then air dried (Zarda et al. 1997). Slides were mounted with Citifluor AF1 immersion oil solution (Citifluor Limited, London, UK) and examined with a Zeiss Axiolab microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence microscopy with a high-pressure metal halide lamp, filter set F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, and D460/50) for DAPI detection, and filter set F41 (AHF Analysentechnik; HQ535/50, Q565LP, and HQ610/75) for Cy3 detection.

Results

At the three San Marcos River sites, we obtained upper and lower gut samples from 32 fish representing 6 species. Sixteen suckermouth catfish *Hypostomus plecostomus* and one largemouth bass *Micropterus salmoides* were taken from site 1. Three largemouth bass, one longnose gar Lepisosteus osseus, one common carp *Cyprinus carpio,* and one suckermouth catfish were taken from site 2. Three channel catfish *Ictalurus punctatus*, two longear sunfish *Lepomis megalotis*, one longnose gar, one American eel *Anguilla rostrata*, one common carp, and one largemouth bass were taken from site 3.

Amplicons were not detected in any of the preenrichment cultures with PCR analysis of the *inv*A fragments. Instead, the first semiselective enrichment culture and occasionally the second semiselective enrichment culture were prerequisites for the detection of amplicons and, consequently, *Salmonella* (Fig. 4.2). For water and benthic

sediment samples collected at sites 1 and 2, *inv*A amplicons were not detected in the first or second semiselective enrichment culture. However, *inv*A amplicons were detected in both semiselective enrichment cultures from only the upper guts of three suckermouth catfish (18% of the 17 fish sampled) from site 1. For site 2, *inv*A amplicons were detected in both semiselective enrichment cultures from the lower gut of one largemouth bass (17% of the 6 fish sampled). For site 3, *inv*A amplicons were detected in one water sample (positive in both semiselective enrichment cultures) and one benthic sediment sample (positive in the second semiselective enrichment culture). In addition, *inv*A amplicons were detected in both semiselective enrichment cultures from the lower guts of two channel catfish and one common carp (33% of the 9 fish sampled) and in the second semiselective enrichment culture from the upper gut of that same common carp.

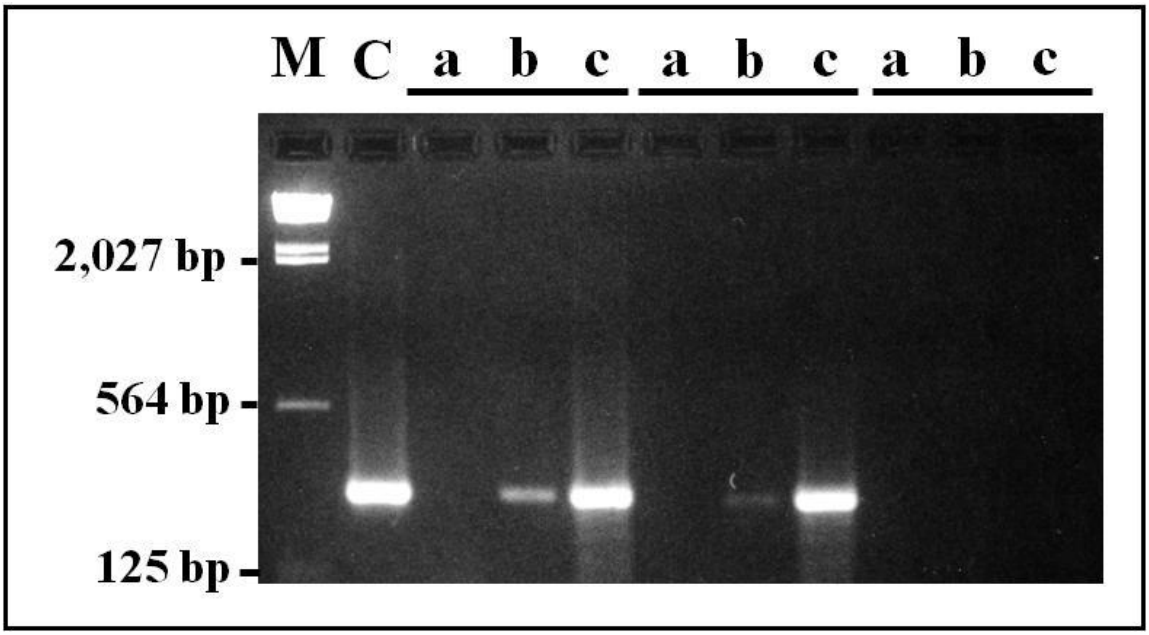

Figure 4.2. Gel picture of selected results. Polymerase chain reaction analyses of *Salmonella* invasion gene (*inv*A) fragments in preenrichment (lane a), first enrichment (lane b), and second enrichment (lane c) cultures from gut samples of three fish collected in 2007 from the San Marcos River, Texas, indicating the presence of *Salmonella* in two of three samples. Lane M represents a Lambda Hind III size marker; lane C represents the *inv*A fragment from *Salmonella typhimurium* ATCC (American Type Culture Collection) 14028.

The PCR-based detection of *Salmonella* in water, benthic sediments, and gut samples was confirmed for all samples by isolation of *Salmonella* from positive enrichment cultures and by subsequent physiological and immunological characterizations performed by the Texas Department of State Health Services. Among the four positive gut samples from sites 1 and 2, three serotypes were identified by isolation: *S. enterica enterica* serovar Thompson in two suckermouth catfish at site 1, serovar Rubislaw in a third suckermouth catfish at site 1, and serovar Typhimurium in the largemouth bass at site 2 (Fig. 4.3). Among the five positive samples from site 3, at least seven serotypes were identified by isolation: *S. enterica enterica* serovar Newport from the water sample, *Salmonella* spp. 4,5,12:i:– from the benthic sediment sample, *S.*

enterica enterica serovar Duesseldorf in the upper gut of the single common carp, serovar Give in the lower gut of that common carp, serovar Berta in the lower gut of one channel catfish, and serovars Mississippi and Typhimurium (with the potential of two Typhimurium strains) in the lower gut of the second channel catfish.

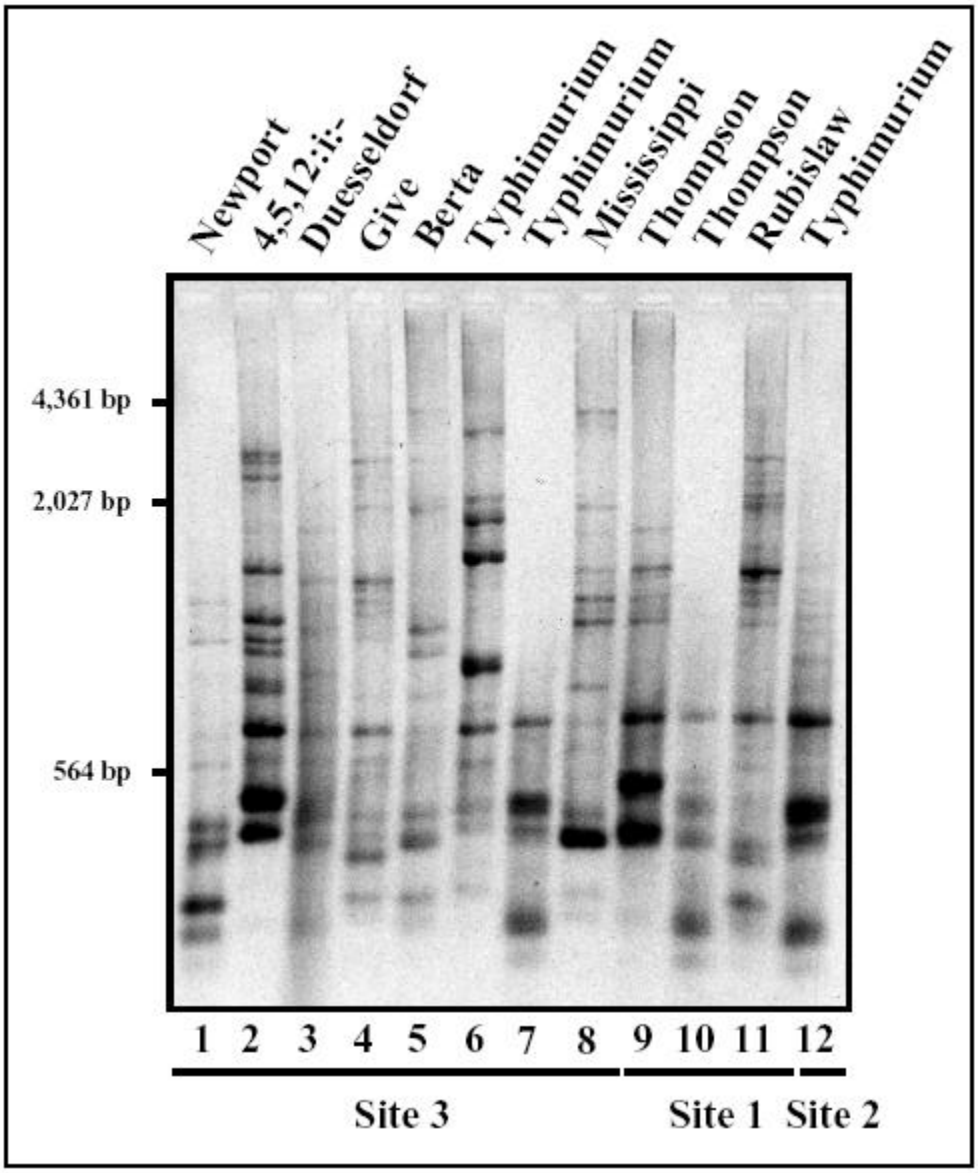

Figure 4.3. Gel picture of rep-PCR profiles of isolates. Repetitive sequence-based polymerase chain reaction profiles of *Salmonella* isolates obtained in 2007 from water (lane 1), sediment (lane 2), and fish gut (lanes 3–8) samples from site 3 and fish gut samples from site 1 (lanes 9– 11) and site 2 (lane 12) of the San Marcos River, Texas (Fig. 4.1). Names at the top of the figure correspond to *Salmonella* serovar characterizations provided by the Texas Department of State Health Services, Austin. Representative fragment sizes (base pairs [bp]) of a Lambda Hind III size marker are indicated.

In contrast to PCR-based analyses that relied on semiselective enrichment of *Salmonella*, *in situ* hybridization allowed us to immediately detect *Salmonella* in samples

of intestinal contents without enrichment. *In situ* hybridization results on these original samples confirmed results obtained by PCR on enrichment cultures, because *Salmonella* were only detected in original samples that had PCR-positive results. *Salmonella* in the original samples were usually found in clumps of cells associated with particulate material (Fig. 4.4). Numbers of cells were highly variable among clumps, ranging from an occasional cell to innumerable cells that dominated the surface of the particulate material.

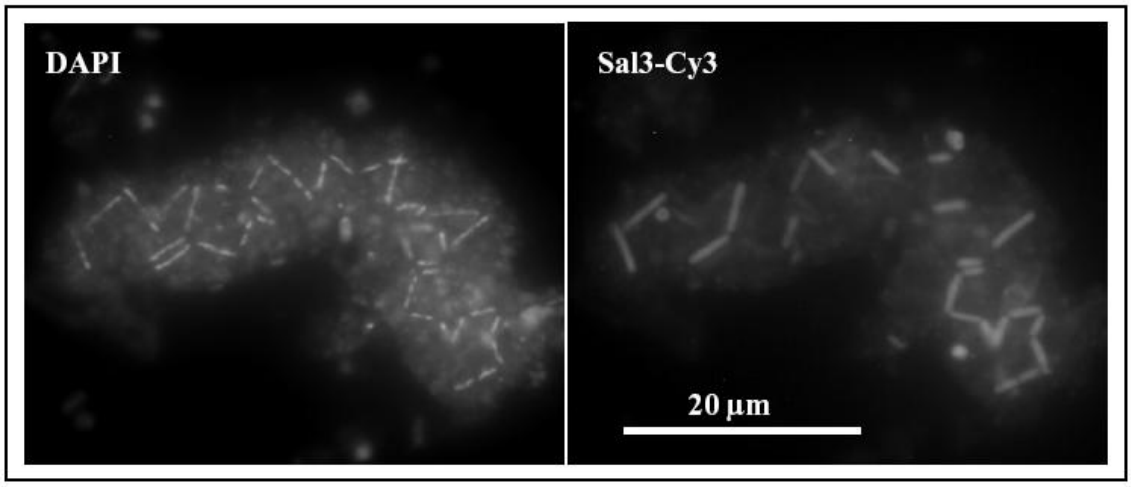

Figure 4.4. Microscopic image of *Salmonella* marked with DAPI and Sal3 probe. Detection of *Salmonella* in fish gut samples from the San Marcos River, Texas (collected in 2007), after *in situ* hybridization with the cyanine-3 (Cy3) labeled oligonucleotide probe Sal3 and analysis by epifluorescence microscopy (right panel) and after staining with 40,6-diamadino-2-phenylindole (DAPI), a DNA-intercalating dye used to detect all organisms (left panel).

Discussion

The percent occurrence of *Salmonella* ranged from 17% to 33% of fish sampled at three sites in the San Marcos River. These values are larger than those reported for fishes (0.0–2.3%) in circulating and noncirculating aquaculture systems, even those receiving treated wastewater effluents (Khalil & Hussein 1997, Pullela et al. 1998, Newaj-Fyzul et

al. 2006). In a sewage-fed pond, however, all individuals (100%; n = 7) of six fish species contained high amounts of *Salmonella* in their intestines, and detritivorous species like common carp (198 x 10^2 cells/g of gut content) and Mozambique tilapia *Oreochromis mossambicus* (113 x 10^2 cells/g) exhibited higher numbers than zooplanktivorous species, such as rohu *Labeo rohita* (75 x 10^2 cells/g) and catla *Catla catla* (87 x 10^2 cells/g) (Balasubramanian et al. 1992). In that study, the overall load of bacteria was reduced by up to 78% in the intestine when fish were transferred to freshwater and maintained there for 20 d. These results suggested that sewage (i.e., organic matter) affected bacterial loads in fish intestines in general and that particulate organic matter affected the presence of *Salmonella* in particular (Balasubramanian et al. 1992). Such results corroborate those of the current study, in that *Salmonella* were associated with particulate material in only certain locations of the intestine as opposed to being randomly distributed throughout the intestine.

The detection of *Salmonella* in fish was not related to the presence of *Salmonella* in water or sediments. Other studies have similarly observed that the presence of *Salmonella* in fish was not related to the abundance of *Salmonella* in water (Newaj-Fyzul et al. 2006). It has been proposed that *Salmonella* are part of the natural flora present in aquatic environments, at least in tropical regions (Reilly & Twiddy 1992). Our sampling sites were relatively close to the headwaters of the San Marcos River, where previous studies did not detect *Salmonella* in water and sediment samples (Hahn et al. 2007, Gaertner et al. 2008). However, as mentioned previously, *Salmonella* were frequently encountered in association with the common musk turtles in Spring Lake (Hahn et al. 2007, Gaertner et al. 2008). Serotypes retrieved from the common musk turtles (i.e.,

serovars Rubislaw, Newport, Gaminara, and Thompson) were largely different from those detected in fish at downstream sites, although serovar Thompson was also isolated from two fish at site 2, which is close to the headwaters. These data suggest that animals such as turtles provide habitats that allow *Salmonella* to persist in the environment but do not play a significant role in the dissemination of *Salmonella*.

In addition to interactions with animals, associations with biofilms (Armon et al. 1997, Barker & Bloomfield 2000) or sediments (Hendricks 1971, Marsh et al. 1998) were assumed to help *Salmonella* survive in the environment. Detection of *Salmonella* in biofilms on the carapaces of common musk turtles tends to support the first assumption (Hahn et al. 2007, Gaertner et al. 2008). Results of the current study also support the second assumption even though *Salmonella* were not detected in sediments from two out of three sites. Both sites at which *Salmonella* were absent were relatively close to (i.e., about 5 km downstream from) the headwaters. The water of the headwaters is characterized by a high mineral nutrient availability, which supports a large macrophyte population (Groeger et al. 1997). These conditions provide optimal habitats for a wide variety of animals that feed on these plants and for saprophytic microorganisms that live on decaying plant material. Since *Salmonella* detected in the intestines of fish were associated with particulate material and were only found at certain locations within the intestine, one might speculate that *Salmonella* were taken up with detritus by feeding fish and were merely passing through the intestine. At the upper two sites, *Salmonella* were detected in only a few fish and were not detected in water or sediments. This is possibly due to the shallow depths and swift currents of this spring-influenced portion of the river, which is dominated by run-type geomorphic units that allow less particulate material to

precipitate into the sediment. This is in contrast to the San Marcos River site located downstream of the Blanco River confluence; this site is characterized by more diverse riverine habitats, a greater number of backwater and pool geomorphic units, deeper water, and slower current velocities than are found at the other two sites. *Salmonella* were present in a higher proportion of fish at site 3 than at sites 1 and 2 and were also detected in sediments and water at site 3. It is possible that higher pathogen loads entered the system through the Blanco River, which is part of a very different watershed with different overall quality than the San Marcos River. However, data supporting this hypothesis are not available.

The presence of *Salmonella* in fish in this natural river system raises questions of potential concern for fisherman. Although *Salmonella* were detected in high numbers in fish at certain sites, their presence in the intestine should not be a human health concern if proper evisceration procedures are followed. The ability to detect *Salmonella* directly in fish by in situ hybridization provides an additional opportunity for using fish in ecosystem health assessments and potential non-point-source pollution monitoring. To contain the spread of diseases and to eliminate nonpoint sources of contamination, continuous and accurate assessment of water quality is paramount for communities that are dependent on rivers for drinking water and agriculture. Surveillance for *Salmonella* and other pathogens in water used for recreation and bathing, however, is expensive and time consuming. It is only performed by a few countries and generally only in a small percentage of areas within those countries (Figueras et al. 1997). Water microbiology quality standards are typically based on the use of coliform bacteria as indicators of pathogenic organisms (Cabelli et al. 1982, Polo et al. 1998), and testing for *Salmonella* is

performed only occasionally. The use of coliform bacteria as indicator organisms, however, often fails to detect threats posed by other pathogens such as *Salmonella* (Gales & Baleux 1992, Morinigo et al. 1992, Ferguson et al. 1996, Figueras et al. 1997). The use of fish in monitoring ecosystems for the presence of *Salmonella* could prove to be a useful alternative to coliform counts.

Acknowledgments

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CHAPTER 5

RECOVERY OF *SALMONELLA* FROM BIOFILMS IN A HEADWATER SPRING ECOSYSTEM[1]

Abstract

Salmonella are pathogenic bacteria often detected in waters impacted by human or animal wastes. In order to assess the fate of *Salmonella* in supposedly pristine environments, water and natural biofilm samples along with snails (*Tarebia granifera*) and crayfish (*Procambarus clarkia*) were collected before and up to seven days following four precipitation events from sites within the headwater springs of Spring Lake, San Marcos, TX. The samples were analyzed for the presence of *Salmonella* by polymerase chain reaction (PCR) after semi-selective enrichment. *Salmonella* were detected in one water sample directly after precipitation only, while detection in ten biofilm and two crayfish samples was not related to precipitation. *Salmonella* were not detected in snails. Characterization of isolates by rep-PCR revealed shared profiles in water and biofilm samples, biofilm and crayfish samples, and biofilm samples collected 23 days apart. These results suggest that *Salmonella* are infrequently washed into this aquatic ecosystem during precipitation runoff and can potentially take up residency in biofilms

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which can help facilitate subsequent long-term persistence and eventual transfer through the food chain.

Introduction

Salmonellosis is one of the most important public health disease problems worldwide with up to 1.3 billion cases reported annually (Pang et al. 1995). In the United States it affects 1.4 million people, causing 16,000 hospitalizations and over 500 deaths annually (Mead et al. 1999). The majority of illnesses result from exposure to undercooked animal products or cross-contamination with foods consumed raw (Tauxe 1997, Organization 2002); however, salmonellosis can also result from direct contact with contaminated water (Foltz 1969, Harvey et al. 1969) or infected animals (Sanyal et al. 1997, Wells et al. 2004, Nakadai et al. 2005). The intestinal tract of vertebrates is generally assumed to be the native habitat of *Salmonella* (Woodward et al. 1997) from which the feces then contaminate environments such as fresh- or marine waters, estuarine environments, vegetables, compost, or soils and sediments (Thomason et al. 1975, Polo et al. 1998, Refsum et al. 2002, Tavechio et al. 2002, Martinez-Urtaza et al. 2004). The occurrence of *Salmonella* in these environments is therefore frequently linked to environmental contamination through, e.g., manure or wastewater discharges (Polo et al. 1998, Martinez-Urtaza et al. 2004).

Despite this assumption, however, *Salmonella* have been detected in pristine aquatic systems (Fair & Morrison 1967, Hendricks & Morrison 1967) and shown to persist in the environment for extended periods of time without significant impact from terrestrial animals (Hendricks 1971, Chao et al. 1987). *Salmonella* have even been suggested to be

part of the natural flora present in some aquatic ecosystems (Jimenez et al. 1989). Interactions with animals (Reilly & Twiddy 1992) and associations with biofilms (Armon et al. 1997, Barker & Bloomfield 2000) or sediments (Hendricks 1971, Marsh et al. 1998) are assumed to help *Salmonella* survive in the environment. Despite the evidence that *Salmonella* have complex interactions with the non-host environment, however, little is known about the fate of this organism outside of host organisms.

Recent studies in our laboratory frequently detected *Salmonella* in water, sediments, animals (i.e., fish, turtles) and biofilms even in supposedly clean habitats such as Spring Lake, the spring-fed headwaters of the San Marcos River in San Marcos, Texas (Hahn et al. 2007, Gaertner et al. 2008a, Gaertner et al. 2008b, Gaertner et al. 2009). Spring Lake is a small reservoir (6 ha) that is considered one of the most pristine waters in Texas (Slattery & Fahlquist 1997). It consists of two arms, the Spring arm, characterized by relatively constant environmental conditions in depth and throughout the year due to the permanent supply of water through numerous springs from the Edwards Aquifer, and the slough arm that represents a more lentic environment with slow flow, large seasonal changes in temperature and redox conditions, and large deposition of organic material (Groeger et al. 1997). While the spring arm is bordered at one side by steep slopes of forested limestone and on the other by landscaped parkland, the entire slough arm is surrounded by the Texas State University Golf Course, and connected to the discharge area of Sink Creek. The lake is not affected by point sources of pollution such as sewage treatment facilities which are common sources of impact to Texas Rivers (Groeger et al. 1997), however, due to its location within the urban-rural interface of San Marcos, could be affected by several potential non-point pollution sources for *Salmonella* that include

cattle ranching operations as well as wildlife habitat associated with the adjacent golf course. In our previous studies, detection of *Salmonella* in water and sediments was more pronounced directly after rainfall events (Gaertner et al. 2009), and thus likely a consequence of contamination through livestock or wildlife fecal droppings transported into the aquatic system by strong rainfall events and associated runoff as suggested for other systems (Kinzelman et al. 2004, Arnone & Perdek Walling 2007). The detection of *Salmonella* in the intestine of turtles and fish, and especially in biofilms on the carapace of turtles, however, could not be linked to runoff, and thus, opens the door for speculations on the dissemination and on the fate of *Salmonella* with respect to short- and long-term population establishment in aquatic ecosystems.

The aim of our study was to monitor the presence of *Salmonella* before and after rainfall events in water and sediment samples, to investigate their potential establishment in biofilms, and to assess the potential for their transfer from biofilms into the food chain. The assumption was that biofilms are used as food resource by herbivorous or omnivorous grazers like snails, and these are preyed upon by carnivorous animals such as crayfish (Nystrom 2002). Both snails (Bartlett & Trust 1976, Moore et al. 2003, Tezcan-Merdol et al. 2004) and crayfish (Lovell & Barkate 1969) have been shown to be potential hosts for *Salmonella*, and are abundant in Spring Lake, the headwaters of the San Marcos River, San Marcos, Texas.

Materials and Methods

Sample collection and preparation

Samples were taken from two locations, one each in the spring and slough arms of Spring Lake (Fig. 5.1) at different time intervals distributed before and after four separate rainfall events that happened June 23 (2.5 mm), June 30 (7.6 mm), July 7 (6.4 mm), and July 9 (30 mm) (Fig. 5.2). Samples collected on each date consisted of four water samples, eight biofilm samples, and 10 snails of the species *Tarebia granifera* from each of the spring and slough arms of Spring Lake with the exception of June 24 in which only six biofilm samples were collected from each the spring and slough arm and only two snails were found in the slough arm. Water samples were collected into 50 ml tubes from just below the surface, cells pelleted by centrifugation at 3000 rpm for 15 minutes and transferred to 2 ml cryotubes containing 1 ml buffered peptone water (BPW) (l^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO4, 1.5 g KH_2PO_4, pH 7.2) (Thomason et al. 1977, International Standard Organization 1993). Biofilm samples were taken by scraping films off of the surface of rocks from within each site directly into 2 ml cryotubes containing 1 ml of BPW. Snails (*T. granifera*) were collected by hand and homogenized in 2 ml cryotubes containing 1 ml of BPW.

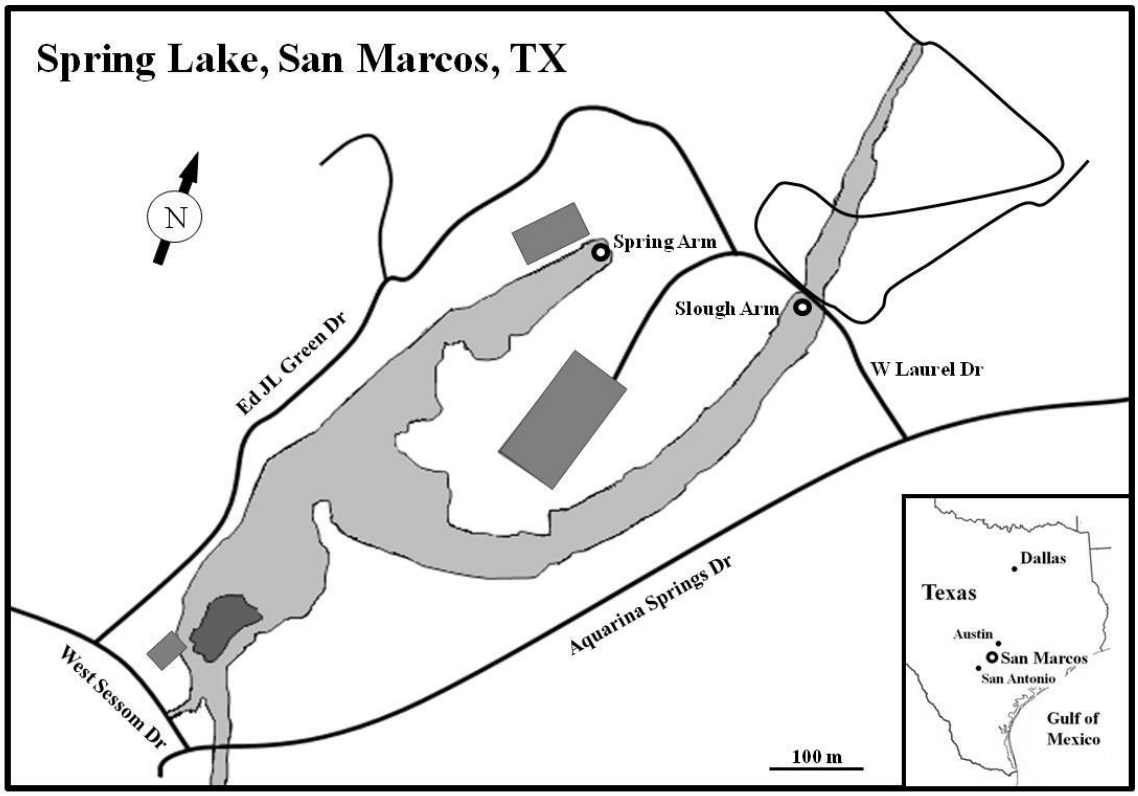

Figure 5.1: Schematic presentation of sampling sites. Sampling sites (open circles) used for collection of samples to be tested for the presence of *Salmonella* in both the spring and slough arms of Spring Lake, San Marcos, TX, USA ($79^{o}53$'N, $97^{o}55$'W).

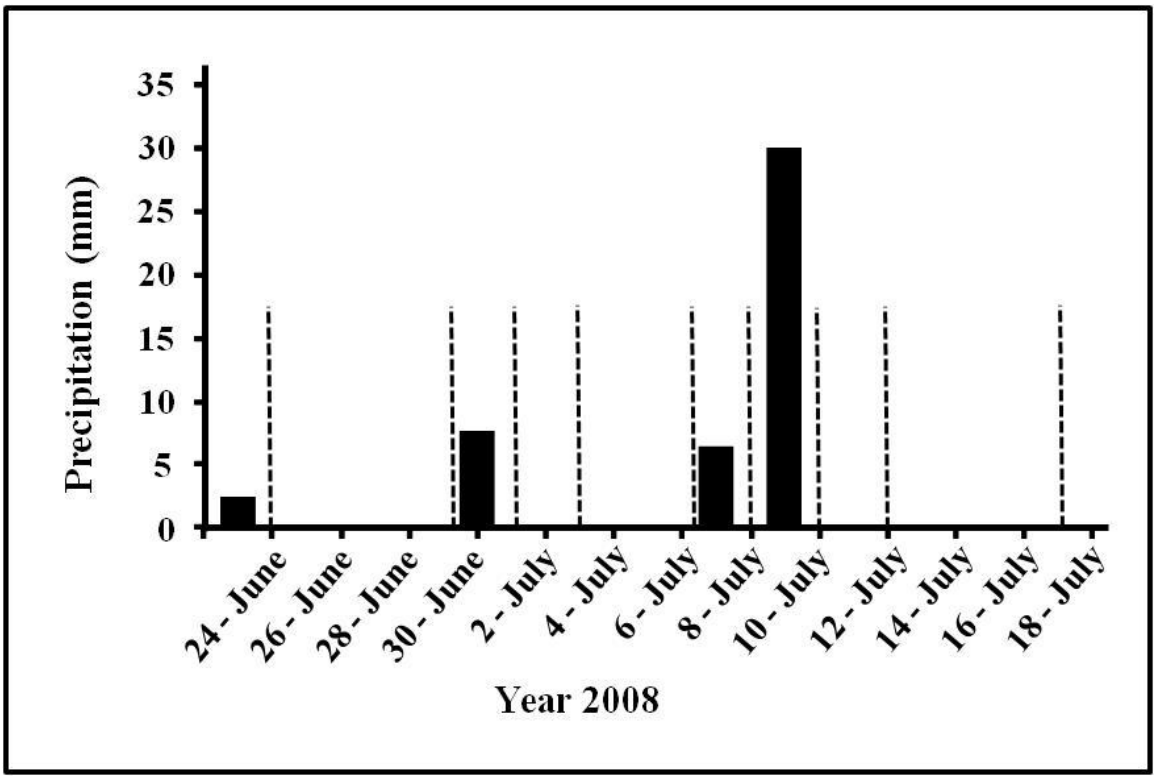

Figure 5.2: Climatic data collected during sampling period. Precipitation data (dark bars) for San Marcos, TX, USA and sampling dates (dashed lines) used for the collection of samples from Spring Lake.

Independent of precipitation events, 21 crayfish (*Procambarus clarkia*) were captured by hand prior to June 23rd in both the spring and slough arms of Spring Lake, and samples from the stomach and intestine separated into 2 ml cryotubes containing 1 ml of BPW.

Enrichment of Salmonella

All samples were incubated in BPW at 37°C for 16-20 hours (International Standard Organization 1993). Aliquots (100-µl) of these cultures were transferred to 2 ml cryotubes containing 1 ml of Rappaport-Vassiliadis Broth (RVS) (l^{-1}: 4.5 g peptone

(soymeal), 29 g $MgCl_2$ x 7 H_2O, 8 g NaCl, 0.4 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) (Vassiliadis et al. 1981). After incubation at 37°C for 24 hours (Vassiliadis et al. 1981), 100-µl sub-samples were transferred to new cryotubes containing RVS media and, again, incubated at 37°C for 24 hours (Gaertner et al. 2009). Aliquots (100 µl) of the final RVS enrichment culture were prepared for polymerase chain reaction (PCR) assisted detection of *Salmonella*, and the remaining samples were mixed with 600 µl of 60% glycerol and stored at -80°C until further use.

PCR-based detection of Salmonella

The presence of *Salmonella* was detected utilizing the amplification of a 284-bp-fragment of the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by *Salmonella* (Suárez & Rüssmann 1998, Khan et al. 2000). This protocol has been acknowledged as the international standard diagnostic method for quality assurance laboratories in epidemiological studies on all *Salmonella enterica* subspecies as well as in *Salmonella bongori* (Malorny et al. 2003). Aliquots of the final enrichment were centrifuged at 14,000 rpm for 2 minutes, the bacterial pellets washed once in sterile distilled water, and the bacteria lysed in 100 µl of 50 mM NaOH by incubation at 65°C for 15 min (Hahn et al. 2007). One microliter of lysate was used as template for PCR amplification with primers 139 ($^{5'}$GTGAAATTATCGCCACGTTCGGGCAA) and 141 ($^{5'}$TCATCGCACCGTCAAAGGAACC) (Rahn et al. 1992) in a final volume of 50 µl containing 1 × PCR buffer (50 mM KCl, 2.5 mM $MgC1_2$, 20 mM Tris/HCI, pH 8.4, 0.1% Triton 100), 0.2 mM dNTPs, 1 U of *Taq* polymerase, and 100 ng of each primer. After an

initial 2-minute denaturation at 96°C, 35 rounds of temperature cycling were performed in a PTC-200 Thermocycler (BioRad, Hercules, CA) with denaturation at 96°C, primer annealing at 64°C, and elongation at 72°C, each for 30 seconds, followed by a final incubation at 72°C for 7 minutes (Malorny et al. 2003). Lysates of *Salmonella typhimurium* ATCC 14028 and sterilized distilled water were used as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 µl ml^{-1}) (Sambrook et al. 1989).

Rep-PCR

Bacteria from the second enrichment of samples positive for *Salmonella* were plated onto RVS Agar (i.e. RVS Broth solidified with 15 g agar l^{-1}) and incubated at 37°C for 24-48 hours. Ten colonies from each plate were sub-cultured in LB medium and checked for the presence of the *inv*A gene by PCR as described above. All positive isolates were further analyzed by rep-PCR using the BoxA1R primer ($^{5'}$CTACGGCAAGGCGACGCTGACG) (Versalovic et al. 1998). A total volume of 25 µl was used for each reaction using 2 µl of lysate (Dombek et al. 2000), 300 ng of primer, 1 x Gitschier buffer, 5 mM dNTPs, 10% di-methyl-sulfoxide (DMSO), 0.0002 µl bovine serum albumin, and 2 U *Taq* polymerase (Rademaker & de Bruijn 1997). Thermocycler conditions consisted of an initial denaturation step of 95°C for 2 minutes followed by 30 rounds of temperature cycling with denaturation at 94°C for 3 seconds and subsequent 92°C for 30 seconds, primer annealing at 50°C for 1 minute, and elongation at 65°C for 8 minutes. This was followed by incubation at 65°C for 8 minutes (Rademaker & de Bruijn

1997, Dombek et al. 2000). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989), and representative profiles documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA).

Results

PCR-based detection of the *inv*A gene was achieved in 13 out of 426 samples (72 water, 140 biofilm, 172 snail, and 42 crayfish samples). While none of the snail samples tested positive for *Salmonella*, amplification of *inv*A gene fragments was obtained for 1 water, 10 biofilm and 2 crayfish samples. The positive water sample was collected from the slough arm on July 1, a day after rainfall (7.6 mm), while 8 of the positive biofilm samples were collected from the spring arm and 2 from the slough arm. Both positive biofilm samples from the slough arm were collected on July 10, one day after a precipitation event. Positive samples from the spring arm were collected on five separate dates including three sampling efforts occurring one day after precipitation (June 24 - 2 positives, July 1 - 3 positives, and July 8 - 1 positive) and two sampling efforts occurring seven days after precipitation (July 7 - 1 positive and July 17 - 1 positive). The two crayfish samples testing positive for *Salmonella* were collected from the spring arm of Spring Lake on June 12 and were from the intestine of one crayfish and the stomach of the other.

Isolates of *Salmonella* were obtained from all 13 positive samples, with all 10 colonies picked at random for each sample being positive for the *inv*A gene, except in the case of a biofilm sample collected from the spring arm on July 1 which yielded only 8

positive colonies. All *Salmonella* colonies for each sample displayed identical rep-PCR profiles, except for *Salmonella* from 4 biofilm samples taken from the spring arm in which two profiles were obtained (July 8, July 17 and two samples on July 1) (Table 5.1). Overall, 12 distinct profiles were obtained (Fig. 5.3).

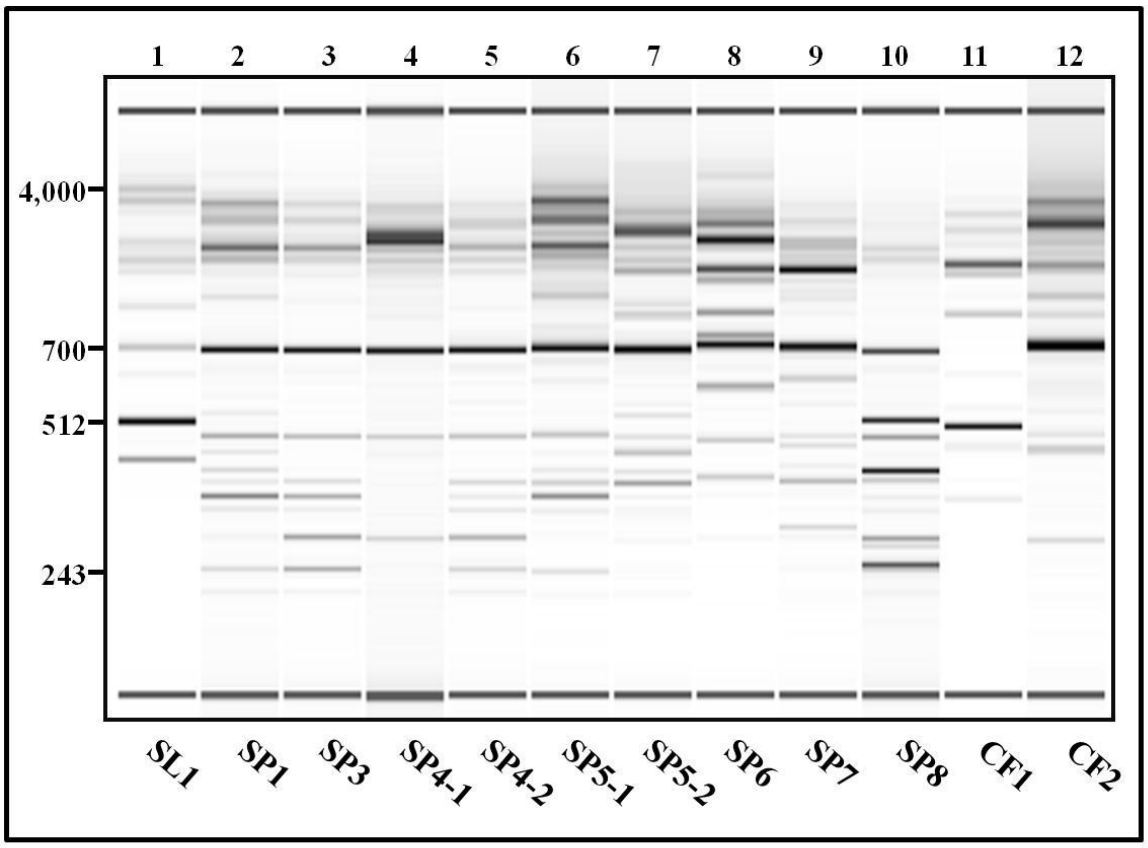

Figure 5.3: Gel picture of rep-PCR profiles of isolates. Representative rep-PCR profiles of *Salmonella* isolates of enrichment cultures from samples taken from water, biofilms and crayfish (*Procambarus clarkia*) (CF) from the slough (SL) and spring (SP) arms of Spring Lake, San Marcos, TX, USA (29°53'N, 97°55'W), and documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA). Profile sources and dates are as follows: Lane 1, water July 1st (SL1) and 2 biofilms July 10th (both SL1); Lane 2, 2 biofilms June 24th (both SP1) and July 17th (SP1); Lanes 3-7, biofilms July 1st (SP#3, SP4-1, SP4-2, SP5-1 and SP5-2); Lane 8, biofilm July 7th (SP6); Lane 9, biofilm July 8th (SP7); Lane 10, biofilm July 17th (SP8); Lane 11 crayfish June 12th (CF1); Lane 12, crayfish June 12th (CF2) and biofilm July 8th (CF2).

Table 5.1: Prevalence and strain identity of *Salmonella* collected during study. Prevalence of *Salmonella* and strain identification in samples collected from water, biofilm and snails (*Tarebia granifera*) from both the spring and slough arms of Spring Lake.

	Spring arm		Slough arm	
Sampling date Sample	**Prevalence**[1]	**Strain**[2]	**Prevalence**[1]	**Strain**[2]
June 24 (last precipitation: June 23 [2.5 mm])				
Water	0/4		0/4	
Biofilm	2/6	SP1	0/6	
Snails	0/10		0/10	
June 30 (last precipitation: June 23 [2.5 mm])				
Water	0/4		0/4	
Biofilm	0/8		0/9	
Snails	0/10		0/10	
July 1 (last precipitation: June 30 [7.6 mm])				
Water	0/4		1/4	SL1
Biofilm	3/8	SP3, SP4-1, SP4-2, SP5-1, SP5-2	0/8	
Snails	0/10		0/10	
July 3 (last precipitation: June 30 [7.6 mm])				
Water	0/4		0/4	
Biofilm	0/8		0/8	
Snails	0/10		0/10	
July 7 (last precipitation: June 30 [7.6 mm])				
Water	0/4		0/4	
Biofilm	1/8	SP6	0/8	
Snails	0/10		0/10	
July 8 (last precipitation: July 7 [6.4 mm])				
Water	0/4		0/4	
Biofilm	1/8	SP7, CF2	0/8	
Snails	0/10		0/10	
July 10 (last precipitation: July 9 [30 mm])				
Water	0/4		0/4	
Biofilm	0/8		2/8	SL1
Snails	0/10		0/10	
July 14 (last precipitation: July 9 [30 mm])				
Water	0/4		0/4	
Biofilm	0/8		0/8	
Snails	0/10		0/10	
July 17 (last precipitation: July 9 [30 mm])				
Water	0/4		0/4	
Biofilm	1/8	SP8, SP1	0/8	
Snails	0/10		0/10	

[1]Prevalence: detection of *Salmonella* per sample/total number of samples

[2]Strain assignments are based on distinct rep-PCR profiles (see Fig. 5.3)

Several profiles were shared among isolates from samples collected during the study (Table 5.1), however, none of them was identical to any of those obtained in previous studies (data not shown). All *Salmonella* isolates from the three positive samples collected from the slough arm of Spring Lake had identical rep-PCR profiles, i.e., profile SL1. These samples included a water sample collected on July 1 and two biofilm samples collected on July 10. Isolates from two biofilm samples collected from the spring arm on June 24 shared an identical rep-PCR profile with isolates from a biofilm sample collected 23 days later on July 17 (profile SP1). Finally, profiles from isolates from a crayfish sample collected on June 12 matched those of a biofilm sample collected on July 8, both collected from the spring arm (Fig. 5.3, profile CF2).

Discussion

Salmonella were detected in water, biofilm and crayfish samples from Spring Lake, San Marcos, TX, after semi-selective enrichment and subsequent detection on *inv*A gene fragments by PCR as described previously (Hahn et al. 2007). Most samples testing positive for *Salmonella* were obtained a day after rainfall events (i.e., 9 out of 13) suggesting runoff from terrestrial systems or upwelling as potential sources of contamination despite the rainfall events being relatively weak with 5 to 30 mm per day. However, in contrast to previous studies that reported significant contamination of water and sediment samples in Spring Lake after extreme flashfloods with up to 740 mm precipitation per day (Gaertner et al. 2009), the current rainfall events resulted in only 1 water sample being contaminated with *Salmonella* indicating lessened effects of runoff or upwelling. The short residence time of *Salmonella* in water indicated by the detection of

Salmonella in samples obtained a day after rainfall events was also evident in our previous study (Gaertner et al. 2009), as well as in those of others (Baudart et al. 2000, Haley et al. 2009, Jokinen et al. 2009) indicating the transient nature of the contamination.

Most samples testing positive for *Salmonella* in this study represented biofilms in which *Salmonella* were detected 1 day after rainfall, but also in samples taken up to 7 days after rainfall. Since the location of the sampling sites, i.e., rocks collected near the headwaters of both the spring arm and slough arm of Spring Lake, San Marcos, TX, excludes potential contamination from upstream water, runoff from rainfall events (Gaertner et al. 2009, Haley et al. 2009), in addition to potential small scale contamination by animals, is the most likely source for potential contamination (Geldreich 1996). Since rainfall events were small and detection of *Salmonella* low in water, it is unlikely that these *Salmonella* were detected as a consequence of runoff or upwelling. It is more likely that *Salmonella* from the terrestrial environment are infrequently moved into the aquatic environment by runoff from precipitation and subsequently take up residency in naturally occurring biofilms (Byappanahalli et al. 2009).

While *Salmonella* have the ability to form biofilms on highly diverse surfaces such as those of plastic, cement, stainless steel and even Teflon (Austin et al. 1998, Joseph et al. 2001), the simple detection of *Salmonella* in biofilms does not provide sufficient information to assess whether *Salmonella* are growing as or in biofilms as suggested for *E. coli* (Byappanahalli et al. 2003) or are just adhering to the biofilm. Biofilms in aquatic systems represent highly active and heterogeneous communities of auto- and

heterotrophic microorganisms that include diatoms, green algae, protozoa, fungi and bacteria, attached to rocks or concrete lining the streambed (Geesey et al. 1978). Microorganisms are embedded in an extracellular matrix (Sabater & Admiraal 2005) that has been shown to increase their survival by providing protection from toxic compounds, and reducing thermal stress and predation pressure (Johnson 2008). Biofilms are also hot spots of rapidly available carbon resources for heterotrophic organisms (Geesey et al. 1978, Augspurger et al. 2008), created by retaining dissolved organic matter from the water (Fischer et al. 2002, Romani et al. 2004) or by releasing labile carbon exudates produced by autotrophic organisms such as diatoms or green algae (Sundh & Bell 1992). Enteric pathogens such as *Salmonella* or *E. coli* have been shown to survive for extended periods of time in different habitats (Domingo et al. 1989, Smith et al. 1994, Ishii et al. 2006, Semenov et al. 2009), including biofilms (Ksoll et al. 2007) and algal mats (Ishii et al. 2006, Englebert 2008, Byappanahalli et al. 2009). Biofilms might therefore provide habitats suitable for long term survival of *Salmonella* in aquatic systems, as discussed for other pathogens (Watnick & Kolter 1999, Yildiz & Schoolnik 1999), or even growth as suggested for other environments such as soil (Topp et al. 2003, You et al. 2006). While the first assumption is supported by the detection of *Salmonella* with identical rep-PCR profiles in water samples taken on July 1 and two separate biofilm samples taken nine days later, as well as of samples of biofilms taken 23 days apart, the latter suggestion remains highly speculative and requires additional quantitative studies on the development of populations of *Salmonella* in biofilms through time.

Independent of *Salmonella* persisting or growing in biofilms, the long term survival of *Salmonella* in biofilms provides additional human health concerns. Since the densities

of pathogens in biofilms can be much higher than in water (Hall-Stoodley & Lappin-Scott 1998, September et al. 2007), the release of pathogens from biofilms can effectively increase the infective dose in the environment and thus increase the incidence of disease in humans with contact to contaminated water (Purevdorj 2002, Marsollier et al. 2004). Biofilms might also provide a potential avenue for transfer of this pathogen through the food chain. Although *Salmonella* was not detected in snails in this study, snails have been shown to be potential reservoirs of the pathogen (Bartlett & Trust 1976, Obi & Nzeako 1980). *Salmonella* ingested during grazing on biofilms by snails could then be transferred to crayfish or fish feeding on snails as indicated by matching rep-PCR profiles of *Salmonella* isolated crayfish collected on June 12 and a biofilm sample collected on July 8. This potential route of contamination, however, needs to be confirmed in more controlled settings in which defined strains of *Salmonella* are systematically followed as they move through a representative food chain when inoculated into water, establishing in biofilms, taken up by snails and then transferred to crayfish or fish prior to any opportunity of consumption by humans.

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CHAPTER 6

DETECTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN ENDEMIC SALAMANDER SPECIES FROM CENTRAL TEXAS[1]

Abstract

A nested PCR protocol was used to analyze five endemic salamander species from Central Texas for the presence of the emerging pathogen chytrid fungus (*Batrachochytrium dendrobatidis*). Chytrid fungus was detected from samples of each of the five species sampled with low abundance in the Texas salamander (*Eurycea neotenes*) (1 positive out of 16 individuals tested; 1/16), the Blanco River Springs salamander (*E. pterophila*) (1/20), the threatened San Marcos salamander (*E. nana*) (1/17), and the endangered Barton Springs salamander (*E. sosorum*) (1/7). Much higher abundance was obtained for the Jollyville Plateau salamander (*E. tonkawae*) (6/14) which has recently been petitioned for addition to the USA endangered species list. With one exception, sequences of PCR products were identical to the 5.8S rRNA gene, and nearly so for the flanking ITS regions of *B. dendrobatidis* which confirmed the detection of Chytrid fungus, and thus demonstrated the presence of this pathogen in populations of endangered species in Central Texas. These confirmations were obtained from non-

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consumptive tail clippings which confirms the applicability of historically collected samples from other studies in the examination of the fungus across time.

Detection of *Bartachochytrium dendrobatidis* in endemic salamander species from Central Texas

During the last 20 years significant declines in populations of amphibians have been observed worldwide (Stuart et al. 2004, Lips et al. 2005). Although factors such ashabitat loss, climate change and overexploitation have been implicated in declines (Davidson et al. 2001, Hero & Morrison 2004, Daszak et al. 2005), some of the blame for recent losses is also given to the emerging infectious fungus *Batrachochytrium dendrobatidis* (Chytrid fungus) (Bosch et al. 2001, Lips et al. 2003, Herrera et al. 2005, Scherer et al. 2005). Chytridiomycosis has been suggested to be the proximal and ultimate cause of extinctions for several species (Daszak et al. 2003, Schloegel et al. 2006). While many reports on species that were severely affected by chytridiomycosis deal with anurans (Skerratt et al. 2007), much less is known on the occurrence of *B. dendrobatidis* on other amphibians, e.g., salamanders. Although studies have linked *B. dendrobatidis* with mortalities of Fire salamanders (*Salamandra salamandra)* (Bosch & Martinez-Solano 2006) and Bolitoglossine salamanders (*Bolitoglossa dofleini)* (Pasman et al. 2004), others have detected *B. dendrobatidis* on salamanders with no apparent signs of illness both in the lab (Davidson et al. 2003) and in wild populations (Cummer et al. 2005, O'Donnell et al. 2006).

Although the presence of *B. dendrobatidis* on amphibians has been confirmed for many states, its presence is not well documented for Texas which provides habitat for

several threatened or endangered amphibians such as the Houston toad (*Bufo houstonensis*) (Hillis et al. 1984), or the Texas Blind salamander (*Eurycea rathbuni)* (Potter & Sweet 1981, Hillis et al. 2001). Central Texas also hosts many species –some of which are threatened or endangered- that have extremely small distribution ranges, which in some cases is limited to only a few springs (Baker 1961, Sweet 1982). Examples include the Texas salamander (*Eurycea neotenes*), the Blanco River Springs salamander (*E. pterophila*), the threatened San Marcos salamander (*E. nana*), the endangered Barton Springs salamander (*E. sosorum*), and the Jollyville Plateau salamander (*E. tonkawae*) (Chippindale et al. 2000). Toe clippings of Jollyville Plateau salamanders have recently been found positive for *B. dendrobatidis* from all 40 individuals that were collected at 16 sites across the entire range of this salamander in an area northwest of Austin, Texas (O'Donnell et al. 2006). None of the salamanders tested, however, showed symptoms of chytridiomycosis or noticeable health effects (O'Donnell et al. 2006).

That report demonstrated the occurrence of *B. dendrobatidis* on salamanders in Central Texas, notably with 100% infection rate on one endemic salamander with a very small distribution range. The occurrence of *B. dendrobatidis* on asymptomatic salamanders makes these a potential reservoir host for the fungus which could pose a potential threat to other amphibians, especially because salamanders have been shown to transmit *B. dendrobatidis* to frogs (Davidson et al. 2003). We were therefore interested to determine whether the occurrence of *B. dendrobatidis* was limited to Jollyville Plateau salamanders, or whether other salamanders with similarly small distribution ranges in Central Texas were also infected. Our analyses took advantage of the availability of nucleic acid extracts from tail clippings of different *Eurycea* species that had been

retrieved for recent population studies on members of this genus (Lucas 2006). Since tail rather than toe clippings were used for the extraction of nucleic acids, tail clippings from Jollyville Plateau salamanders known to harbor *B. dendrobatidis* were used to evaluate the methodology and validate the results of the analyses.

In addition to Jollyville Plateau salamanders (n=14 individuals from three sites), nucleic acid extracts were obtained from San Marcos salamanders (n=17 individuals), Texas salamanders (n=16 individuals from two sites), Blanco River Springs salamanders (n=20 individuals from three sites), and Barton Springs salamanders (n=7 individuals) (Table 6.1). Salamanders were collected by hand at sites in Comal, Travis and Hays counties in Central Texas (Fig. 6.1) between October 2004 and August 2005. A small portion of the tail (25 mg) was cut off and preserved in 96% ethanol (Lucas 2006). DNA was extracted from these samples using the Qiagen DNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol for purification of DNA from animal tissues. DNA was 200 μL of distilled water and stored at -80°C until further use.

Table 6.1: Salamander samples obtained from Central Texas sites.

Species	Origin	Collected/Bd-positive Samples
Eurycea nana (San Marcos Salamander)		
	Spring Lake (29º53’33”N/97º56’1”W)	17/1
Eurycea neotenes (Texas Salamander)		
	Comal Springs (29º42’48”N/98º8’7”W)	11/0
	Hueco Springs (29º45’56”N/98º8’31”W)	5/1
Eurycea pterophila (Blanco River Springs Salamander)		
	Ott’s Spring (29º55’22”N/98º9’3”W)	10/0
	Fernbank Springs (29º59’38”N/97º59’45”W)	5/1
	Jacob’s Well (30º2’3”N/98º7’20”W)	5/0
Eurycea sosorum (Barton Springs Salamander)		
	Barton Springs (30º15’49”N/97º46’13”W)	7/1
Eurycea tonkawae (Jollyville Plateau Salamander)		
	Jollyville-Stillhouse (30º22’24”N/97º45’54”W)	6/3
	Jollyville-Mainstem7 (30º22’13”N/97º45’46”W)	5/1
	Jollyville-Tributary 6 (30º25’31”N/97º48’53”W)	3/1

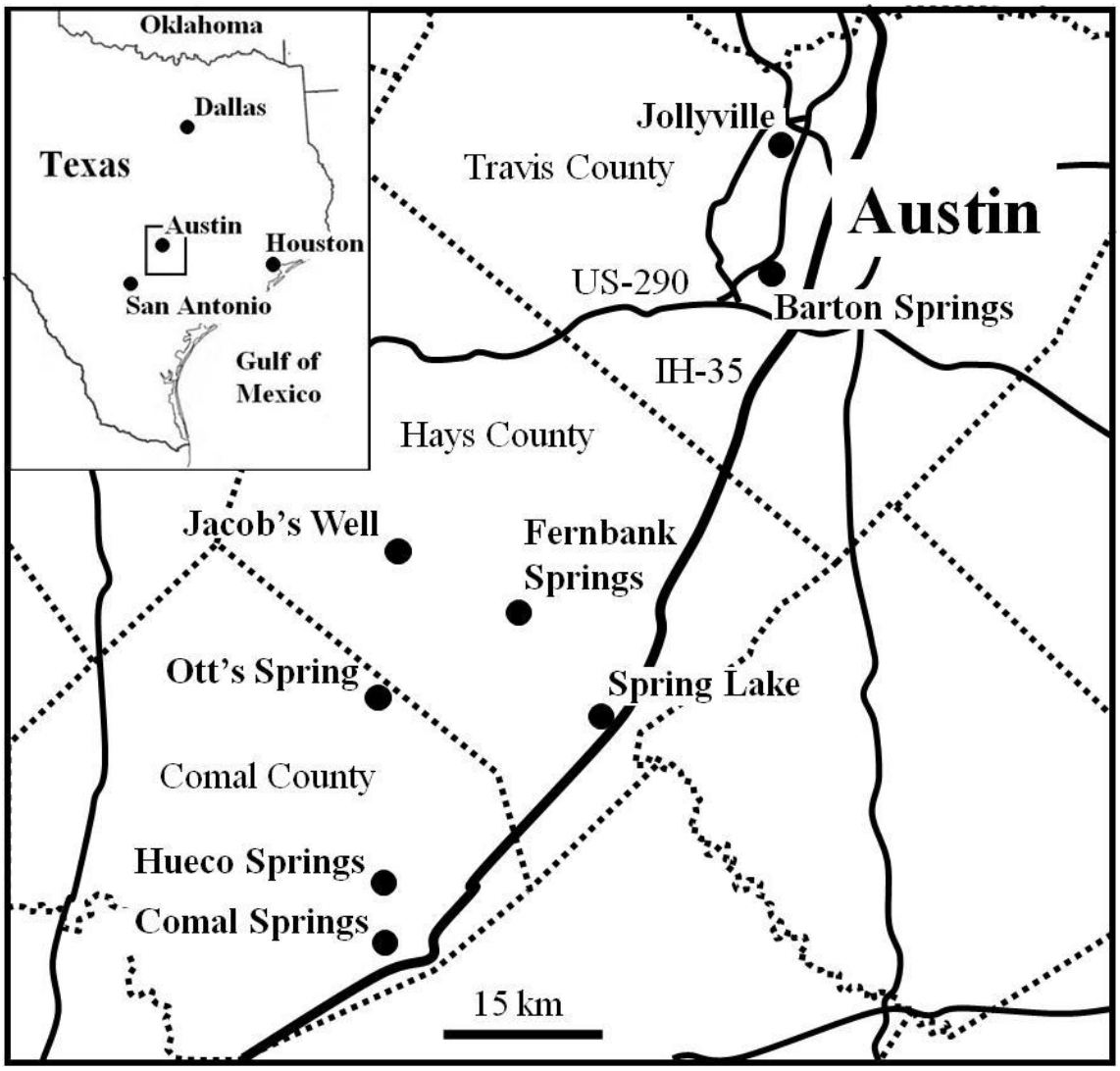

Figure 6.1: Map of sampling sites used in study. Schematic presentation of sampling sites for salamanders in the Central Texas area. Samples were taken from 4 sites in Austin, Travis County including Jollyville (Stillhouse Hollow, Mainstem 7-11, and Tributary 6) and Barton Springs, 3 sites near San Marcos, Hays County (Spring Lake, Fern Bank and Jacobs Well), and 3 sites near New Braunfels, Comal County (Comal Springs, Hueco Springs, and Ott's Spring).

One μL of DNA extract was used as template for PCR amplification using primers Bd1a (5'-CAG TGT GCC ATA TGT CAC G) and Bd2a (5'CAT GGT TCA TAT CTG TCC AG) (Annis et al. 2004). This primer set has shown high specificity for *B. dendrobatidis* when tested against other closely related species (Annis et al. 2004).

PCR was performed in a total volume of 50 µL containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µL dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µL *Taq* polymerase (GeneScript, Piscataway, NJ; 5 U μL^{-1}), 1 µL of each primer (100 ng μL^{-1}), 8 µL bovine serum albumin (BSA; 30 mg mL^{-1}), and 1 µL DNA extract (Widmer et al. 1999). After an initial 10-min-denaturation at 94°C, and subsequent addition of *Taq* polymerase (hot-start PCR), thirty rounds of temperature cycling was performed in a PTC-200 Thermocycler (MJ Research, Waltham, MA) with denaturation at 93°C for 45 seconds, primer annealing at 60°C for 45 seconds, followed by elongation at 72°C for 60 seconds (Annis et al. 2004). This was followed by incubation at 72°C for seven minutes. DNA from sample TK04 (*E. tonkawae*) that was positive for *B. dendrobatidis* and sterilized distilled water were used as positive and negative controls, respectively. The presence of fragments of about 300 bp (Annis et al. 2004) was examined by agarose gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989).

In addition to the positive control, only two samples, both of which originated from Jollyville Plateau salamanders, resulted in the production of amplification products which indicated the presence of *B. dendrobatidis* on tails of these salamanders. However, the low rate of detection (14%) compared to previous studies that used toe clippings (100%) (O'Donnell et al. 2006) indicated methodological issues of using tail clippings. Lower keratinization of tails might result in lower densities of *B. dendrobatidis* and therefore in fewer templates for PCR-based detection of *B. dendrobatidis.* In order to adjust for fewer templates and consequently increase sensitivity of detection, a nested PCR approach was performed. In this approach, amplification products generated using

primers ITS1f (5'-CTT GGT CAT TTA GAGC GAA GTA) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC) that target conserved regions of the 28S and the 18S rRNA gene and amplify the 5.8S rRNA gene along with the flanking internal transcribed spacer (ITS) of all fungi (White et al. 1990) were used as the starting template for subsequent amplification using the specific primer set Bd1a/Bd2a.

The initial reaction using primer set ITS1f/ITS4 was also performed in a total volume of 50 µL with conditions as described above except for denaturation and annealing temperatures that were at 94°C and 57°C, respectively. PCR products were purified using the MoBio Ultraclean 15 Gel Extraction Kit (MoBio, Carlsbad, CA) following the manufacturer's instructions, and re-suspended in 20 µL of water. One µL of these purified PCR products was then used as template for the subsequent PCR reaction with the specific primer set Bd1a/Bd2a as described above.

Nested PCR analyses of tail clippings from 74 salamanders for *B. dendrobatidis* retrieved a total of 9 samples that were positive for the fungus as emphasized by the detection of amplification product of the appropriate size of about 300 bp (Fig. 6.2). These samples included at least one sample from each of the five species tested, that is, the Texas salamander (1 positive out of 16 individuals tested; 1/16), the Blanco River Springs salamander (1/20), the San Marcos salamander (1/17), the Barton Springs salamander (1/7), and the Jollyville Plateau salamander (6/14) (Table 6.1). For the first four species, only one individual was found positive for *B. dendrobatidis*, and thus this fungus could not be detected in individuals from all sites (Table 6.1). In contrast, *B. dendrobatidis* was found on Jollyville salamanders at all three sites sampled (Table 6.1), with a total rate of detection of 43% which, however, did not match the 100% obtained in

the previous study using toe clippings (O'Donnell et al. 2006). Only one site (Stillhouse Hollow, Austin) resulted in more than one positive detection from any locality (3 out of 6 samples). This site is considerably more impacted by urban development than other sites (Bowles et al. 2006, O'Donnell et al. 2006). Spinal deformities and reduced population size have been reported at this site and been attributed to poor water quality (i.e., high nitrate levels) (O'Donnell et al. 2006).

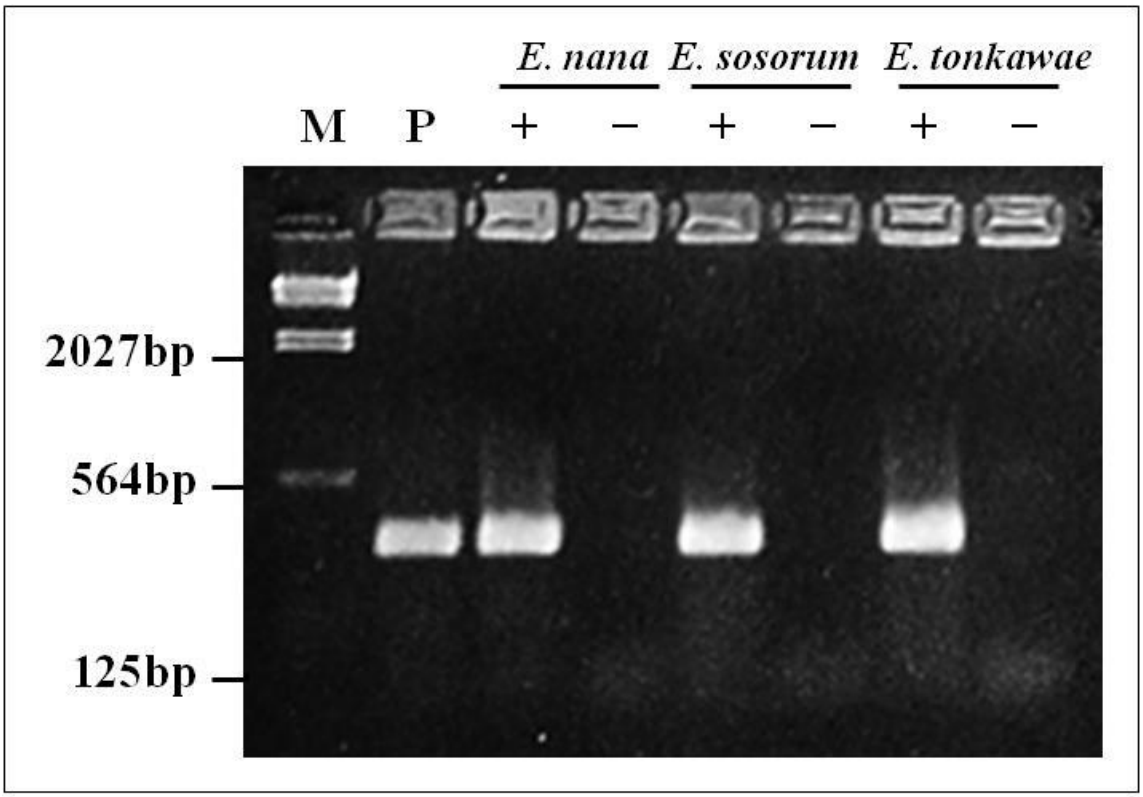

Figure 6.2: Gel picture of PCR product of selected samples. Agarose gel showing typical pattern of fragment detection by PCR using the *Bd* primer set with DNA extracted from tail clips obtained from individuals of *Eurycea nana*, *Eurycea sosorum*, and *Eurycea tonkawae*. Lane M represents a fragment size marker (lambda HindIII), and lane P a positive control.

The high incidence of detection of *B. dendrobatidis* from Jollyville Plateau salamanders at Stillhouse Hollow, Austin, supports the notion that environmental factors

can influence the prevalence and pathogenicity of *B. dendrobatidis* on host organisms. Environmental factors such as temperature and pollutants have been shown to affect the severity of chytidiomycosis (Carey et al. 1999, Davidson et al. 2001, Hopkins & Channing 2002). The differences in severity may be in part due to the effects of those environmental factors on the release and efficiency of antimicrobial peptides (Matutte et al. 2000) which have shown to provide some resistance to *B. dendrobatidis* (Rollins-Smith et al. 2002). These peptides are secreted through glands located in the outer layer of the skin by ranid frogs and some other amphibians, including salamanders (Fredericks & Dankert 2000, Davidson et al. 2003). Salamanders in environments without noticeable stressors might therefore be protected more effectively against infection by *B. dendrobatidis* than salamanders in disturbed or more contaminated environments. This does not mean that *B. dendrobatidis* is absent on salamanders in undisturbed environments since PCR-based detection of *B. dendrobatidis* was achieved on all Jollyville Plateau salamanders independent of the severity of anthropogenic impacts, i.e., on salamanders from sites representing undisturbed rural to disturbed urban areas (O'Donnell et al. 2006).

While nested PCR approaches have been shown to increase sensitivity and to provide sufficient accuracy in diagnostic analyses (e.g., (Arias et al. 1995, Beyer et al. 1995, Llop et al. 2000, Miller & Sterling 2007), all 300-bp-fragments obtained were sequenced using the CEQ 8800 Quickstart Kit with the addition of 5% DMSO to the reaction mix on a CEQ 8800 sequencer (BeckmanCoulter, Fullerton, CA) in order to verify the retrieval of fragments representing *B. dendrobatidis*. These sequences (GenBank accession numbers EU779859 to EU779867, see Fig. 6.3 for details) were first

validated against the GenBank/EMBL databases using BLASTn (Pearson & Lipman 1988). Most sequences of the 300 bp amplicons retrieved by nested PCR were identical to the published sequences of the 5.8S rRNA gene, and to most of the flanking ITS regions of *B. dendrobatidis* (AY997031). However, one sequence (TK01) displayed a one-base-mismatch in the 5.8S rRNA gene and three others insertions of different length at the same position relative to the reference sequence, i.e., a 5-bp insertion in amplicon PT12 obtained from *E. pterophila*, a 6-bp insertion in amplicon TK09 obtained from *E. tonkawae*, and a 1-bp insertion in amplicon TK01 from another individual of *E. tonkawae*. In addition, three sites (postions 23, 25, 161 relative to the reference sequence) show single substitutions (C to T in amplicons SS01 from *E. sosorum* and PT12; A to T in amplicon SS01; and A to G in amplicon TK01, respectively). Obviously, these small sequence differences indicate that they are effectively nearly identical to each other which was not unexpected given the low overall diversity found for widespread sampling of the fungus (Morehouse et al. 2003).

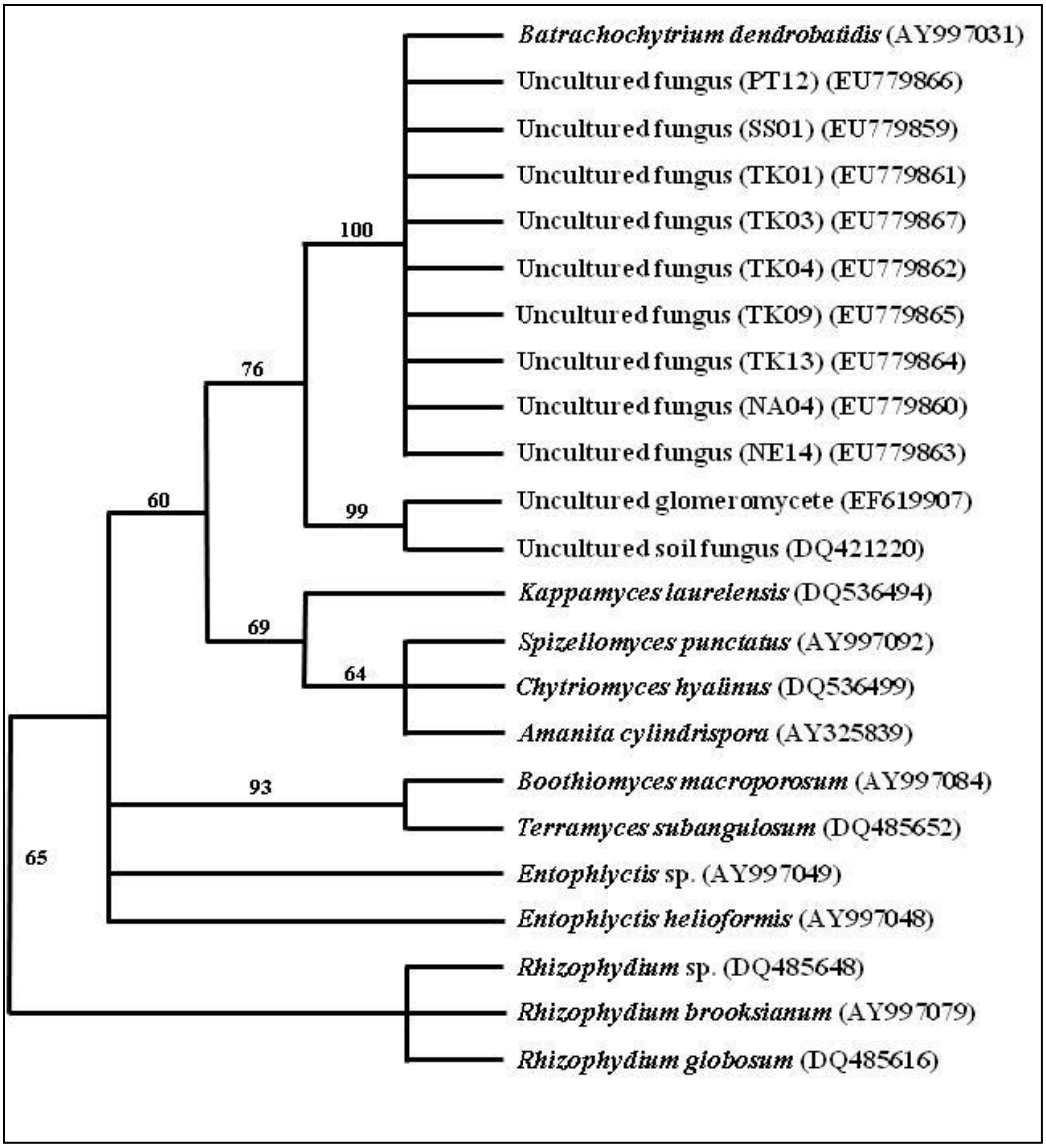

Figure 6.3: Phylogenetic analysis of 5.8s rRNA gene. Maximum likelihood tree topology from 5.8S rRNA gene sequences for amplicons obtained from samples of *E. tonkawae* (acronyms TK), *E. pterophila* (PT), *E. neotenes* (NE), *E. nana* (NA), *E. sosorum* (SS) and closely related fungi including *B. dendrobatidis* (AY997031). Numbers reflect bootstrap support (BS) measures generated in PAUP* 4.0b and only include those measures over 60%. The outgroup consists of different *Rhizophydium* species.

While not strictly required given the very high sequence identity recovered, sequences representing related fungal species of the genera *Rhizophydium*, *Boothiomyces*, *Terramyces*, *Entophylyctis*, *Kappamyces*, *Spizellomyces*, *Chytriomyces*, and *Amanita*, as

well as two uncultured soil fungi high sequence similarity (Fig. 6.3) were analyzed phylogenetically. A maximum likelihood bootstrap with rate heterogeneity, invariant sites, and empirical base frequencies that was applied to clarify monophyly of the novel chytrid sequences using the RaxML online servers (Stamatakis et al., 2008) recovers this group as predictably monophyletic with the 5.8S rRNA gene sequence of the reference organism *B. dendrobatidis* (AY997031). Likewise a parsimony bootstrap (2500 iterations) with identical sequences removed provides very strong support (99/100) for the clade with the novel chytrid sequences and that of the available reference (Fig. 6.3). Similar analyses of the ITS sequences alone did not provide any supported relationships among the samples. Notably the addition of the sequences recovered here does provide documentation of sequence variation for the ITS region in this fungus.

These results demonstrate that *B. dendrobatidis* can be detected in samples from tail clippings of asymptomatic endemic salamanders within a small geographic range in Central Texas. These samples were not originally meant to be used for the analysis of *B. dendrobatidis* which is usually analyzed from swabs of the feet or ventral sides or from whole toe clips (Hyatt et al. 2007). While our results provide evidence for the presence of *B. dendrobatidis* in all salamander species tested, statements on sensitivity and accuracy of detection of *B. dendrobatidis* on tail clippings, e.g., with respect to the detection of true negative and positive signals will require additional analyses and direct comparison to detection on toe *versus* tail clips from the same animal. As such toe clips remain the sample of choice for future analyses of *B. dendrobatidis* on amphibians, but alternative tissues sources may provide an opportunity for use of historical tissues in the examination of the past prevalence where available.

The lack of symptoms of chytridiomycosis in these salamanders is fortunate because many are threatened or endangered and already face small population sizes and home ranges. It will require subsequent monitoring and, if available, comparison with a temporal breadth of samples to enable determination that the taxa remain asymptomatic carriers. Additional quantitative analyses of salamanders and of other amphibians need to be conducted in order to assess the extent of infection with *B. dendrobatidis* and examine the potential threat the presence of *B. dendrobatidis* might cause on amphibian populations in Central Texas.

Acknowledgments

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CHAPTER 7

SEASONAL VARIATION IN THE DETECTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN A TEXAS POPULATION OF BLANCHARD'S CRICKET FROG (*ACRIS CREPITANS BLANCHARDI*)[1]

Abstract

A population of Blanchard's cricket frogs (*Acris crepitans blanchardi*) located at the headwaters of Barton Creek near Dripping Springs, Texas, was tested for *Batrachochytrium dendrobatidis* in May and July, 2008. Climatic conditions of the 30 days preceding each sampling effort differed in mean temperature and total precipitation with 21.6 ± 0.6ºC and 57.1 mm for May 18, and 27.6 ± 0.4ºC and 18.8 mm for July 18, respectively. In May, nested PCR detected *B. dendrobatidis* on 83% of the samples collected from 30 individuals while none of the samples from 27 individuals collected in July tested positive for *B. dendrobatidis*. The July samples included one recapture that had tested positive for *B. dendrobatidis* in May. These results confirm seasonal variation of infection by *B. dendrobatidis* and suggest that environmental conditions such as temperature or water availability might impact infection on anurans in the wild.

[1] Authors: James P. Gaertner, Michele A. Gaston, Dana Spontak, Michael R.J. Forstner *and* Dittmar Hahn. Publication: Herpetological Review, 40(2):184-187 (2009).

Seasonal variation in the detection of *Batrachochytrium dendrobatidis* in a Texas population of Blanchard's cricket frog (*Acris crepitans blanchardi*)

Blanchard's cricket frog (*Acris crepitans blanchardi*), is a small anuran with a broad distribution in North America (Conant & Collins 1998), and recently has been suggested for elevation to full species (Gamble et al. 2008). Cricket frog populations are in decline throughout much of the northern portion of their range, although causes for these declines are unclear at present (Lannoo 2005). However, *A. c. blanchardi* is often the most abundant and conspicuous member of anuran breeding assemblages throughout much of the year in eastern and central Texas. Central Texas supports a particularly robust population, with cricket frogs found in virtually any natural or artificial aquatic environment, including ephemeral streams and pools, spring outflows, stock tanks, roadside ditches, and along riverbanks. At the eastern edge of the Edward's plateau, *A. c. blanchardi* is sympatric with a number of endangered, threatened, or endemic amphibians including the Houston Toad (*Bufo* [*Anaxyrus*]} *houstonensis*) (Hillis et al. 1984) and several species of plethodontid salamanders (*Eurycea nana, E. neotenes, E. pterophila, E. rathbuni, E. sosorum*) (Chippindale et al. 2000) each with increasing conservation concern in this rapidly developing region.

Amphibian populations are declining at an alarming rate worldwide (Houlahan et al. 2000, Stuart et al. 2004), and infection by *Batrachochytrium dendrobatidis* (*Bd*) is frequently implicated as a factor in documented cases of population decline (Daszak et al. 1999, Daszak et al. 2000). *Bd* seems to be widespread in North America (Ouellet et al. 2005), and has been detected in both captive and wild populations of *A. c. blanchardi* in several states in the midwestern United States (Steiner & Lehtinen 2008, Zippel &

Tabaka 2008). Detection of *Bd* on individuals of *A. c. blanchardi* was not restricted to regions with declining populations, and also not accompanied by any mortality or signs of chytridiomycosis (Steiner and Lehtinen 2008). Previous studies indicate that *Bd* can have variable effects on amphibian populations, with potential impacts ranging from no mortality to mass die-offs without recovery as the most extreme effects (Daszak et al. 1999, Daszak et al. 2003). The susceptibility of amphibians to *Bd*, however, can vary across species, developmental stage, and temperature regimes (Lamirande & Nichols 2002, Davidson et al. 2003, Berger et al. 2004, Daszak et al. 2004). In addition, seasonal variation in prevalence and severity of infection by *Bd* within individual populations has been noted (Bradley et al. 2002, Woodhams & Alford 2005, Kriger & Hero 2007), suggesting effects of environmental conditions on the severity of infection.

The purpose of this study was to determine the rate of infection by *Bd* within a population of *A. c. blanchardi* at the headwaters of Barton Creek, located approximately 11 km west of Dripping Springs and 30 km west of Austin, Texas (30.2248611°N, 98.1687306°W), adjacent to habitat for several endemic and endangered salamanders (Sweet 1982, Chippindale et al. 2000). We conducted a second sampling and subsequent analysis of frogs captured later in the season at the same site during times with higher mean temperature and lower water availability and we examined seasonality of the infection across a relatively narrow time scale.

Frog sampling was conducted on 18 May and on 18 July 2008. Weather data collected for the 30 days preceding sampling differed significantly between sampling events, with a mean temperature of 21.6 ± 0.6ºC and total precipitation of 57.1 mm for the May sampling date, and a mean temperature of 27.6 ± 0.4ºC and total precipitation of

18.8 mm for the July sampling date (Fig. 7.1). In May, the creek was flowing and several pools of considerable size (10 m^2 to 50 m^2) provided habitat for large numbers of cricket frogs. In July, sampling sites along the creek were virtually dry, with only a few predominantly mud-filled patches of habitat remaining. Frogs were much less abundant than in May, and two weeks after the July sampling frogs were completely absent from the sample site.

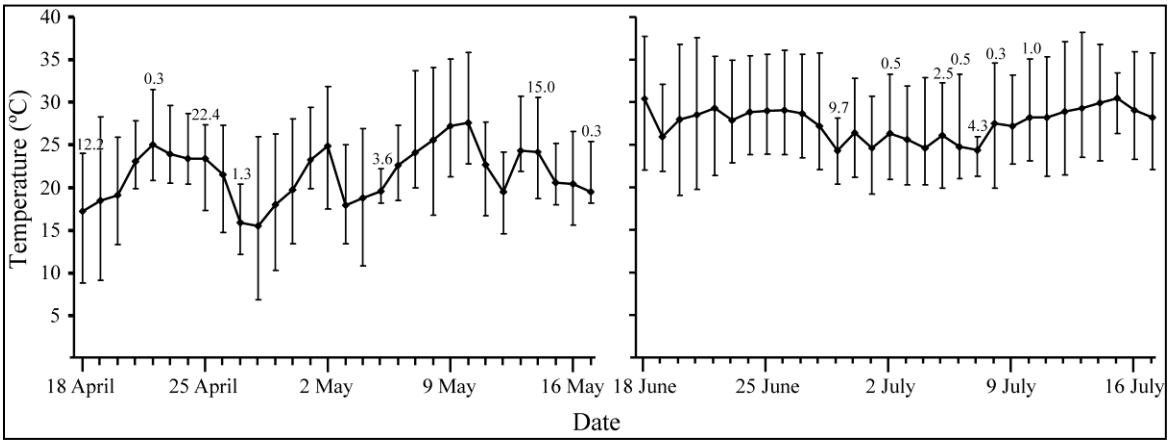

Figure 7.1: Climatic data collected for study. Average temperature for each day with error bars showing the minimum and maximum daily temperatures for the 30 days preceding each sampling event (18 May and 18 July 2008) at the headwaters of Barton Creek near Dripping Springs, Texas, USA. Numbers adjacent to error bars depict total daily precipitation in millimeters.

For the May sampling, which was part of a larger population genetics study of *Acris*, 30 frogs were captured by hand and uniquely marked using a series of toe clips (Hero 1989). These toes as well as toes from 27 individuals obtained in July were retained and stored in 95% EtOH until used for DNA extraction. DNA extracts were tested for the presence of *Bd* using a nested PCR approach that has shown high sensitivity of detection and is outlined in detail in (Gaertner et al. 2008). This procedure uses primers ITS1f (5'-CTT GGT CAT TTA GAGC GAA GTA) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC) to amplify the 5.8S rRNA gene and the flanking internal

transcribed spacers (ITS) within conserved regions of the 28S and 18S rRNA genes of all fungi (White et al. 1990). A portion of this reaction was then used as a template in a subsequent PCR using primer set Bd1a (5'-CAG TGT GCC ATA TGT CAC G) and Bd2a (5'-CAT GGT TCA TAT CTG TCC AG) (Annis et al. 2004) which amplified fragments of about 300 bp that included the 5.8S rRNA and flanking ITS regions of *Bd* (Annis et al. 2004). A *Bd*-positive DNA sample from a previous study (Gaertner et al. 2008) and sterilized distilled water were used as positive and negative controls in all reactions, respectively.

Agarose gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989) of the nested PCR revealed that 83% of the samples obtained in May were positive for *Bd*. None of the samples collected in July, however, tested positive for *Bd* even though the positive controls amplified. Individuals collected in July included one recapture (AD03, marked 3 using the method of Hero 1989) that tested positive for *Bd* in May. The clipped toe was almost fully healed and showed no evidence of regrowth. Additional toes were removed for the second *Bd* test, resulting in a new unique mark for this individual (8093; Hero 1989). In order to verify the detection of *Bd*, six randomly selected 300-bp-fragments were sequenced using the CEQ 8800 Quickstart Kit (5% DMSO added to the reaction mix) on a CEQ 8800 sequencer (Beckman Coulter, Fullerton, CA). These sequences (GenBank accession numbers FJ373880 to FJ373885) were validated against the GenBank/EMBL databases using BLASTn (Pearson & Lipman 1988) and were identical to the published sequence of the 5.8S rRNA gene, and virtually identical to the published flanking ITS regions of *Bd* (AY997031). These data demonstrate *Bd* infection of large numbers of individuals of *A. c. blanchardi* in late spring (May), and absence of

infection in the same population including a previously positive recapture in summer (July). Since sampling times differed with respect to basic environmental conditions (temperature and water availability), the data suggest seasonal effects of environmental conditions on the infection of *A. c. blanchardi* by *Bd.*

Seasonal variation in the prevalence of disease is not uncommon, as documented for several diseases of both humans and wildlife (Hosseini et al. 2004, Pascual & Dobson 2005, Kriger & Hero 2007). For *Bd*, significantly higher detection rates have been reported in winter than in summer (Retallick et al. 2004, McDonald et al. 2005, Ouellet et al. 2005). Kriger and Hero (Kriger & Hero 2007) detected *Bd* infection in a population of stony creek frogs (*Litoria wilcoxii*) in up to 60% of samples collected in winter and spring, but at much lower rate (0 – 13%) in summer for the same population. As in our study, *L. wilcoxii* that were positive for *Bd* initially were shown to be negative for this pathogen upon subsequent recapture (Kriger & Hero 2006).

The effects of temperature on *Bd* are well documented in laboratory studies. *Bd* can grow *in vitro* in the lab from 6 - 28ºC, with an optimum at 23ºC (Longcore et al. 1999). Frogs in laboratory studies have shown large differences in susceptibility to the fungus as a function of temperature, with the highest mortalities occurring at 23ºC and with decreasing pathogenicity at higher temperatures (Lamirande & Nichols 2002, Berger et al. 2004). Infected frogs could be cured of *Bd* in the laboratory at temperatures above 32ºC (Johnson et al. 2003, Woodhams et al. 2003). The laboratory results suggest that the disappearance of *Bd* from the *A. c. blanchardi* population in the headwaters of Barton Creek during the season could very well be a function of increasing temperatures. However, currently we have no direct evidence for a causal relationship between the

disappearance of *Bd* and higher temperature. Other seasonally variable environmental conditions such as microhabitat temperature differences, relative humidity, and precipitation/water availability are closely related to temperature, and synergistic effects of these factors are difficult to quantify.

Although it is well known that *Bd* is spread via aquatic zoospores and prefers moist environments (Longcore et al. 1999, Ron 2005), a significant inverse relationship between precipitation and prevalence of *Bd* has been found (Kriger & Hero 2007). These authors acknowledge that this finding is counterintuitive to the life history of this pathogen, and suggest that this negative relationship may be an artifact of seasonal weather patterns in southeastern Queensland, Australia (warm wet summers, cool dry winters), with a positive relationship between precipitation and *Bd* infection being more the global norm. Our results support the latter hypothesis, in that *Bd* detection was 0% in the samples collected during drier conditions.

The overall pattern in *Bd* detection with respect to temperature is the same in our study as has been found in previous work in wild amphibian populations (e.g., (Kriger & Hero 2007) decreasing prevalence of infection with increasing temperature). However, the mean 30-day temperature for our May sampling date (21.6ºC) was within the range of highest 30-day mean temperatures recorded in Kriger and Hero (Kriger & Hero 2007). These authors found that *Bd* prevalence ranged from 0 - 13.3% when temperatures ranged from 19.5 - 22.2ºC, a temperature range that our data suggest could be optimal for infection in central Texas. This lack of concordance emphasizes the need for studies that carefully consider multiple environmental variables in examining seasonal variation in *Bd* prevalence within populations.

Our findings indicate that high temperature, drying of aquatic habitats or an interaction between these factors may reduce *Bd* prevalence in semi-aquatic amphibians to very low levels in ecosystems characterized by hot, dry summers and/or ephemeral water sources. The diurnally active *A. c. blanchardi* sampled in this study may have been exposed to temperatures in excess of 30ºC (Fig. 7.1), approaching the thermal range known to cure *Bd* in laboratory studies (Johnson et al. 2003). This hypothesis might explain the decline of populations of *A. c. blanchardi* in the northern parts of the range where populations experience temperatures that are amenable to *Bd* for a much larger part of the year than further south where infections may be regularly cleared by higher temperatures and the lack of water which have both been suggested to limit the distribution of *Bd* (Skerratt et al. 2008).

Further research is necessary to determine whether the sharp declines in *Bd* prevalence observed in cricket frogs are mirrored in the entirely aquatic plethodontid salamanders that occur in the region, and thereby the utility of these frogs as a proxy or indicator of *Bd* in central Texas aquatic ecosystems.

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and Wildlife Department (TPWD, permit SPR-1005-1515 and SPR-0890-234). We thank Alicia Diehl for allowing us access to the sampling location.

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CHAPTER 8

ANNUAL VARIATION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE HOUSTON TOAD (*BUFO HOUSTONENSIS*) AND A SYMPATRIC CONGENER (*BUFO NEBULIFER*)[1]

Abstract

The Houston toad (*Bufo* [= *Anaxyrus*] *houstonensis*) is endemic to south-central Texas with suitable habitat currently occupied in seven counties only (Forstner & Dixon 2010). Population sizes are in steady decline, and the populations in Bastrop County are considered the most robust and sustainable of the remaining populations (Seal 1994), with current estimates of less than 500 adult individuals (M.R.J. Forstner, unpublished). The Houston toad was listed as endangered in 1970, and has been referred to as "probably the rarest and most endangered amphibian in the United States" (USFWS 1978). Factors such as habitat alteration (Alford & Richards 1999), global environmental change (Gibbs & Breisch 2001), environmental contaminants (Sparling et al. 2001), introduced species (Kiesecker & Blaustein 1997), emerging infectious diseases (Jancovich et al. 1997, Berger & Speare 1998, Blaustein & Kiesecker 2002) or combinations thereof have been linked to declines of amphibians globally, and are likely

[1]James P. Gaertner, Diana McHenry, Michael R. J. Forstner, and Dittmar Hahn. Submitted: Herpetplogical Review (2010).

primary threats to the Houston toad as well (Peterson et al. 2004). While habitat destruction and alteration, predation, interspecific competition and hybridization, contamination by herbicides, pesticides and fertilizers and prolonged drought have been identified as potential threats (Seal 1994), published information on infectious diseases in this endangered taxon is still lacking.

Annual variation of *Batrachochytrium dendrobatidis* in the Houston toad (*Bufo houstonensis*) and a sympatric congener (*Bufo nebulifer*)

Chytridiomycosis is a recently emerged infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). It has been implicated as a significant contributor to amphibian population declines worldwide (Berger et al. 1998, Garner et al. 2005, Ouellet et al. 2005). The presence of *Bd* has been confirmed in many states of the USA (Davidson et al. 2003, Ouellet et al. 2005, Rothermel et al. 2008) including south-central Texas near Bastrop County where it was detected in several endemic salamander species (*Eurycea nana*, *E. neotenes*, *E. pterophila*, *E. sosorum*, and *E. tonkawae*) and in cricket frogs (*Acris crepitans blanchardi*) (Gaertner et al. 2009a, Gaertner et al. 2009b).

The aim of this study was to expand the previous research on *Bd* in south-central Texas in order to assess the potential threat that the presence of *Bd* might pose to Houston toads. These studies took advantage of existing DNAs from toes of *B. houstonensis* and a sympatric toad, *Bufo nebulifer* (coastal plain toad) originally sampled for a long-term conservation study on *B. houstonensis* in Bastrop County and available from the M.R.J. Forstner Frozen Tissue Catalogue held at Texas State University-San Marcos. Toads were sampled opportunistically and via drift fences around small ephemeral and semi-

permanent reservoirs on the Griffith League Ranch, Bastrop State Park, and several other sites in Bastrop County, Texas. In Bastrop County (Fig. 8.1), Houston Toad habitat is characterized by patches of loblolly pine and mixed deciduous woodland fragmented by open, grassy areas and agriculture, on deep sandy soils.

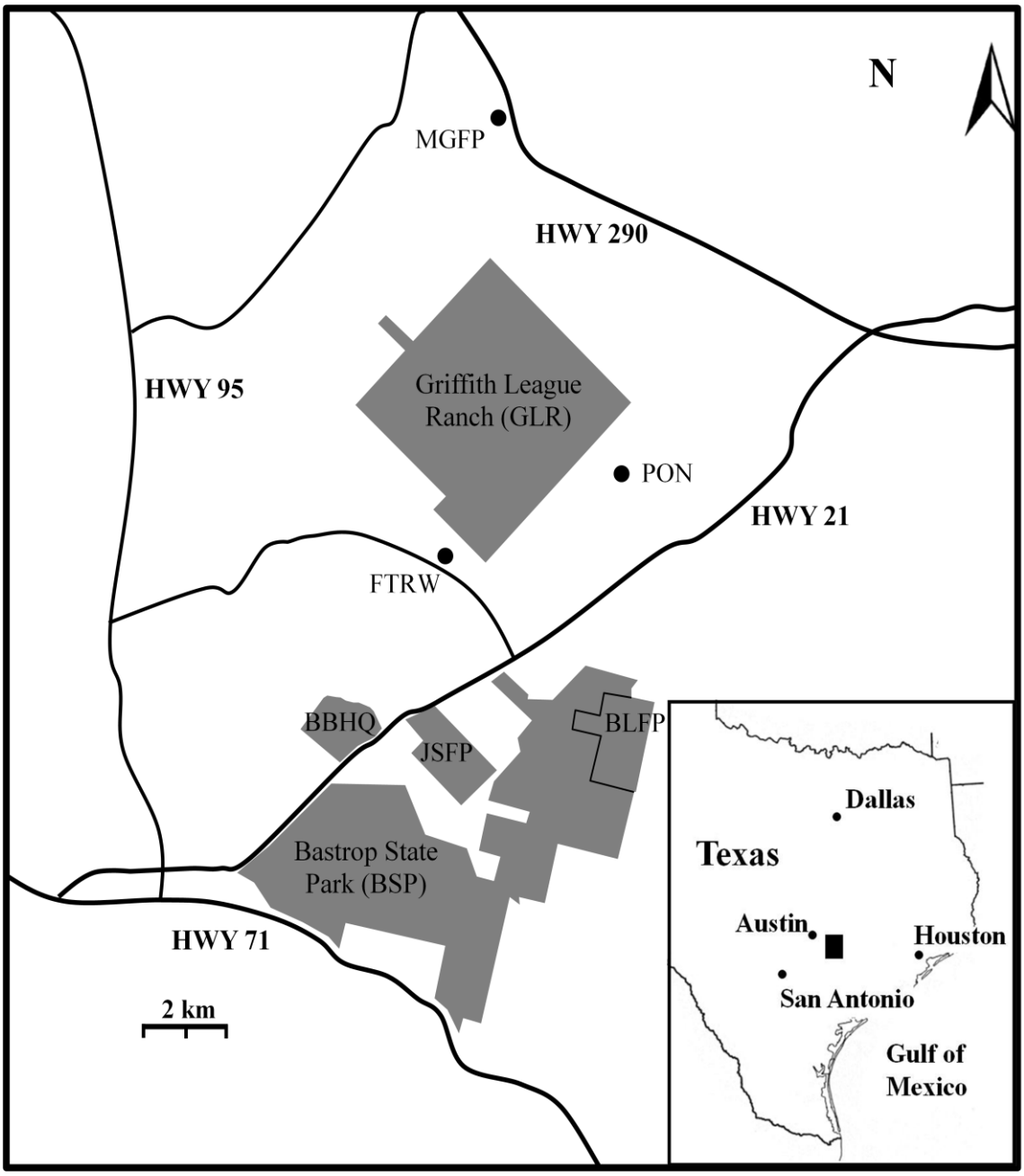

Figure 8.1: Schematic presentation of the sampling sites within Bastrop County, Texas. Sampling sites used for the collection of toads from the species *Bufo*

houstonensis and *B. nebulifer* to be tested for infection by *Batrachochytrium dendrobatidis*. Shaded areas and filled circles represent sampling sites MGFP, Musgrave family property; PON, Ponderosa Drive; FTWR Firetower Road; BBHQ, Bluebonet Electric headquarters; JSFP, Jim Small family property; and Bob Long family property.

Toes were collected between 2001 and 2007 and stored in 95% ethanol at -80^{0}C: 105 *B. houstonensis* (2001, N = 8; 2002, N = 24; 2003, N = 16; 2004, N = 2; 2005, N = 7; 2006, N = 24; 2007, N = 24) and of 96 *B. nebulifer* (2001, N = 21; 2002, N = 3; 2003, N = 6; 2004, N = 18; 2006, N = 24; 2007, N = 24) (Table 8.1). DNA was isolated using a Wizard® SV 96 Genomic DNA Purification System (Promega) on a Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter), a DNeasy® Tissue Kit (QIAGEN Inc.), or a standard phenol-chloroform method (Sambrook et al. 1989). We tested for *Bd* using a nested PCR approach (Gaertner et al. 2009). The nested PCR used primers ITS1f and ITS4 targeting conserved regions of the 28S and 18S rRNA to amplify the 5.8S rRNA gene and the flanking internal transcribed spacer (ITS) of all fungi (White et al. 1990). Purified PCR products were then used as template for the second PCR reaction using the primer set Bd1a and Bd2a which is specific for *Bd* (Annis et al. 2004). Reactions were then examined for the presence of a 300-bp fragment (Annis et al. 2004) using gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989).

Of the 201 specimen tested, a total of 29 (14.4%) were positive for the 300-bp fragment, indicating the presence of *Bd* (Table 8.1). Six of these were from *B. houstonensis* (out of a total of 105) and 23 from *B. nebulifer* (out of 96). More than 80% of these *Bd*-positive samples were obtained in year 2006 with a total of 24 positive detections. Samples obtained in 2003 (23 tested) and 2005 (7 tested) were all *Bd*-negative, while years 2001 (2/29 or 6.9%), 2002 (1/28 or 3.6%), 2004 (1/20 or 5.0%), 2006 (24/48 or 50%) and 2007 (1/48 or 2.1%) had positive samples though mostly at low

percentages (Fig. 8.2, Table 8.1). All amplicons of the appropriate size were purified, and then sequenced at the DNA Sequencing Facility of the Institute for Cellular and Molecular Biology at the University of Texas at Austin, TX. Sequences were compared to GenBank/EMBL databases and aligned using BLAST (Pearson & Lipman 1988) and alignment functions in Geneious 4.8.3 (Drummond et al. 2009). Sequence diversity was limited. Two haplotypes were discovered that shared more than 99% similarity to sequences in the databases representing *Bd*. Haplotype A (GenBank Accession HM153084) was detected in toads sampled in 2001, 2002, 2004 and 2006 and in both *B. houstonensis* and *B. nebulifer*. Haplotype B (HM153085) was not detected in samples collected prior to 2006 and was detected only in *B. nebulifer* (Table 8.1). These results demonstrate the presence of *Bd* in populations of *B. houstonensis* and *B. nebulifer* as early as 2001 and within federally designated Critical Habitat of the Houston toad. Any further statements about the significance of this observation and consequences for current and future conservation efforts for *B. houstonensis* specifically remain speculative, and require additional studies.

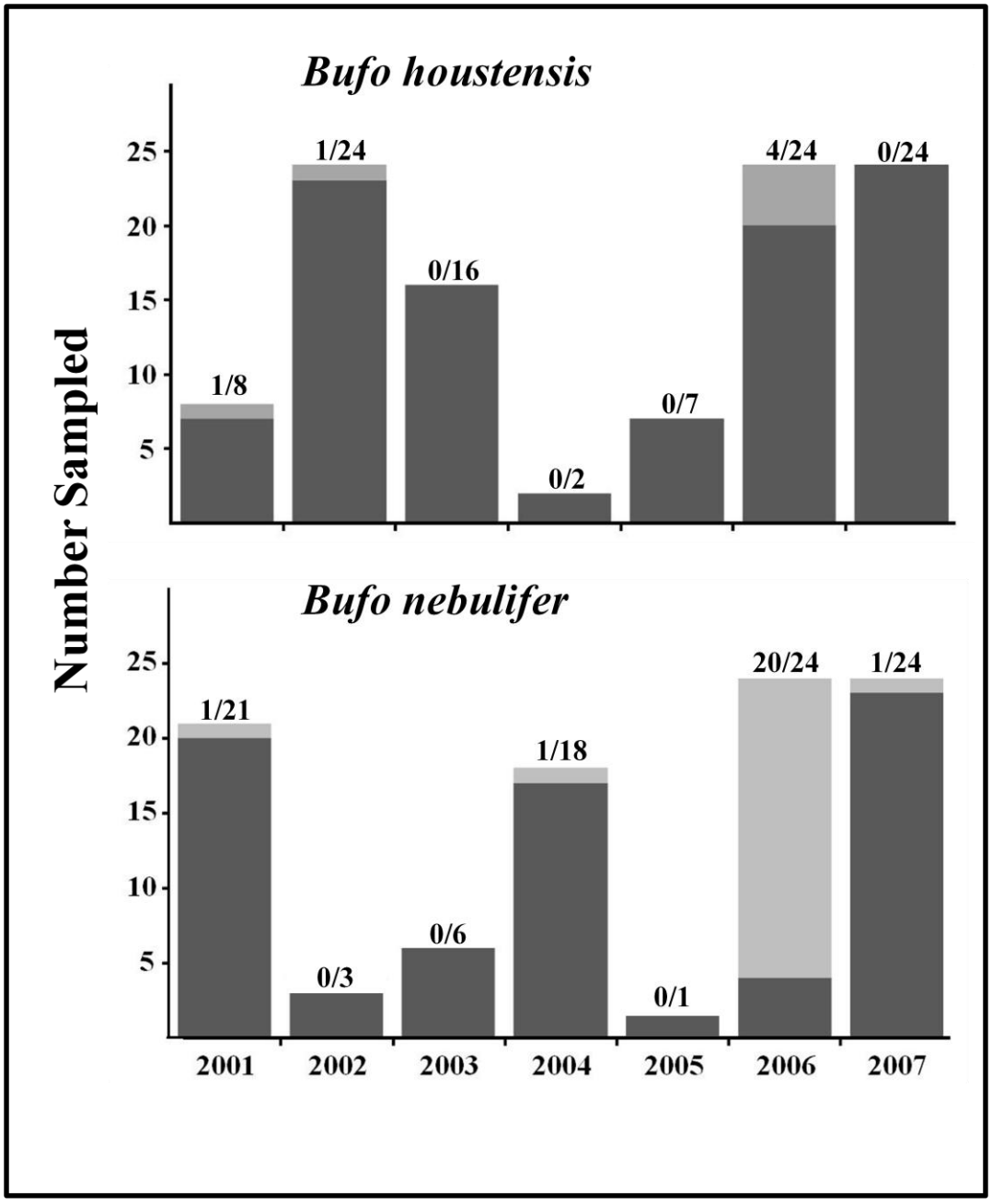

Figure 8.2: Prevalence of *Bd* in years tested. Detection of *Batrachochytrium dendrobatidis* in samples taken from *Bufo houstonensis* (n=105) and *B. nebulifer* (n=97) during the 2001 to 2007 breeding season. Bars show the number of negative samples (lower dark portion) and number of positive samples (top lighter portion) for each year.

Table 8.1: Prevalence of *Batrachochytrium dendrobatidis* in *Bufo houstonensis* and *B. nebulifer* at localities in Bastrop County, Texas, from 2001 to 2007.

Species by year	Locality[1]	Prevalence (infected/total)	Fungal haplotype (number infected)
2001			
B. houstonensis	BAN02p	1/5	A (1)
	BAN04p	0/1	
	BAN15t	0/1	
	BAN18t	0/1	
B. nebulifer	BAN02p	0/7	
	BAN12t	1/8	A (1)
	BAN15t	0/2	
	BAN19t	0/2	
	BAN34t	0/1	
	BAN35t	0/1	
	GLR	0/1	
2002			
B. houstonensis	BAN02p	1/17	A (1)
	BAN12t	0/1	
	BAN14t	0/1	
	BAN19t	0/2	
	GLR	0/3	
B. nebulifer	BAN02p	0/2	
	GLR	0/1	
2003			
B. houstonensis	BAN01p	0/1	
	BAN12t	0/1	
	BAN13t	0/1	
	BAN21t	0/1	
	BAN23t	0/1	
	BAN24t	0/1	
	BAN26t	0/1	
	BAS06p	0/3	
	GLR	0/6	
B. nebulifer	BAN40s	0/6	
2004			
B. houstonensis	BAN13t	0/1	
	BAS15p	0/1	
B. nebulifer	BAN02p	0/7	
	BAN04p	0/2	
	BAN14t	1/4	A (1)
	BAN17t	0/1	

	BAN23t	0/1	
	BAN24t	0/1	
	BAN36t	0/1	
	GLR	0/1	
2005			
B. houstonensis	BAS10t	0/1	
	BAS11t	0/3	
	BAS12t	0/2	
	BAS13t	0/1	
B. nebulifer	n/a	0/0	
2006			
B. houstonensis	BAS01p	1/4	A (1)
	BAS07p	1/4	A (1)
	BAS09p	1/8	A (1)
	BAS15p	0/1	
	BAS17p	0/6	
	BAS18p	1/1	A (1)
B. nebulifer	BAN02p	5/5	A (3), B(2)
	BAN09p	2/4	B (2)
	BAPp	3/3	A (1), B (2)
	BAS01p	1/1	B (1)
	BAS09p	9/11	B (9)
2007			
B. houstonensis	BAN29s	0/3	
	BAS04p	0/6	
	BAS07p	0/6	
	BAS08p	0/6	
	BAS18p	0/3	
B. nebulifer	BAN09p	1/3	B (1)
	BAN11p	0/2	
	BAN28p	0/4	
	BAN38p	0/2	
	BAS01p	0/1	
	BAS14p	0/4	
	BAS15p	0/4	
	BAS18p	0/4	

[1]Sites with the prefix BAN (Bastrop Co. 'north', see Forstner & Dixon [2010]) are located 8-11 km W and WSW of the TX21/US290 junction. Site BAPp (Bastrop County 'p12') is about 10 km WSW of TX21/US290 junction. Sites with the prefix BAS (Bastrop Co. 'south') are within a 9 km radius E of TX21/TX95 junction, mostly between TX21 and TX71, with few sites W of TX21.

Detection here of *Bd* in zero or only a few individuals during most years may depend on seasonal and environmental conditions as is the case in other wild amphibians (Kriger & Hero 2006, Gaertner et al. 2009b, Voordouw et al. 2010). Although this situation does not correspond with the commonly viewed perception of disease dynamics, populations of frogs with low numbers of infected individuals have been reported (Voordouw et al. 2010). Populations of *Xenopus* frogs in South Africa, for example, have carried the fungus with a relatively low prevalence (about 2.7%) for as long as 65 years (Weldon et al. 2004). Alternatively, these results could underestimate the presence of *Bd* due to sampling bias as a consequence of low numbers or of preferential predation on infected toads in nature. Assuming a low prevalence of infection, approximately 60 individuals would need to be tested to provide a 95% certainty of detecting *Bd* on at least one animal (Skerratt et al. 2008).

Of particular interest are the results from year 2006. Despite populations from all other years lacking detections or with relatively low numbers of positive samples, 50% of the samples collected in 2006 (n=48) were *Bd*-positive. Differences in the prevalence of *Bd* in anuran populations have been seen frequently but were attributed to seasonal differences in temperature (Retallick et al. 2004, McDonald et al. 2005, Ouellet et al. 2005, Kriger & Hero 2007) or precipitation (Ron 2005, Gaertner et al. 2009b). Weather data retrieved for 2001-2007 breeding seasons (December to June) from the National Climatic Data Center using the average for three sites surrounding Bastrop (Smithville, Station ID 20024578; Cedar Creek, Station ID 20024579 and Elgin, Station ID 20024696) reveal that 2006 was notably hotter and dryer for the period, with an increase in temperature by 1.5^{0}C and a decrease in precipitation by 256 mm from the average for

the period (Table 8.2). The conditions were the inverse necessary for optimal growth of *Bd* (Lamirande & Nichols 2002, Berger et al. 2004), even though a significant negative relationship between the occurrence of *Bd* and precipitation has been reported (Kriger and Hero 2007). That relationship, however, was suggested to be affected more by low temperature conditions than by the concomitant dry conditions. The increase in occurrence of *Bd* during 2006 in our study might therefore not be a consequence of the environmental conditions in this year alone, but may be significantly affected by the environmental conditions in the preceding drought in 2005 with 204 mm below average precipitation. Environmentally stressed toads could be more susceptible to *Bd* which may consequently result in an increase in occurrence of *Bd* the following year. A similar scenario was discussed in one of our previous studies that suggested high occurrence of *Bd* on salamanders at specific sites was caused by environmental stress from pollution originating from urban runoff (Gaertner et al. 2009a). Relief from the drought with precipitation 91 mm above average during year 2007 and thus reduction in environmental stress could then have resulted in a reduction in occurrence of *Bd.* The fluctuation in occurrence of *Bd* on toads, however, could also be a function of sample size and sampling strategy that were originally designed to retrieve information on the genetic diversity of toads. Low sample numbers, but also the inability to conduct repetitive sampling of sites with high numbers of infected toads in consecutive years might therefore have influenced the detection of *Bd* through time.

Table 8.2: Historical climate data for Bastrop County, Texas. Data collected from the National Climatic Data Center. Tables show the departure from average temperature and rainfall for each year's *Bufo houstonensis* breeding season (December through June) 2001 through 2007. Values were obtained by averaging three sites surrounding the town of Bastrop (Smithville, Station ID 20024578; Cedar Creek, Station ID 20024579 and Elgin, Station ID 20024696). Monthly average temperature and precipitation also shown.

Departure from Average Temperature (C)								
	DEC	JAN	FEB	MAR	APR	MAY	JUN	Average
Monthly Average Temperature	10.6	9.4	11.6	15.8	19.8	23.7	27.2	16.87
2001	-2.22	-0.61	2.78	-2.61	1.39	1.33	0.44	0.07
2002	1.50	1.22	-1.83	-1.33	3.11	1.00	0.28	0.56
2003	0.83	-0.28	-0.44	-0.50	1.17	2.72	0.56	0.58
2004	0.50	1.89	-1.56	2.72	-0.22	0.17	-0.56	0.42
2005	-0.17	3.56	1.56	-0.50	-0.78	-0.39	0.94	0.60
2006	-0.50	3.67	-0.50	2.61	3.83	1.33	0.06	1.50
2007	0.89	-1.61	-0.44	2.72	-1.72	0.00	-0.44	-0.09

Departure from Average Rainfall (cm)								
	DEC	JAN	FEB	MAR	APR	MAY	JUN	Cumulative
Monthly Average Rainfall	7.2	6.9	5.9	6.5	7.6	13.0	9.3	56.5
2001	-1.7	2.6	-3.9	8.2	-6.2	4.5	-4.7	-1.3
2002	7.0	-2.8	-3.7	-2.9	-0.6	-10.8	-0.7	-14.6
2003	6.2	-2.9	6.8	-2.2	-7.6	-10.4	-3.3	-13.5
2004	-5.2	2.0	6.0	-2.6	5.3	-7.9	11.4	9.1
2005	-6.6	-0.2	1.1	-5.5	-1.7	-1.6	-5.9	-20.4
2006	-6.7	-6.9	-1.3	-4.9	-1.4	-6.9	2.5	-25.6
2007	1.2	2.3	-5.9	13.0	-0.5	0.1	-0.9	9.3

Data obtained for 2006 showed not only much higher occurrence of *Bd* on toads, but also species-specific differences in occurrence, with a much higher detection in *B. nebulifer* (83% of the samples, 20 out of 24) than in *B. houstonensis* (17%, 4 out of 24). Differences in occurrence of *Bd* in sympatric amphibian species have been observed (Retallick et al. 2004, Peterson et al. 2007, Goka et al. 2009), with few studies linking the occurrence of *Bd* to the disease (Retallick et al. 2004). The increase in occurrence of *Bd* in 2006 was coincident with the identification of a new haplotype (haplotype B) that

differed from the previously identified one (haplotype A) by a 10 bp insertion after base number 64. Haplotype B was identical to the most prominent *Bd* haplotype found in *Eurycea* salamanders from central Texas (GenBank Accession numbers EU779859, EU779862, EU779864, and EU779867) (Gaertner et al. 2009a) suggesting strain-specific differences affect occurrence and potential virulence as demonstrated in other studies (Berger et al. 2005, Retallick & Miera 2007, Goka et al. 2009). Because haplotype B was not found in *B. houstonensis*, but only in *B. nebulifer* so far, differences in host specificity might be present. This may also be evidence of *B. nebulifer* acting as a vector for *Bd* similar to assumptions for the bullfrog (Daszak et al. 2004, Hanselmann et al. 2004) as *B. nebulifer* dispersed into Houston toad habitats during the spring of 2006 as the drought left no breeding ponds in the grassland patches.

In any case, these speculations need additional studies with more samples and a sampling strategy directed towards repeated and seasonal analyses of *Bd* at a site with high occurrence of *Bd*. These studies should include comparative analyses of water and sediment samples for presence and abundance of *Bd* and the respective haplotypes as a function of environmental characteristics during the season.

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CHAPTER 9

SPATIAL AND TEMPORAL ANALYSES OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN POPULATIONS OF *ACRIS CREPITANS BLANCHARDI*[1]

Abstract

Populations of Blanchard's Cricket Frog (*Acris crepitans blanchardi*) were sampled monthly for the period of one year at six ponds in close proximity, and were analyzed for the presence and abundance of *Batrachochytrium dendrobatidis* (*Bd*), using a TaqMan *q*PCR procedure. Water and sediment samples were collected at the same sites as frogs. While both water and sediment samples remained negative for *Bd* at all ponds at all times, *Bd* was detected on 89% of all individuals of *A. crepitans blanchardi* tested (n=572), with no significant differences in infection rate between ponds and during the season. None of the infected individuals, however, showed clinical signs of infection. Seasonal differences were observed for abundance of *Bd* cells on individuals, with up to 2 orders of magnitude higher numbers during spring (March to May with up to 9400 cells per individual) than during the rest of the season. Higher abundance of *Bd*, however, was not related to changes in the body condition index of *A. crepitans blanchardi*. The seasonal pattern in abundance was more pronounced in deeper ponds than in shallow or

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ephemeral ponds and was correlated to the consistently lower nutrient levels in deeper ponds, but also to the 30 day air temperature. This study provides evidence on differences in *Bd* abundance on frogs as a function of environmental conditions in their habitat, but also on seasonal effects with temperature as an environmental driver.

Introduction

Amphibians are experiencing severe and widespread population declines on a global scale (Houlahan et al. 2000, Stuart et al. 2004). While a wide variety of factors are implicated in these declines, the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is recognized worldwide as a major contributor (Daszak et al. 1999, Speare & Berger 2000, Gewin 2008). However, despite the contribution of *Bd* to declines (Ron & Merino 2000, Beebee & Griffiths 2005) or even local extirpation of some populations of amphibians (Daszak et al. 2003, Ron et al. 2003, Burrowes et al. 2004), other populations are left seemingly unaffected by *Bd* (Retallick et al. 2004, Kriger & Hero 2006). As a consequence, infection by *Bd* and the severeness of disease (chytridiomycosis) seem to be influenced by complex interactions between the host, the pathogen and local environmental conditions (Pounds et al. 2006, Kriger & Hero 2007b, Gaertner et al. 2008).

Many of studies examining the link between environmental factors and the presence and pathogenicity of *Bd* on amphibians deal with climatic or species effects. Despite evidence that *Bd* can infect a wide range of amphibians (Speare & Berger 2000), obvious differences in the susceptibility of species to chytridiomycosis have been noted in the laboratory (Blaustein et al. 2004, Daszak et al. 2004) and in wild populations (Lips 1999, Carey 2000, Retallick et al. 2004). Furthermore, in contrast to the pathogen's

ability to persist under a wide variety of climatic conditions, under controlled conditions temperature strongly effects the outcome of infection (Berger et al. 2004). The severity of chytridiomycosis has been linked to seasonal differences in climate (Berger et al. 2004, Kriger & Hero 2007b) as well as to long-term climatic changes (Pounds et al. 2006, Bosch et al. 2007). Differences in susceptibility to *Bd* could be the result of variations in the amphibian species' innate abilities to combat infections (Rollins-Smith & Conlon 2005), weather conditions that favor the pathogen (Berger et al. 2004, Pounds et al. 2006) or environmental stressors having direct effects on the immune system of individuals (Carey 1993, Reading 2007). Despite the fact that amphibians are particularly vulnerable to pollutants due to their highly permeable skin and the close contact most species have with water throughout their lives, only few studies have considered the potential for environmental contaminants, such as fertilizers or pesticides, to increase the susceptibility of amphibians to *Bd* (Parris & Baud 2004, Gaertner et al. 2008).

The purpose of this study was to assess the effects of different environmental factors on the presence and abundance of *Bd* in populations of *Acris crepitans blanchardi*. This frog species was sampled for *Bd* monthly over a 1-year period in 6 ponds with different environmental characteristics located in the same geographical area. Presence and abundance of *Bd* was analyzed by quantitative PCR (*q*PCR) and related to a comprehensive set of environmental data characterizing individual ponds.

Materials and Methods

Sampling sites

All sampling sites were chosen within Bastrop County, TX on six ephemeral to semi-permanent ponds on the Griffith League Ranch and the adjacent Welsh property. Habitat in the area consists of deep sandy soils, patches of loblolly pines and mixed deciduous woodlands broken with open grassy areas and land cleared for agriculture. The ponds were chosen for their close proximity to each other (with a maximum distance of about 3 km) and due to their unique characteristics driven primarily by use in cattle ranching operations (ponds 1 and 2), excess use by feral hogs (ponds 3 and 6), or relatively un-impacted by these factors (ponds 4 and 5). Pond size was quantified using a long tape and area estimation based on equivalent geometric shapes (e.g., circles, rectangles), or using a portable GPS device (Map60, Garmin, Olathe, KS) and Geographic Information System (GIS) software (ArcGIS 9.2, ESRI, Redlands, CA). Percent canopy cover was estimated using a concave spherical densitometer (Forestry Suppliers Inc., Jackson, MS). The percent canopy cover was recorded at two to six randomly selected points along the pond edge and averaged. Water depth was recorded at permanent staff gauges located at the deepest point in each pond. Pond size and percent canopy cover were re-estimated using regression equations derived from these and additional monitoring data. The estimates were calculated based on pond depth, which drastically reduced the influence of measurement error in any given sampling period.

Sampling for Bd analyses

Ponds were sampled once monthly for 12 months starting in February of 2009. However because of drought and lack of availability of frogs during the final four months (October, 2009 – January 2010), these data were not considered in analyses. Whenever possible, at least 20 individuals of *A. crepitans blanchardi* were collected by hand or net at each of the six ponds. Frogs were weighed and the snout vent length (SVL) was determined using a digital caliper. The mass and SNV were used to calculate the body condition index (mass [g] / SVL $[cm]^3$) (Jehle & Hodl 1998). A sterile cotton swab was run ten times over each of the four feet, the medial and ventral area of the rear legs, on each of the sides from groin to armpit, and on the ventral surface of the body of each individual (Kriger et al. 2006). Swabs were placed in sterile, 2 ml cryotubes kept at 4°C until returning to the lab, and stored at -80°C until further processing. At each sampling location and time, two water and sediment samples were also collected. Water samples were collected in 50 ml tubes and stored at 4°C. Upon returning to the lab, tubes were centrifuged at 3,000 x g for 15 minutes to pellet cells and the supernatants were discarded. The pellets were re-dissolved and washed in 500 µl sterile water and transferred to 1.5 ml microcentrifuge tubes. The cells were again pelleted by centrifuging at 14,000 x g for one minute and excess water removed before storage at -80°C. Approximately 500 mg of sediment (wet weight) was obtained from the shore line in a 2 ml cryotube and stored at 4°C until returning to the lab. Samples were centrifuged at 14,000 x g for 1 minute and excess water removed before storage at -80°C.

Analysis for Bd

DNA was extracted from swabs using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI) following the protocol for extraction from animal tissue. From water and soil samples, DNA was extracted with the SurePrep™ Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) using a modified lysis procedure, which increases total yield of DNA (Dhabekar et al. 2010). Amphibian and environmental samples were tested for the presence of *Bd* following a TaqMan quantitative real time PCR (*q*PCR) assay using the Chytr MGB2 probe ($^{5'}$6FAM CGAGTCGAACAAAAT - MGBNFQ) in conjunction with the primers ITS1-3 Chytr ($^{5'}$CCTTGATATAATACAGTGTGCCATATGTC) and 5.8s Chytr ($^{5'}$AGCCAAGAGATCCGTTGTCAAA) as described by (Boyle et al. 2004). This assay had been shown to be specific with a detection limit of ≤ 0.1 genomic units (Boyle et al. 2004). Quantitative PCR thermocycler (Eppendorf *realplex*^{2} Hauppauge, New York) conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute (Boyle et al. 2004). Test samples were run in triplicate 20 µl reactions containing 10 µl *q*PCR FastMix™ (Quanta Biosciences, Gaithersburg, MD), PCR primers ITS1-3 Chytr and 5.8s Chytr at 900 nM, the Chytr MGB2 probe at 250 nM, and 1 µl of template DNA. Quantification of samples was based on a standard curve created using a serial dilution of a standardized DNA control with known copy number provided by CSIRO Livestock Industries, Geelong, Australia. One µl of sterile water, run in triplicate, served as a negative control for each reaction.

Sampling for environmental analyses

Water and sediment samples were obtained from two, initially randomly selected, points at each pond, with the sampling locations held constant among months. Two water samples were collected within 1 m of the pond edge in 1 L Nalgene® collection bottles; a sediment core (approximately 5 cm deep) was taken at the shore line. Samples were stored at 4°C for a maximum of 24 hours before further processing. The cumulative precipitation and average temperature of the 30 days preceding sampling dates were collected using information downloaded from the National Climatic Data Center. Because no station was available for Bastrop, daily values from the three surrounding stations (Elgin-412820, Smithville-418415 and Cedar Creek-411541) were averaged (Table 9.1).

Table 9.1: Climatic data collected for the study. Average temperature and precipitation for the 30 days preceding sampling events at six ponds in Bastrop County, Texas, USA during the spring and summer of 2009. Values were obtained from the National Climatic Data Center and represent an average of the three closest surrounding stations (Elgin-412820, Smithville-418415 and Cedar Creek-411541).

30 Day Mean Air Temperature (°C) and Precipitation (mm)							
	Feb	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**
Temperature	12.1	15.4	17.1	23.8	25.4	31.2	31.2
Precipitation	21	55	35	82	61	3	42

Environmental analyses

Within 24 hours of collection, pond water was filtered through Paul Gelmann A/E glass-fiber filters (1 µm pore size) (ThermoFisher Scientific, Waltham, MA, USA). Residues on the filters were used to determine chlorophyll *a* (Chl *a*), non-volatile suspended solids (NVSS), and organic matter (OM) concentrations, and the filtrate was analyzed for dissolved organic carbon (DOC), soluble reactive phosphorus (SRP), nitrate

(NO_3^-), and ammonium (NH_4^+). Total phosphorus (TP) and pH were analyzed in unfiltered water samples. Samples were refrigerated or frozen prior to analyses, and water samples were preserved in 250 ml Nalgene® bottles with 188 µl 85% sulfuric acid. pH was obtained using a SympHony 5B70P pH meter (VWR, Weschester, PA, USA). Chl *a* was extracted by adding the filter to 20 ml acetone into a clean film canister and storing at 4°C for four hours. Samples were allowed to adjust to room temperature and were analyzed with a Turner Designs Trilogy fluorometer (Turner Designs Inc, Sunnyvale, CA, USA). For quantification of NVSS and OM in the water column, water was filtered through pre-combusted (450°C for 4 hours) and pre-weighed filters and allowed to dry at 60°C for 48 to 72 hours or until a consistent weight (total suspended solids) was recorded. Filters were then combusted at 550°C for 4 hours, and re-weighed on a Mettler-Toledo MX5 microbalance (Mettler-Toledo Inc., Columbus, OH, USA) to determine NVSS and OM (Heiri et al. 2001). Sediment samples were dried, and subsequently combusted at 550°C for 4 hours to retrieve information on sediment organic matter (SOM).

DOC was analyzed in filtered water, using a Shimadzu TOC-V_{CSH} total organic carbon analyzer (Shimadzu Scientific Instruments, Columbia, MD), which measures combustion products with a non-dispersive infrared gas analyzer. SRP, TP, NO_3^-, and NH_4^+ were analyzed with a Varian Cary 50 Ultraviolet-Visible light spectrophotometer (Varian Inc., Palo Alto, CA, USA) and SRP and TP were measured using the molybdenum blue method (Wetzel & Likens 2000). To estimate TP, samples were digested with potassium persulfate ($K_2S_2O_8$) before quantification. NO_3^- concentrations were determined using second-derivative UV spectroscopy (Crumpton et al. 1992), while

NH_4^+ was quantified using the phenol-hypochlorite method (Wetzel & Likens 2000). Water samples were analyzed in duplicate, and the average of the replicates used for this study.

Statistical methods

Temporal patterns in environmental data were assessed with principle components analysis (PCA). Monthly data for each pond were analyzed using CANOCO for Windows 4.5 (Braak & Smilauer 1997) for ordination. The relationship between 30 day temperature and precipitation as well as PCA species scores were compared to the presence of *Bd* and monthly average number of *Bd* cells collected from frogs at each pond and were assessed with the Pearson product-moment correlation (Zar 1999).

Results

Batrachochytrium dendrobatidis was detected overall on 89% (n=572) of the frogs collected during the duration of the experiment while all of the sediment and water samples remained negative. None of the infected individuals, however, showed clinical signs of infection while handling them (lethargy, lack of righting reflex, excessive sloughing of skin etc). During the entire year, the overall monthly percentage of frogs infected remained high and ranged from a minimum of 78% (n=67) in June to a maximum of 98% (n=80) in July (Table 9.2). Although the values from each pond in the same months were in agreeance during November (100% of individuals tested were positive at ponds 1, 2, 4 and 6), other Values taken from different ponds within the same

month varied from as little as 15% (pond 1 [100%] and pond 5 [85%] in March) to as much as 42% (pond 2 [100%] and pond 4 [58%] in February) (Fig. 9.1 and Table 9.2).

Table 9.2: Percent frogs infected. Monthly percent and 95% confidence interval of Blanchard's cricket frogs (*Acris crepitans blanchardi*) infected with *Batrachochytrium dendrobatidis* from six ponds located in Bastrop County, Texas, USA during the spring and summer of 2009. No frogs were available in September, October, December and January and all frogs collected in November were positive (ponds 1, 2, 4 and 6).

	Percent Frogs Infected							
	Feb	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Average**
Pond 1	90 ±13	100	88 ±23	100	nd	nd	nd	90
Pond 2	100	100	85 ±16	100	nd	nd	nd	89
Pond 3	nd	92 ±16	86 ±26	85 ±16	80 ±18	100	100	88
Pond 4	58 ±22	100	65 ±21	100	80 ±18	95 ±10	75 ±19	88
Pond 5	100	85 ±16	85 ±16	90 ±13	65 ±21	nd	94 ±11	89
Pond 6	95 ±10	93 ±13	75 ±42	100	100	100	88 ±16	73
Average	89	94	80	95	78	98	89	89

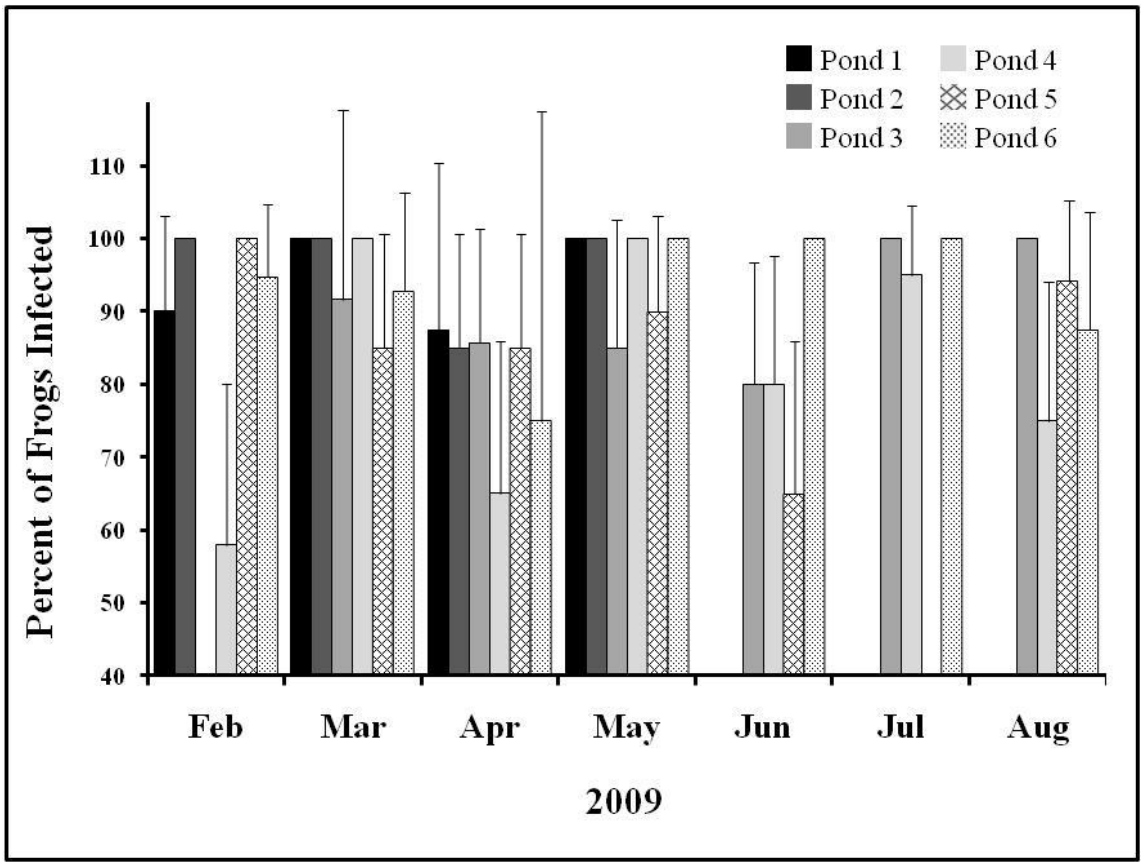

Figure 9.1: Monthly percent frogs infected. Monthly percent of Blanchard's cricket frogs (*Acris crepitans blanchardi*) infected with *Batrachochytrium dendrobatidis* at six sites located in Bastrop County, Texas, USA during the spring and summer of 2009. Error bars represent the positive value for the 95% confidence interval.

The number of *Bd* cells detected on frogs ranged from 0 to $3.0x10^5$ cells per individual with the overall average being 2400 cells per individual. While small variations in numbers of *Bd* cells were noted on frogs collected within ponds during some sampling events (e.g. 0-54 cells for pond 4 in February and 0-153 cells for pond 3 in June), differences as large as five orders of magnitude were not uncommon (e.g. 0-$3.0x10^5$ cells for pond 6 in March and 0-$1.2x10^5$ for pond 5 in April). Average values for the number of *Bd* cells detected on frogs at each pond during each sampling event ranged from a minimum of 12 cells per individual at pond 4 in February to over 3.0×10^4 cells

per individual at pond 6 in March (Fig. 9.2 and Table 9.3). Large seasonal variations in numbers of *Bd* cells on frogs collected throughout the study were detected with monthly average intensities peaking in March at 9400 *Bd* cells per individual and decreasing through the summer to a minimum of 85 *Bd* cells per individual in June (Table 9.3).

Table 9.3: Average number of *Bd* cells detected. Monthly average number and 95% confidence interval of *Batrachochytrium dendrobatidis* cells detected in samples collected from Blanchard's cricket frogs (*Acris crepitans blanchardi*) at six sites located in Bastrop County, Texas, USA during the spring and summer of 2009.

	Average Number of *Bd* Cells							
	Feb	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Average**
Pond 1	270	780	670	5500	nd	nd	nd	1200
	±120	±1000	±790	±9600				
Pond 2	150	270	120	85	nd	nd	nd	180
	±67	±170	±57	±47				
Pond 3	nd	6600	1300	3300	38	180	200	1600
		±7400	±850	±3100	±17	±26	±40	
Pond 4	12	2100	1600	170	40	120	210	870
	±6	±1400	±850	±36	±20	±36	±65	
Pond 5	1300	9100	13,000	360	100	nd	160	3800
	±980	±10,000	±12,000	±340	±63		±47	
Pond 6	2200	30,000	1500	2400	280	220	140	5200
	±2100	±43,000	±2600	±2700	±310	±23	±55	
Average	760	9400	4000	1400	85	174	180	2400

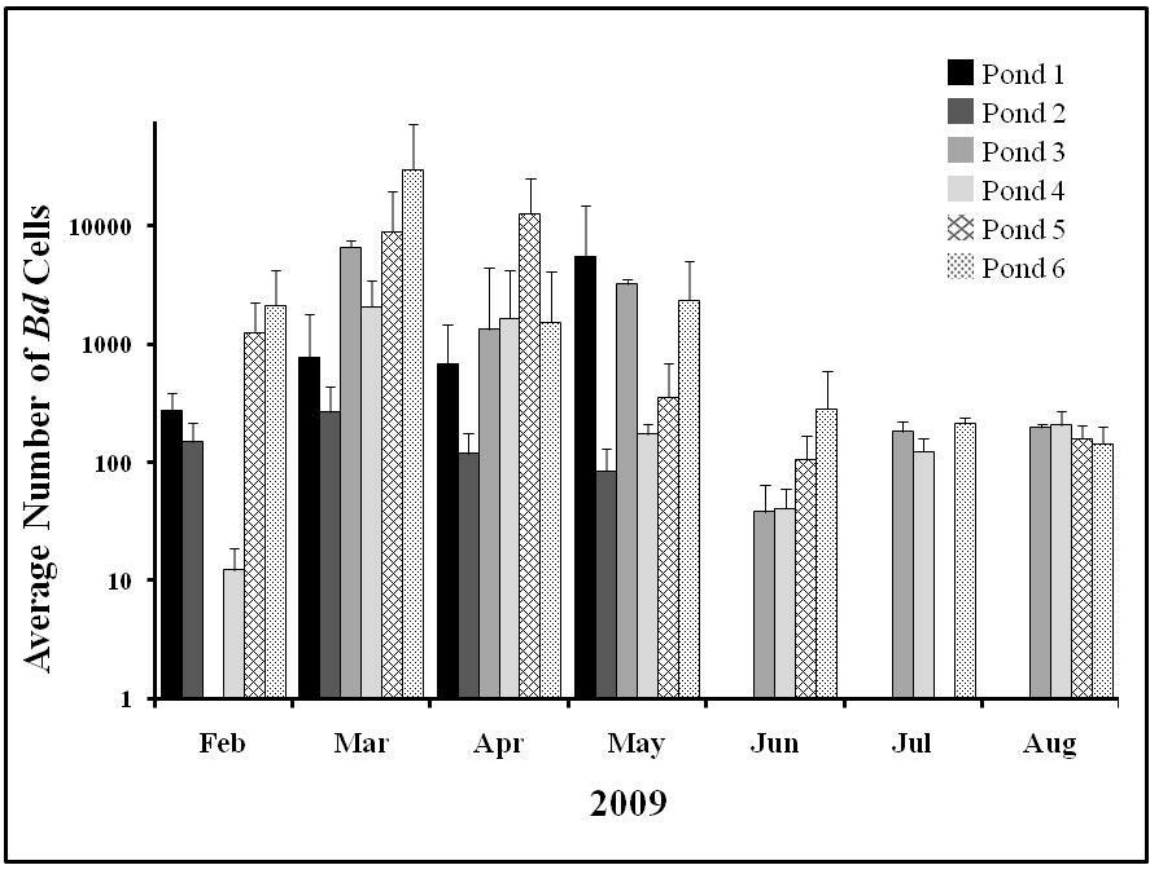

Figure 9.2: Monthly average number of *Bd* cells detected. Monthly average number of *Batrachochytrium dendrobatidis* cells detected in samples collected from Blanchard's cricket frogs (*Acris crepitans blanchardi*) at six sites located in Bastrop County, Texas, USA during the spring and summer of 2009. Error bars represent the positive value for the 95% confidence interval.

Ponds differed along environmental gradients of data collected (Fig. 9.3). The first two principal components explained 47% of the variation in the data collected at each pond. The first PC explained 29% of the variation and described a gradient from relatively deep ponds with consistently lower nutrient levels to ponds that were shallow and thereby more prone to nutrient loading including organic matter (OM), total phosphorous (TP), non-volatile suspended solids (NVSS), dissolved organic carbon (DOC) and soluble reactive phosphorous (SRP). The second PC explained 18% of the variation and contrasted aquatic habitats dominated by high respiration (high ammonium

and nitrate and low pH) to those of high primary production (high dissolved organic carbon and chlorophyll a).

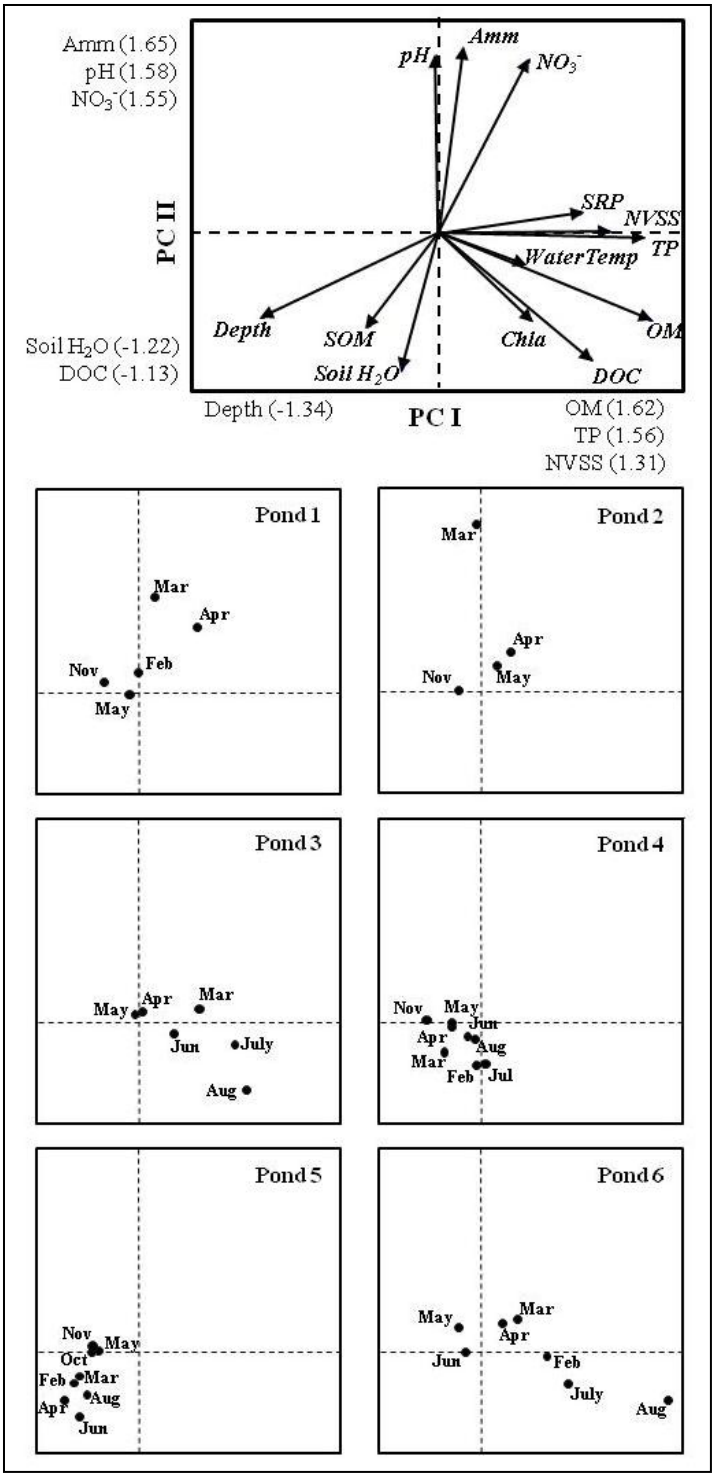

Figure 9.3: Biplots of PCA results. Plots of species scores and sample scores from six sites on principal component axis I and II of environmental data (H+ [*pH*], ammonium [*Amm*], nitrates [NO_3^-], soluble reactive phosphorus [*SRP*], non-volatile suspended solids [*NVSS*], total phosphorus [*TP*], water temperature [*WaterTemp*], organic matter [*OM*], chlorophyll *a* [*Chla*], soil moisture [$SoilH_2O$], soil organic matter [*SOM*], and depth [*Depth*]) collected monthly from ephemeral ponds located in Bastrop County, Texas.

When plotted, the sample scores of the first and second principle components for ponds 4 and 5 at each one of the sampling events were more closely aggregated as apparent by visual inspection of the biplots (Fig. 9.3) when compared with the other ponds which are shallower and more affected by seasonal variations. The number of *Bd* cells per individual was correlated to the sample scores of the first principal component of these two ponds ($r^2=0.25$, $P=0.04$) while this relationship did not exist for the remainder of the ponds. For all ponds, a significant correlation of the number of *Bd* cells per individual was obtained for the 30 day mean air temperature ($r^2=0.01$, $P=0.02$) but not for precipitation. The presence of *Bd* on individual frogs, however, was not correlated with 30 day precipitation or temperature when examined for the ponds taken individually or the combined monthly value for all ponds. Furthermore, there was no correlation found between the number of *Bd* cells per individual and the snout-vent length (SNV), weight or calculated body condition index.

Discussion

This study confirms previous investigations demonstrating that *Bd* is established in central Texas (Gaertner et al. 2008, Gaertner et al. 2009). These studies have demonstrated a wide distribution of *Bd* on salamanders in the genus *Eurycea* at ponds in central Texas (Gaertner et al. 2008) as well as a population of *A. crepitans blanchardi* with 83% of individuals testing positive for the pathogen in one sampling (Gaertner et al. 2009). Our number of 89% of all frogs being infected throughout the year closely approximates this number and further supports the supposition that *Bd* occurs with a relatively high prevalence in the area. However, our previous studies also documented

seasonal changes in infection rate with no *Bd* detections, and cured individuals, during summer (Gaertner et al. 2009). While these results seem to contradict our current investigation with high *Bd* detections throughout the year, the differences between both studies are most likely due to different sensitivities of the detection methods. Our current study was based on *q*PCR that is about 2 to 5 times more sensitive than the nested PCR approach in our previous study. The low numbers detected during summer by *q*PCR (i.e., usually less than 10 cells) might therefore not have been detectable by nested PCR. Nevertheless, although no differences in the detection rate of frogs during the season were retrieved, a clear reduction in *Bd* cells per individual was obvious, and related to temperature increases as already discussed in our previous study (Gaertner et al. 2009).

Similar to our previous study, no clinical effects were observed on frogs, independent of intensity of infection, i.e. the presence of high or low numbers of *Bd* cells per individual. The introduction of *Bd* into amphibian communities can have a wide range of outcomes ranging from high rates of mortality and population declines (Lips et al. 2006, Schloegel et al. 2006) to infection without any clinical signs of disease (Daszak et al. 2004). Central Texas has a high density of endangered and endemic amphibians (Chippindale et al. 2000, Forstner & Dixon 2010) and, because of this, information on potential impacts of infection is important to conservation efforts. Our study used a body condition index (Jehle & Hodl 1998) calculated from SVL and mass to estimate the health of organisms tested, expanding on previous studies of *Bd* in the area. The body condition index did not show any correlation with the presence or intensity of infection by *Bd* and therefore provides no evidence that the pathogen causes a reduction in the health of *A. crepitans blanchardi* tested.

Seasonal variation in the dynamics of pathogens and their hosts is common to a wide variety of systems (Hosseini et al. 2004, Pascual & Dobson 2005). Seasonal variation in the prevalence of *Bd* on amphibian hosts has been documented (Kriger & Hero 2007b, Gaertner et al. 2009), however, contradictory results have been reported as well (Green et al. 2002). Differences noticed in the seasonality of infection by *Bd* could be due to any number of factors including differences in the species, habitat or intensity of seasons in the area studied. In this study, the 30 day mean temperature before each of the sampling events was significantly correlated with the average monthly intensity of infection of all frogs sampled ($r^2=0.01$, $P=0.02$) and a seasonal trend was apparent. The combined average monthly intensity of infection of all six sampling ponds for this study peaked at 9400 *Bd* cells per individual in March. That number remained relatively high measuring on average 4000 *Bd* cells per individual in April, but was quickly reduced in May to 1400 and finally extremely low in June (85 cells per individual) and remained low throughout the remainder of the study (Fig. 9.2). Previous studies have shown the peak prevalence of disease levels at temperatures less than 19.4°C (Kriger & Hero 2007b) and 21.6°C (Gaertner et al. 2009). These temperatures are in agreement with those in this study with the monthly peak overall intensity of infection averaged for all sites occurring in March and April with temperatures at 15.3°C and 17.1°C, respectively (Table 9.1).

Although this pattern was true for the overall average, not all ponds followed this trend when examined separately. The PCA analysis of environmental variables measured at each of the ponds helped to identify two distinct types of ponds (Fig. 9.3). Two of the ponds were characterized by relatively stable year-round environmental characteristics and a larger volume of water (pond 4 and pond 5) than the others. The seasonality of

infection by *Bd* was fairly predictable in these ponds with the average intensity of infection rising to a peak in March and April and then declining through the summer months. The remaining ponds were characterized by much more dynamic environmental characteristics due to their small size and the concomitant impact of evaporation and precipitation events (ponds 1-3 and pond 6). Consequently, the value of environmental variables measured in these ponds fluctuated widely between monthly sampling events, and so did the intensity of infection by *Bd*. Thus, the more complex temporal pattern of infection in these ponds is therefore presumably a function of the dynamic environment.

The number of *Bd* cells on frogs collected at pond 2 differed from that on the other ponds in several ways. Pond 2 showed a reduced seasonal pattern if any at all. No significant differences were detected in the monthly average number of *Bd* cells per individual for this pond which ranged from a maximum of 266 observed in March down to a minimum of 85 in May. Pond 2 also showed a dramatic reduction in the overall number of *Bd* cells per individual collected throughout the year. The average number of *Bd* cells detected from all samples collected at pond 2 throughout the entire study was only 180 *Bd* cells per individual and the monthly average never increased above 270, and thus low compared to the overall average of other ponds which ranged from 870 to 5400. The differences observed at this pond were noted even despite being located approximately 1,000 m to a maximum of 2,600 m from the other ponds. One possible explanation for the reduced intensity of infection and lack of seasonal differences in this pond could be due its ephemeral nature. Although the soil retained sufficient moisture to maintain amphibian populations, this pond lacked any standing water in February and again in June through August. Previous studies have linked the availability of standing

water to the prevalence of *Bd* in populations of amphibians (Gaertner et al. 2009, McCracken et al. 2009). The aquatic zoospore of *Bd* cannot survive desiccation (Johnson et al. 2003) which may prevent it from causing significant infections at sites without standing water despite there being enough moisture to support amphibian populations. These speculations are supported by results of other studies that demonstrated that amphibians breeding in permanent sources of water had a much higher likelihood of infection by *Bd* than those collected from ephemeral sources (Kriger and Hero (2007a). *Bd* was detected on 42% of individuals collected from permanent streams and on 27% of those collected from permanent ponds while all amphibians collected at ephemeral sources remained negative for the pathogen (Kriger & Hero 2007a).

This study provides evidence on differences in *Bd* abundance on frogs as a function of environmental conditions in their habitat, but also on seasonal effects with temperature as an environmental driver. These findings may be significant in areas such as central Texas in which high temperatures, as well as semi-permanent and ephemeral sources of water provide unfavorable conditions for *Bd* for most of the year. Since these water sources represent a significant amount of available breeding habitat for amphibians, the presence of *Bd* in small amounts on amphibians in these habitats throughout the season does not seem to represent a significant threat for these. The results of this study could help conservation efforts on amphibians and could be particularly useful in selection of sites for reintroduction of captive-bred individuals.

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