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Ecological and Geochemical Aspects of Terrestrial Hydrothermal Systems

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Ecological and Geochemical Aspects of Terrestrial Hydrothermal Systems

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy

in

Oceanography

by

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2013

The Dissertation of Matthew James Forrest is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013

DEDICATION

This thesis is dedicated to my mother Virginia Forrest, who always believed in me and supported me throughout the trials and tribulations of graduate school and life. I also dedicate this thesis to my grandfather Byron Darling who taught me to seize opportunities and never give up, but also to stop and smell the roses. California Byron!

EPIGRAPH

"Springs are the aquatic ecologist's natural constant temperature laboratory. Because of the relative constancy of the chemical composition, velocity of water, and temperature, in comparison with lakes, rivers, marine environments, and terrestrial communities, springs hold a position of importance as study areas that is out of proportion to their size and number."
—Eugene Pleasants Odum (1971)

"From the naturalist's standpoint and from that of the nature-lover as well it is distressing to record that all except the most inaccessible springs, or those included within the boundaries of National Parks, have been either converted to natatoria, sanatoria for arthritics, radium baths and the like, or have been diverted into irrigation ditches, sometimes with the aid of dynamite, to supply a few desolate ranches with water for cattle and alfalfa. For this reason the fauna and the flora of these thermal springs is destined soon to be wiped out over considerable areas by the advance of commercialism."
—Charles T. Brues (1928)

TABLE OF CONTENTS

SIGNATURE PAGE .... **iii**

DEDICATION .... **iv**

EPIGRAPH .... **v**

TABLE OF CONTENTS .... **vi**

LIST OF FIGURES .... **viii**

LIST OF TABLES .... **xi**

ACKNOWLEDGEMENTS .... **xiv**

MATERIAL SUBMITTED FOR PUBLICATION IN THE DISSERTATION .... **xv**

CURRICULUM VITAE .... **xvii**

ABSTRACT OF THE DISSERTATION .... **xx**

GENERAL INTRODUCTION **1**

CHAPTER 1: Hydrothermal contamination of public supply wells in Napa and Sonoma Valleys, California **19**

ABSTRACT 20

INTRODUCTION 20

METHODS 22

RESULTS 23

DISCUSSION 28

REFERENCES 35

CHAPTER 2: Nothing a Hot Bath Won't Cure: Infection Rates of Amphibian Chytrid Fungus Correlate Negatively with Water Temperature under Natural Field Settings **36**

ABSTRACT 37

INTRODUCTION 37

RESULTS 39

DISCUSSION 40

MATERIALS AND METHODS 43
REFERENCES 44
CHAPTER 3: First reports of amphibian chytrid fungus infections in the Ash Meadows National Wildlife Refuge 46
ABSTRACT 47
INTRODUCTION 47
METHODS 49
RESULTS 53
DISCUSSION 54
REFERENCES 64
CHAPTER 4: Habitat loss and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* may threaten the Dixie Valley toad, a narrowly distributed endemic species 69
ABSTRACT 70
INTRODUCTION 71
MATERIALS AND METHODS 73
RESULTS 75
DISCUSSION 77
REFERENCES 98
CHAPTER 5: Biogeographic patterns of desert springs in the Great Basin with an emphasis on regional aquifer thermal springs as refugia for vulnerable species 102
ABSTRACT 103
INTRODUCTION 104
MATERIALS AND METHODS 115
RESULTS 119
DISCUSSION 124
REFERENCES 151

LIST OF FIGURES

Figure I-1: Plot of $\delta^{13}C$ and $\delta^{15}N$ (in ‰) of "Vent" organisms collected near shallow water marine hydrothermal vent in Punta Banda Mexico (red symbols) and "Non-vent" organisms collected in Scripps Canyon in La Jolla California (black symbols) 13

Figure 1-1: Geologic map of the North San Francisco Bay study unit with locations of sampled wells (from Kulongoski et al., 2010) 21

Figure 1-2: Dendrogram of the results of hierarchical clustering analyses with the well samples on the x-axis, and the y-axis defining the similarity levels (in Euclidean distances) at which samples or groups are considered to have fused 28

Figure 1-3: Multidimensional Scaling (MDS) Map. The proximity of the well samples to each other indicates how similar they are to each other 29

Figure 1-4: Principal components analysis (PCA) ordination of well samples…....... 29

Figure 1-5: Diagram showing mixing proportions from The Multivariate Mixing and Mass-balance model (M3) 33

Figure 1-6: Piper diagram of sampled wells 34

Figure 2-1: Occurrence of *Bd* in lowland leopard frogs as a function of water temperature 39

Figure 2-2: Schematic of Mammoth Hot Well showing approximate locations of amphibians and water temperatures 41

Figure 2-3: Schematic of Secret Spring showing approximate locations of amphibians and water temperatures 42

Figure 2-4: Lowland leopard frog in Muleshoe Hot Spring 43

Figure 3-1: Map of Ash Meadows National Wildlife Reserve with locations of sites where amphibians were sampled for *Batrachochytrium dendrobatidis* 59

Figure 3-2: Picture of a Woodhouse's toad (*Anaxyrus woodhousii*) on the left and a western toad (*Anaxyrus boreas*) on the right 60

Figure 3-3: Close-up picture of a Woodhouse's toad (*Anaxyrus woodhousii*) showing elongate parotoid glands and the presence of a cranial crest, a ridge that frames the inner rim of the upper eyelid 61

Figure 3-4: Close-up picture of a western toad (*Anaxyrus boreas*) showing oval-shaped parotoid glands and the lack of a cranial crest 62

Figure 4-1: Image of adult Dixie Valley Toad sampled in 2009 from Dixie Meadows... 86

Figure 4-2: Map of the Dixie Valley, Nevada area showing locations of sampling sites for Dixie Valley toads, western toads *Anaxyrus* (=*Bufo*) *boreas*, and American bullfrogs *Lithobates catesbeianus* 87

Figure 4-3: Image of adult western Toad *Anaxyrus* (=*Bufo*) *boreas* sampled in 2009 from McCoy Ranch 88

Figure 4-4: Map of line transect survey locations for Dixie Valley toads from 2009 89

Figure 4-5: Distribution of SVL from western toads *Anaxyrus* (=*Bufo*) *boreas* sampled at McCoy Ranch from 2009-2012... 90

Figure 4-6: Distribution of SVL from Dixie Valley toads sampled in Dixie Meadows from 2009-2012 91

Figure 4-7: Dixie Valley toads in amplexus underwater and egg masses in the perennial pond in Dixie Meadows.. 92

Figure 4-8: Developmental stages of Dixie Valley Toads recorded using the Gosner method (Gosner, 1960) during 2011 93

Figure 4-9: Snout-Vent Lengths (SVL) of Dixie Valley Toads recorded during investigations of developmental stages during 2011 94

Figure 5-1: Multidimensional Scaling (MDS) plot of biological and physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles).. 135

Figure 5-2: Multidimensional Scaling (MDS) plot of only physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles) 136

Figure 5-3: Principle Components Analysis (PCA) plot of biological and physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles) 137

Figure 5-4: Principle Components Analysis (PCA) plot of only physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles) 138

LIST OF TABLES

Table I-1: δ15N and δ13C values for bacterial mat samples (PBBM) and muscle tissue from five blackeye gobies *Rhinogobiops nicholsii* collected at the shallow water hydrothermal vent near Punta Banda (PBRn). 12

Table 1-1: Trace elements for all 44 wells used in statistical analyses (in µg/L)......... 24

Table 1-2: Values for temperature, pH, isotope ratios, concentrations of major and minor elements, and Total Dissolved Solids (TDS) for all 44 wells used in statistical analyses (in mg/L).. 25

Table 1-3: Mean (first row) and median (second row in bold text) values of constituents important to the classifications of the wells from this study........ 26

Table 1-4: Results of non-parametric Spearman's method analysis of correlations between selected water-quality constituents and groundwater temperature and ^{3}He/^{4}He ratio. 27

Table 1-5: Coefficients in the linear combinations of variables making up PC's (Eigenvectors), and % Variation explained by each PC.................................. 30

Table 1-6: Similarity Percentage Analyses (SIMPER) of differences between ground water from public supply wells (Group GW) and hydrothermal wells from Calistoga (Group HC) 31

Table 1-7: Similarity Percentage Analyses (SIMPER) of differences between ground water from public supply wells (Group GW) and mixed hydrothermal/meteoric wells (Group MW). 31

Table 1-8: Similarity Percentage Analyses (SIMPER) of differences between hydrothermal wells from Calistoga (Group HC) and wells from mixed hydrothermal/meteoric wells (Group MW) 32

Table 1-9: Similarity Percentage Analyses (SIMPER) of differences between hydrothermal wells from Calistoga (Group HC) and the hydrothermal well from Sonoma (HSON)... 32

Table 1-10: Mixing proportions from The Multivariate Mixing and Mass-balance model, M3 using the following reference waters: HOT-05 as the reference hydrothermal well from Calistoga (HC), WG03T as the reference well for groundwater (GW), and VP-36 as the reference well for saline water (SW)... 33

Table 2-1: Sites sampled, Latitude and Longitude (Datum=WGS 84), dates sampled, range of water temperatures measured during sampling, species sampled, number of individuals, and Bd prevalence (percent infected) per species sampled 38

Table 2-2: Logistic binary regression of infection status (0 = not infected; 1 = infected with *Batrachochytrium dendrobatidis*) of lowland leopard frogs *Rana* (*Lithobates*) *yavapaiensis* (N = 198; 3 missing SVL)........ 40

Table 3-1: Locations, species and *Batrachochytrium dendrobatidis* prevalence of amphibians sampled in the Ash Meadows National Wildlife Refuge in April 2012........ 63

Table 4-1: Population survey data from 2009-2012 for western toads *Anaxyrus* (=*Bufo*) *boreas* at McCoy Ranch........ 95

Table 4-2: Population survey data from 2009-2012 for Dixie Valley Toads in Dixie Meadows........ 96

Table 4-3: Results from sampling for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) during 2011 and 2012 97

Table 5-1: Mean values of certain physicochemical parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold)........ 139

Table 5-2: Mean values of certain disturbance parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold) 139

Table 5-3: Mean values of certain habitat parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold) 140

Table 5-4: Mean values of certain biological parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold) 140

Table 5-5: Eigenvalues of the individual PC’s from the combined physicochemical and biological data from springs in the Great Basin 141

Table 5-6: The eigenvectors for each of the first 10 Principle Components (PCs) from the combined physicochemical and biological data from springs in the Great Basin 142

Table 5-7: Eigenvalues of the individual PC's from only the physicochemical data from springs in the Great Basin 143

Table 5-8: Eigenvectors for each of the first 10 Principle Components (PCs) from only the physicochemical data from springs in the Great Basin. 144

Table 5-9: SIMPER analyses of the differences between biological and physicochemical parameters from geothermal local aquifer (Local Hot) springs and cold local aquifer (Local Cold) springs...................................... 145

Table 5-10: SIMPER analyses of the differences between biological and physicochemical parameters from geothermal local aquifer (Local Hot) springs and regional aquifer thermal (Regional Aquifer) springs 146

Table 5-11: SIMPER analyses of the differences between biological and physicochemical parameters from cold local aquifer (Local Cold) springs and regional aquifer thermal (Regional Aquifer) springs.............................. 147

Table 5-12: SIMPER analyses of the differences between physicochemical parameters from geothermal local aquifer (Local Hot) springs and cold local aquifer (Local Cold) springs 148

Table 5-13: SIMPER analyses of the differences between physicochemical parameters from geothermal local aquifer (Local Hot) springs and regional aquifer thermal (Regional Aquifer) springs 149

Table 5-14: SIMPER analyses of the differences between biological and physicochemical parameters from cold local aquifer (Local Cold) springs and regional aquifer thermal (Regional Aquifer) springs.............................. 150

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Chapter 2, in full, is a reprint of the material as it appears in PLoS ONE: Forrest MJ, Schlaepfer MA (2011) Nothing a Hot Bath Won't Cure: Infection Rates of Amphibian Chytrid Fungus Correlate Negatively with Water Temperature under

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Chapter 3, in full, was prepared as an internal report for Ash Meadows National Wildlife Refuge: Forrest MJ, Jaeger JR (2012) First reports of amphibian chytrid fungus infections in the Ash Meadows National Wildlife Refuge. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Ecological and Geochemical Aspects of Terrestrial Hydrothermal Systems

by

Matthew James Forrest

Doctor of Philosophy in Oceanography

University of California, San Diego, 2013

Richard Norris, Chair

Hydrothermal systems are important for a myriad of reasons. Life itself may have begun in hydrothermal ecosystems, and in more modern times these environments are serving as refugia for many unique and vulnerable species. In the Great Basin region of North America, regional aquifer hydrothermal springs support a disproportionate number of endemic species, some of which live in only one hydrothermal spring. Many of these organisms appear to be relict species that may have had far wider distributions in the past when climatic conditions were wetter and large pluvial lakes covered vast areas within the Great Basin. Today, regional aquifer hydrothermal springs provide important information regarding regional biogeography, paleohydrological connectivity, and climate change. However, these unique ecological hotspots also face many anthropogenic threats including

groundwater depletion and pollution, flow diversions, and the introduction of exotic species.

The unique physical and geochemical characteristics of hydrothermal systems may also protect some vulnerable amphibian species from pathogens. In Arizona and Nevada, several species of frogs and toads inhabit hydrothermal ecosystems, and the elevated temperatures in these environments appear to be protecting them from the amphibian chytrid fungus *Batrachochytrium dendrobatidis*, which causes the potentially fatal disease chytridiomycosis. The Dixie Valley toad, a yet undescribed species currently restricted to four thermal spring-fed wetlands within Dixie Valley NV, appears to be a relict member of the western toad (*Anaxyrus boreas*) species group. We surveyed populations of Dixie Valley toads between 2009-2012, and also tested individuals for *Bd* in 2011-12. While none of the toads tested positive for *Bd*, the prevalence of *Bd* infections among nearby populations of introduced American bullfrogs increased significantly. This unique and vulnerable species also faces threats from increased exploitation of nearby geothermal energy resources.

In Napa and Sonoma Counties, California we used multivariate statistical analyses and mixing models to elucidate the occurrence and extent of contamination of local groundwater used for drinking water by hydrothermal fluids. We found that several public supply wells used for drinking water and irrigation have concentrations of As, F and B that exceed drinking-water standards or notification levels due to contamination by hydrothermal fluids.

GENERAL INTRODUCTION

INTRODUCTION

Spring environments in desert regions are rare, fragile, and endangered ecosystems that often support vulnerable endemic species (Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007). In arid regions, spring environments also represent ‘island’ ecosystems surrounded by ‘seas’ of deserts--often providing the sole source of water for the biota within and around them (Soltz and Naiman, 1978). The desert springs of the Great Basin in western North America represent ideal systems to study biogeographic and evolutionary patterns because they are numerous; have abundant endemic species (species whose entire distributions are limited to one area) with histories that may reflect differential colonization, extinction and local adaptations; may preserve relict species by virtue of their isolation; and particularly in the case of regional aquifer thermal springs, because they have unusual physicochemical properties that may exclude competitors and pathogens. Springs are also among the most threatened ecosystems in the world (Stevens and Meretsky 2008). Primary anthropogenic impacts include groundwater depletion and pollution, alteration of source area geomorphology, flow diversions, and the introduction of exotic species (Hubbs et al., 1974; Sada and Vinyard, 2002; Springer and Stevens, 2009). This is important because springs have significant conservation value due to their roles as refugia for rare and vulnerable species.

The Great Basin is the most arid region in North America, containing only a few, widely dispersed permanent wetland habitats--yet there are hundreds of aquatic species that are endemic to the region (Sada and Vinyard, 2002). The springs of the Great Basin have also been relatively well studied, and researchers have compiled large databases of their biota and chemistry. Consequently, these systems lend themselves to biogeographic studies and multivariate statistical analyses of relationships between the chemical and physical characteristics of the springs and the biological communities present within them. I examined the physicochemical and biological characteristics of desert springs in the Great Basin specifically to investigate the correlates of spring physicochemical characteristics to their retention of endemic taxa. My study is motivated by a desire to elucidate the factors involved in thermal spring systems roles as refugia for vulnerable species, as discussed for amphibians in subsequent chapters (Chapters 2,3,4; Forrest and Schlaepfer, 2011).

Smaller springs are generally autotrophic aquatic systems with little dependence on allochthonous (derived from outside sources) carbon sources (Minshall. 1978; Cushing and Wolf, 1984). In larger springs, energy may enter the system during periodic floods that flush carbon from the surrounding landscape. As a consequence, most spring environments are less variable than other aquatic habitats (e.g., streams, rivers, and lakes), resulting in lower variability in population size and assemblage structures (Minckley 1963, van der Kamp 1995). Thermal springs are generally characterized by simple food webs that are highly reliant on autochthonous (derived from sources within the system) production (Naiman, 1976), but also may receive pulses of allochthonous carbon (Wilson and Blinn, 2007). Typically there are copious amounts of primary producers such as cyanobacteria and thermophilic diatoms in thermal springs, and these organisms may serve as the base of local food webs. In contrast, much of the energy available in non-thermal springs may derive from allochthonous sources, such as leaves, seeds, and other material derived from nearby terrestrial vegetation (Naiman, 1976). Because food webs in thermal springs are usually more reliant on autochthonous sources, they may provide more stable environments that are less likely to be influenced by stochastic events that disrupt the input of allochthonous sources.

Carbon and nitrogen stable isotopes may have great utility in determining the extent that autochthonous sources contribute to food webs in thermal springs. Rounick et al. (1982) showed that allochthonous terrestrial plant material and autochthonous algae were isotopically distinct in small New Zealand streams, thereby providing a basis for identifying food resources used by aquatic animals. Further work by Rounick and James (1984) showed that the $\delta^{13}C$ values of the invertebrates and their algal foods at thermal and cold springs were drastically different as a result of differences in their respective sources of inorganic carbon. In studies in the Jackrabbit Spring system in Ash Meadows gut analyses revealed that filamentous algae were the predominant food item consumed by pupfish inhabiting thermal waters, and the $\delta^{13}C$ signature of Ash Meadows pupfish also suggested an algae-dominated diet (Scoppettone et al., 2013). The upper reaches of the thermal springs were particularly favorable to pupfish because the warm waters promote year-round growth of filamentous algae (Kennedy and Hobbie, 2004; Kennedy et al., 2005). Although filamentous algae appear to be important food sources for pupfish (Naiman, 1975; Kennedy et al., 2005; Scoppettone et al., 2013), some authors have raised questions about its digestibility (Naiman, 1979). Small epiphytic and grazing organisms associated with filamentous algae may be rapidly digested, and therefore not readily detected in gut analysis; however, they may contribute significantly to pupfish diet. In fact, analyses of the trophic position of pupfish using $\delta^{15}N$ indicated primary consumers contributed substantially to their diet (Scoppettone et al., 2013).

I have observed very similar patterns with fish living near shallow water marine hydrothermal vents utilizing the autochthonous resources present around the vents. In April 2006, I performed a stable isotope study around shallow water (25-30m) hydrothermal vents near Punta Banda, Baja California, Mexico. I found that several organisms showed significant dietary contributions from vent microbes--particularly blackeye gobies, *Rhinogobiops nicholsii* (Figure 1). Some of the gobies that were collected for this study were hovering directly over the vent orifices. When they were chased off, the gobies immediately returned to the sites near vents, suggesting that these fish are permanently residing around the hydrothermal vents, which is consistent with their known behavior as harem-forming nest-guarders that defend permanent territories (Cole, 1984). Moreover, the stable isotope values of the gobies (n=3; $\delta^{13}C$ mean = -26.66‰, $\delta^{15}N$ mean = 5.04‰) collected at the vents suggested that they were deriving significant nutritional input from the chemosynthetic bacterial mats, which only grow in areas influenced by hydrothermal fluids (bacterial mats $\delta^{13}C$ = -40.63‰, $\delta^{15}N$ = -7.26‰) (Table 1; samples PBRn1, 2, 3). A single isotope, two end-member linear dietary mixing model (Fry and Sherr,

1984) suggests that these gobies were obtaining 36% of their carbon and nitrogen from the bacterial mats. Because these fish are known to primarily consume small crustaceans and gastropods (Wiley, 1973), a more likely explanation for these results is that the gobies were consuming prey that were primary or secondary predators on organisms that were directly grazing on the bacterial mats. This would also make sense in terms of the $\delta 1^5$N values of the *R. nicholsii* examined, as nitrogen stable isotopes increase approximately 3.4‰ with each trophic level (Minawaga and Wada, 1984). During a subsequent sampling endeavor, two more *R. nicholsii* were collected at a site approximately 2m away from the venting influence at Punta Banda (Table 1; PBRn4, 5). These fish were compared with 5 *R. nicholsii* collected in the Scripps Canyon in La Jolla, California at similar depths (Table 1; SCRn1-5). The isotope signatures of the gobies collected farther away from the hydrothermal vents at Punta Banda indicated that were less influenced by dietary contributions from vent microbes, yet the stable isotope signatures were still significantly different from the gobies collected in Scripps Canyon (Table 1). This pattern of enhanced production from autochthonous sources in both marine and terrestrial thermal springs may help explain why these habitats support a disproportionate number of vulnerable taxa.

Thermal springs also may persist for hundreds of thousands of years (Brock, 1967; Hershler et al., 2007), and they represent constant environments that do not freeze or dry up (Brock, 1967; Brown and Feldmeth, 1971). According to carbon-14 analysis, the average age of water discharging from regional aquifer thermal springs ranges from 4,000 to 14,000 years old (Thomas et al., 1996). Hershler et al. (2007)

also found that molecular clocks based on cytochrome c oxidase subunit I (COI) gene suggest that the springsnail fauna from four thermal springs in Soldier Meadow may be a product of a lengthy history of evolution within the basin. This is also supported by evidence that the thermal spring habitats of these snails, which are associated with geologic faults that are potentially millions of years based on the amount of offset of spring deposits across faults having known slip rates (Coolbaugh et al., 2005). Thermal springs may also be less prone to washing out during storm and/or flood events due to the fact that they are typically located outside of areas that are subjected to heavy flooding such as canyon floors (D. Sada, pers. comm., 2009).

There are several lines of evidence that in addition to harboring endemic species, hydrothermal ecosystems may be providing amphibians with refugia from disease (Schlaepfer et al., 2007; Forrest and Schlaepfer 2011; Chapters 2, 3, 4). Field studies conducted around the world show that infections of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (hereafter referred to as *Bd*) are generally more severe in winter months, and when hosts are found in cooler temperatures (Bradley et al. 2002, Berger et al. 2004, Murray et al. 2009, Voordouw et al. 2010). In the laboratory *Bd* growth ceases at temperatures above 28°C (Piotrowski et al. 2004; Johnson and Speare, 2003). Similarly, *Bd* does not persist in amphibian hosts above certain temperature thresholds. In laboratory experiments, short-term exposure to temperatures between 27 and 37°C successfully cleared *Bd* infections from five different species of adult frogs (Berger et al. 2004; Woodhams et al. 2003; Retallick and Miera 2007; Chatfield and Richards-Zawacki, 2011). Temperature is also strongly linked to amphibian immune system responses, and declines in amphibian immune defenses as temperatures decrease are well documented (Green and Cohen 1977, Zapata et al. 1982, Maniero and Carey 1997, Rollins-Smith et al., 2002). Conversely, at warmer temperatures amphibians may be less susceptible to *Bd* due to greater effectiveness of the immune response (Andre et al., 2008; Murphy et al., 2011). Additionally, studies have demonstrated that resistance to *Bd* is stronger when amphibians experience constant temperatures—as would be present in thermal springs--rather than drastic fluctuations in temperatures typical of cold springs (Raffel et al., 2006).

Despite their role as refugia for vulnerable endemic taxa, springs are among the most threatened ecosystems in the world (Hubbs et al., 1974; Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007; Deacon et al., 2007; Stevens and Meretsky 2008; Springer and Stevens, 2009). Primary anthropogenic impacts include groundwater depletion and pollution, alteration of source area geomorphology, flow diversions, and the introduction of exotic species (Hubbs et al., 1974; Sada and Vinyard, 2002; Springer and Stevens, 2008). For example, Ash Meadows is one of the most important endangered aquatic ecosystems in western North America (Hershler and Sada, 1987). Excessive groundwater pumping threatens the flows and biota of springs in Ash Meadows (Hubbs et al., 1974; Deacon et al., 2007), and approximately 16% (4000 hectares) of the total area within the Ash Meadows National Wildlife Refuge has been perturbed by mining, agriculture, and municipal development--including most of the springs (Hershler and Sada, 1987; U.S.

Fish and Wildlife Service, 1990). The introduction and persistence of exotic species has caused further alteration of spring ecosystems, with invading biota including bullfrogs (*Lithobates catesbeianus*), mosquito fish (*Gambusia affinis*), sailfin mollies (*Poecilia latipinna*), redrim melania snail (*Melanoides tuberculata*), crayfish (*Procambarus clarki*), and salt cedar (*Tamarisk* sp.) (Hubbs et al., 1974; Hershler and Sada, 1987; Sada and Vinyard, 2002). These perturbations have resulted in population declines of virtually all of the endemic taxa within Ash Meadows (Hershler and Sada, 1987).

Effective management of invasive species such as American bullfrogs, mosquitofish, crayfish is an essential part of restoring spring systems, because under certain habitat conditions they can outcompete and all but replace native species (Scoppettone et al., 2013). For example, in the Jackrabbit Spring system in Ash Meadows, spring restorations allowing more rapid water flow sustained the minimum water temperatures required for native pupfish reproduction farther downstream, and also served to reduce marsh-like habitat favoring introduced sailfin molly, mosquitofish, and crayfish (Scoppettone et al, 2005). Minimum water temperature for Amargosa pupfish reproduction is ~25 ºC (Soltz and Naiman, 1978). Thermal springs generally cool as they flow downstream; water temperature in the spring discharge is constantly ~26.9 ºC, but water temperatures downstream can range well over 20 ºC (Scoppettone et al, 2005). Therefore, restoring and channelizing the upper portions of the springs increased flow rate--which in turn caused the water to carry its thermal load further downstream, thereby increasing essential habitat for pupfish, as well as reducing cooler marshy habitat that favored the introduced species (Scoppettone et al., 2013).

Identifying environmental and climactic conditions that provide natural refuges from *Bd* and chytridiomycosis will benefit imperiled amphibian populations (Puschendorf et al. 2009, Walker et al. 2010), and may provide some susceptible species with opportunities to evolve evolutionary responses to the pathogen (Tobler and Schmidt 2010). My own work with amphibians in Arizona demonstrated that thermal springs can provide amphibians with refugia from *Bd* (Forrest and Schlaepfer 2011; Chapter 2). In 2012, I also tested amphibians within the Ash Meadows National Wildlife Refuge for *Bd*. Although 14 western toads (*Anaxyrus boreas*) tested positive for *Bd,* no infections were found in amphibians inhabiting the upper reaches of the thermal springs, where water temperatures above 28°C may provide refuge from the pathogen (Chapter 4). Additionally, the relict leopard frog *Lithobates onca* was once thought to be extinct, but several populations were rediscovered in the 1990's and all naturally occurring *R. onca* populations are now associated with regional aquifer thermal springs with source temperatures exceeding 30°C (Jaeger et al., 2001; Bradford et al., 2004)--including Roger's Spring and Blue Point Spring which were both included in the multivariate analyses in Chapter 5. Within the Greater Yellowstone Ecosystem, *A. boreas* breed predominantly in geothermal ecosystems, which also appear to be protecting them from redleg, a potentially fatal bacterial disease (Hawk 2000). In fact, the only toads that survived a mass mortality episode in Yellowstone National Park were those that hibernated near water warmed by thermal springs (Peterson, C. unpubl. data cited in Carey, 2000). These findings may have important conservation implications because chytridiomycosis has been implicated in drastic declines in some populations of western toads.

In addition to their role as refugia for vulnerable species, hydrothermal springs and wells are also valuable economically, because they can be exploited as energy sources, and are popular for bathing and recreational use. However, in Sonoma and Napa Counties, California, hydrothermal wells are contaminating nearby local public supply aquifers used for drinking and irrigation purposes, and some of these contaminated wells have concentrations of As, F and B that exceed drinking-water standards or notification levels (Forrest et al., 2013; Chapter 1). Hydrothermal contamination of public supply aquifers may have serious ramifications because As has been linked to cancer of the bladder, lungs, skin, kidney, nasal passages, liver and prostate, and elevated F in drinking water may lead to increased likelihood of bone fractures in adults and mottling and pitting in tooth enamel in children (US EPA, 2006).

Table I-1: δ15N and δ13C values for bacterial mat samples (PBBM) and muscle tissue from five blackeye gobies *Rhinogobiops nicholsii* collected at the shallow water hydrothermal vent near Punta Banda (PBRn). Asterisks indicate fish caught closest to venting sites. For comparison, the δ^{15}N and δ^{13}C values of five gobies collected in the Scripps Canyon (SCRn) are also presented. T-test p values are for one-tailed, two-sample equal variance Student's t-tests.

Sample	**δ^{15}N**	**δ^{13}C**
PBBM1	-8.5	-38.4
PBBM2	-8.7	-41.6
PBBM3	-4.6	-41.9
PBRn1*	7.4	-23.7
PBRn2*	3.5	-28.4
PBRn3*	4.2	-27.9
PBRn4	11.3	-19.2
PBRn5	13.0	-19.1
PBmean	**7.9**	**-23.7**
SCRn1	14.1	-16.5
SCRn2	14.3	-15.5
SCRn3	13.8	-16.5
SCRn4	14.8	-14.1
SCRn5	14.1	-15.8
SCmean	**14.2**	**-15.7**
T-test p value	**0.005**	**0.002**

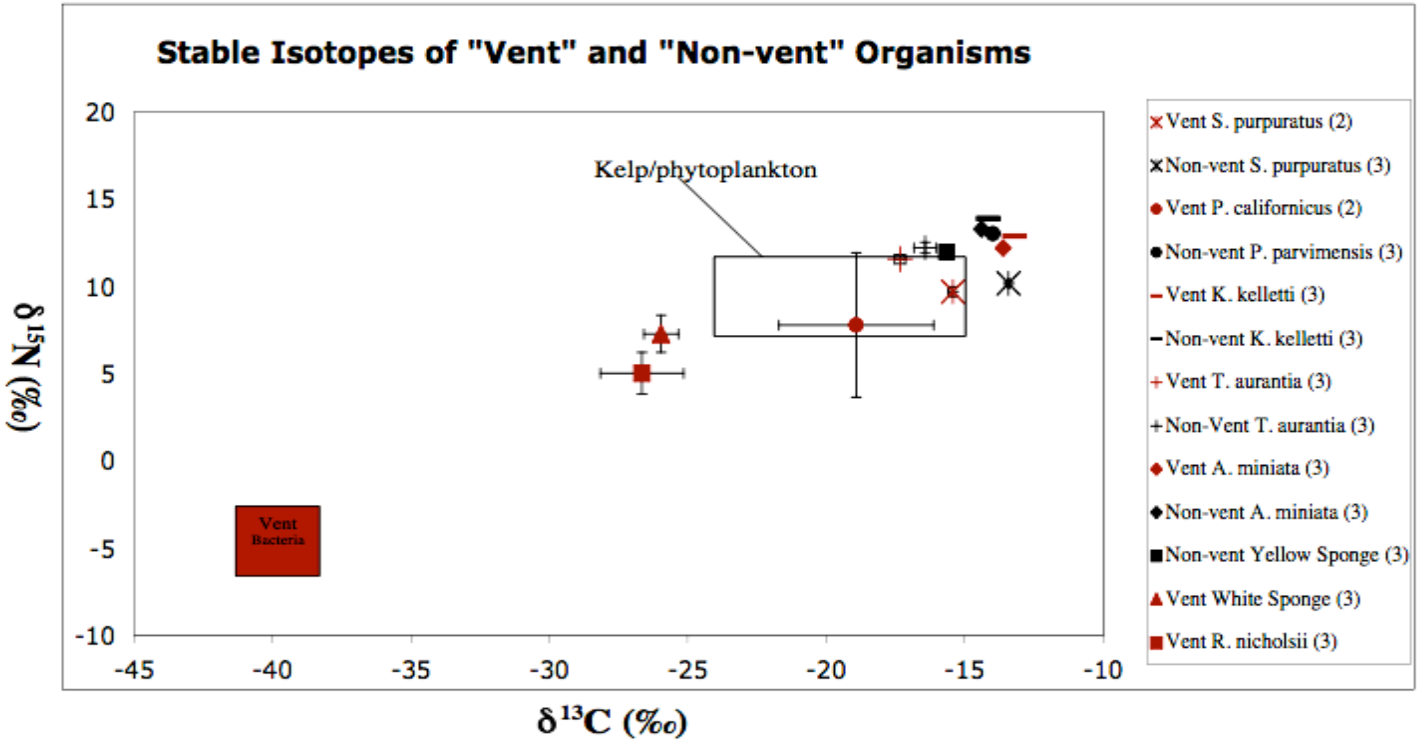

Figure I-1: Plot of δ^{13}C and δ^{15}N (in ‰) of "Vent" organisms collected near a shallow water marine hydrothermal vent in Punta Banda Mexico (red symbols) and "Non-vent" organisms collected in Scripps Canyon in La Jolla California (black symbols) organisms. Numbers next to organisms in the legend refer to the sample sizes. Ranges for the chemosynthetic bacteria from around the vent are represented by the red box, while the black rectangle represents the ranges typical of kelp and phytoplankton. Error bars represent standard errors.

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CHAPTER 1: Hydrothermal contamination of public supply wells in Napa and Sonoma Valleys, California

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Hydrothermal contamination of public supply wells in Napa and Sonoma Valleys, California

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ABSTRACT

Groundwater chemistry and isotope data from 44 public supply wells in the Napa and Sonoma Valleys, California were determined to investigate mixing of relatively shallow groundwater with deeper hydrothermal fluids. Multivariate analyses including Cluster Analyses, Multidimensional Scaling (MDS), Principal Components Analyses (PCA), Analysis of Similarities (ANOSIM), and Similarity Percentage Analyses (SIMPER) were used to elucidate constituent distribution patterns, determine which constituents are significantly associated with these hydrothermal systems, and investigate hydrothermal contamination of local groundwater used for drinking water. Multivariate statistical analyses were essential to this study because traditional methods, such as mixing tests involving single species (e.g. Cl or SiO_2) were incapable of quantifying component proportions due to mixing of multiple water types. Based on these analyses, water samples collected from the wells were broadly classified as fresh groundwater, saline waters, hydrothermal fluids, or mixed hydrothermal fluids/meteoric water wells. The Multivariate Mixing and Mass-balance (M3) model was applied in order to determine the proportion of hydrothermal fluids, saline water, and fresh groundwater in each sample. Major ions, isotopes, and physical parameters of the waters were used to characterize the hydrothermal fluids as Na–Cl type, with significant enrichment in the trace elements As, B, F and Li. Five of the wells from this study were classified as hydrothermal, 28 as fresh groundwater, two as saline water, and nine as mixed hydrothermal fluids/meteoric water wells. The M3 mixing-model results indicated that the nine mixed wells contained between 14% and 30% hydrothermal fluids. Further, the chemical analyses show that several of these mixed-water wells have concentrations of As, F and B that exceed drinking-water standards or notification levels due to contamination by hydrothermal fluids.

1. Introduction

Groundwater quality in the ~2600 km^2 North San Francisco (NSF) Bay study unit (Fig. 1) was investigated from August to November, 2004 and again in November 2007 as part of the Priority Basin Project of the California State Water Board's Groundwater Ambient Monitoring and Assessment (GAMA) Program (Belitz et al., 2003; http://ca.water.usgs.gov/gama/). Details of sample collection, analyses and quality-assurance procedures are described by Kulongoski et al. (2006). Here, the data from 44 wells in Napa, Sonoma and Marin Counties were used to identify the primary constituents associated with deep hydrothermal fluids.

Multivariate analyses were applied in order to elucidate constituent distribution patterns, determine which constituents are significantly associated with hydrothermal systems, and quantify the contribution of the hydrothermal system to groundwater used for drinking-water supply. Multivariate statistical methods can help elucidate groundwater flow in complex aquifer systems, particularly when integrated with information regarding geological and hydrological settings (e.g. Farnham et al., 2000; Stetzenbach et al., 2001; Cloutier et al., 2008). Principal Components Analyses (PCA) have been applied to several large and complex water quality datasets obtained from groundwater monitoring studies (e.g. Cruz and Franca, 2006; Kouras et al., 2007). Multi-sample graphical techniques such as Piper plots, used in tandem with PCA and Cluster Analyses, can also facilitate the classification of large numbers of water samples into major groups, help determine the factors affecting groundwater quality, and assist with the identification of water–rock interaction and groundwater redox conditions (Melloul and Collin, 1992; Schot and van der Wal, 1992; Güler et al., 2002).

Relatively few studies have attempted to quantify mixing between deep hydrothermal systems and public supply aquifers. Near the geothermal field of Los Azufres in Mexico, Birkle and Merkel (2000) investigated the lateral extent and distribution of constituents

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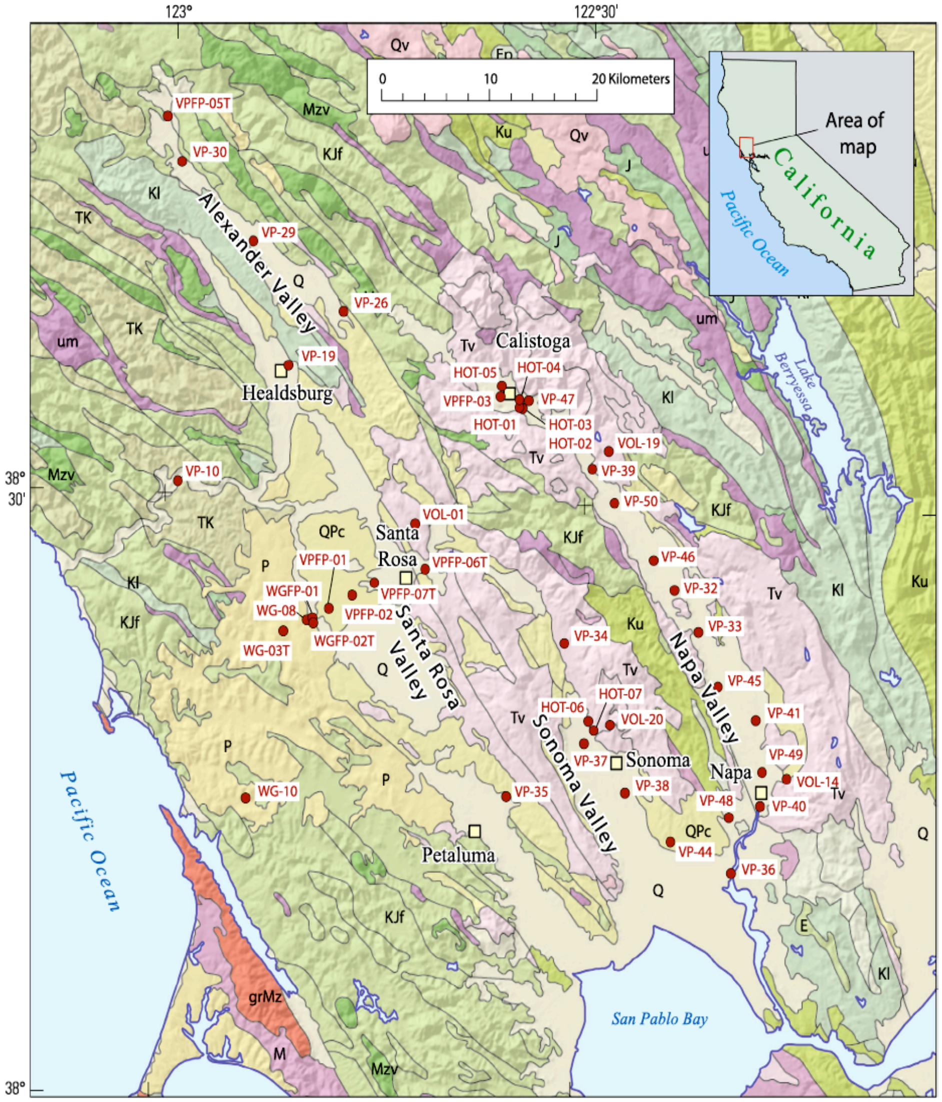

Fig. 1. Geologic map of the North San Francisco Bay study unit with locations of sampled wells (from Kulongoski et al., 2010).

associated with the hydrothermal system, finding that W, Li, Cs, Mo, As and B are suitable tracers of hydrothermal contamination, and that rivers discharge these elements as far as 10 km away. In Ethiopia's central Rift Valley Region, Reimann et al. (2003) documented that the deepest drinking water wells have elevated concentrations of As, B, F, Ge, I, Li. Mo, Na, Rb, Sb, Ta, U and W due to a hydrothermal component in the groundwater. Aksoy et al. (2009) investigated shallow groundwater contamination in the Balcova Geothermal system in Izmir, Turkey, and found that the cold groundwater in the alluvial aquifer mixes with hydrothermal fluids, resulting in elevated concentrations of As, Sb and B. Near the Akarcay Basin in Turkey, Dogdu and Bayari (2005) investigated the impact of thermal water on ground and surface waters, and found that mixing with hydrothermal fluids results in higher electrical conductivity, warmer temperatures, and elevated concentrations of Na, Cl, Fe, Li, B, Br, Mn, Al, I and As.

The elements F and As are often useful in determining whether hydrothermal contamination is occurring, despite the fact that they may be affected by water–rock interaction (Mahon, 1970; Aiuppa et al., 2003). Fluoride is often highly enriched in hydrothermal fluids (Ellis and Mahon, 1967; Dissanayake, 1991; Reimann et al., 2003; Shitumbanuma et al., 2007), commonly exceeding the US EPA Maximum Contamination Level (MCL) for drinking water of 4.0 mg/L. Water at high temperatures can also leach As from rock, which explains the high concentrations of As in many hydrothermal fluids – often exceeding the MCL for drinking water of 10 µg/L. Angelone et al. (2009) found that the occurrence of As in groundwater of the Cimino-Vico volcanic area in central Italy is mainly connected with upflowing fluids from deep hydrothermal systems, with the highest As concentrations in thermal springs and wells.

Hydrothermal contamination of public supply aquifers may have serious ramifications because As has been linked to cancer of the bladder, lungs, skin, kidney, nasal passages, liver and prostate, and elevated F in drinking water may lead to increased likelihood of bone fractures in adults and mottling and pitting in tooth enamel in children (US EPA, 2006). Boron has also been classified as a pollutant of drinking water in national and international drinking water legislation (WHO, 1998 Guidelines: 2.4 mg/L, EU Directive, 1998 98/83/EC: 1 mg/L). The California Department of Public Health Drinking Water Program Notification Level for B is 1.0 mg/L. Boron also affects the health and production of certain plants (Nable et al., 1997), and becomes toxic for sensitive crops (e.g. avocado, citrus fruits) at concentrations >1 mg/L in irrigation water (Kloppmann et al., 2005).

1.1. Hydrogeologic setting

The NSF study unit (Fig. 1) extends from Alexander Valley in the north to the San Francisco Bay (San Pablo Bay) in the south, and includes most of the alluvium-filled basins that result from a series of NW-trending structural depressions in the southern part of the Coast Ranges of northern California (Bailey et al., 1964; Fox, 1983). Mountain ranges 300–1200 m in altitude bound the study area to the north and east, and separate the Napa Valley, Sonoma Valley and the Santa Rosa Plain. The San Francisco Bay bounds the study area to the south, and the Wilson Grove Formation Highlands bounds the study area to the west. The valleys consist of a relatively thin cover of Quaternary alluvium overlying a thick section of Neogene volcanics and sedimentary rocks, Cretaceous sedimentary rocks, Franciscan Complex sedimentary and metamorphic rocks, and Jurassic serpentinite (Bailey et al., 1964; Fox, 1983). In these valleys, the main water-bearing unit is the alluvium and underlying Neogene sedimentary and volcanic rocks. The thickness of alluvium increases from north to south and from the margin of the valleys towards the rivers. In most valleys, the thickness of the alluvium ranges from less than 3 m to more than 90 m. Water in the alluvium is generally unconfined and moves under a natural hydraulic gradient, reflecting the surface topography (Faye, 1973). Groundwater recharge to the alluvial aquifers occurs by stream-channel infiltration beneath the major rivers and their tributaries, and by direct infiltration of precipitation on alluvial fans.

The climate in the study area is characterized by warm, dry summers and cool, moist winters. The National Climate Data Center station in Sonoma reports the average annual temperature of 15 °C, and the average annual precipitation of 0.76 m, occurring as rain during the winter and early spring. However, the distribution of precipitation across the study area depends on the topography, as precipitation increases with altitude.

1.2. Napa Valley-Calistoga hydrothermal system

The hydrothermal system near Calistoga is thought to be two or more hydrothermal convection systems (Youngs et al., 1980) that are associated with faulting along the axis of the upper Napa Valley at Calistoga (Fig. 1). In this system it appears that heated meteoric water ascends along fault or fracture zones to near surface depths. Measured temperatures of wells and springs range from 20 to 130 °C (Murray, 1996). Drilling and well tests by Taylor et al. (1981) support this hypothesis, and show the highest temperature surficial waters coinciding with projected traces of faults. Detailed gravity surveys identified two negative gravity anomalies in the upper Napa Valley, the larger of which is located approximately 1 km SW of Calistoga (Chapman et al., 1982). According to Murray (1986), the gravity anomaly near Calistoga may represent partially molten silicic volcanic rock intruded into the higher density rock of the Franciscan Formation at a depth of 1–2 km. Murray et al. (1985) also suggested that these gravity anomalies may represent shallow magma chambers that were the source of the late Pliocene–Pleistocene volcanic sequences in the Calistoga-Clear Lake region, and that residual heat from these, now-crystallized, shallow intrusive rocks, may be the heat source for the hydrothermal activity at Calistoga. Murray (1996) found that the upper Napa Valley hydrothermal fluids are distinguished by elevated concentrations of Na (>170 mg/L), Cl (>180 mg/L), B (>8 mg/L), and F (>7 mg/L), while the non-hydrothermal waters of the shallow unconfined aquifer are Ca–Mg–HCO_3 type waters with relatively high concentrations of Mg, Ca, Fe, SO_4 and HCO_3 (Murray, 1996; Kulongoski et al., 2010).

1.3. Sonoma Valley hydrothermal system

In Sonoma Valley, hydrothermal fluids with temperatures ⩾20 °C, have been identified in wells and thermal springs across an area that extends north from Sonoma (Fig. 1), and includes Fetter's Hot Springs, Boyes Hot Springs, and Agua Caliente (Waring, 1915; California Division of Mines and Geology, 1984). The north Sonoma hydrothermal system was constrained to depths from 15.2 to 167.6 m (50–550 ft) below land surface based on temperature gradient data from wells (Farrar et al., 2006). Hydrothermal fluids in the southern part of Sonoma Valley may be separate from the northern Sonoma hydrothermal system, and could be related to upflow along fractures in the Rodgers Creek Fault Zone (Farrar et al., 2006). The Eastside fault is thought to form the western boundary for the hydrothermal systems (California Division of Mines and Geology, 1984).

Hydrothermal fluids in the Sonoma area generally are Na–Cl type waters and often contain As, B, F and Li in concentrations that exceed drinking-water standards (California Division of Mines and Geology, 1984; Farrar et al., 2006; Kulongoski et al., 2010). Hydrothermal fluids are significant components in some wells in the Sonoma Valley, particularly in the area between Fetters Hot Springs and the city of Sonoma (Farrar et al., 2006). The source and movement of hydrothermal fluids in the Sonoma Valley may be similar to those in the upper Napa Valley, where hot mineralized waters upwell along faults or fractures extending from depth to near land surface (Murray, 1996). The most mineralized hydrothermal fluids in the Sonoma Valley, characterized by the composition of samples from wells HOT-07 and HOT-06 from this study, may coincide with the topographic axis of the valley, as in the upper Napa Valley.

2. Methods

Water samples were collected from 96 wells in 2004 using standard USGS protocols (Kulongoski et al., 2006), and 21 of these wells were resampled in November of 2007 using the same protocols. Samples were analyzed for major and minor ions, trace elements, TDS, isotopes ($^3He/^4He$, $\delta^{18}O$, δD), pH, DO, SC and water temperature. Complete data were available for 44 wells used in this study, except for the well VP-44, which was missing data for $^3He/^4He$. Seven wells identified as hydrothermal wells by Kulongoski et al. (2010) were sampled, four in northern Napa Valley near the city of Calistoga (HOT-01, HOT-02, HOT-03, HOT-04 and HOT-05) and two in Sonoma Valley near Agua Caliente (HOT-06 and HOT-07) (Fig. 1). The hydrothermal well samples ranged in temperature from 29.5 °C to 97.5 °C, the hottest from a well near the active geyser 'Old Faithful' in Calistoga (HOT-05).

2.1. Statistical methods

Non-parametric statistical methods were used to test the significance of correlations between water temperature, constituent concentrations, isotopes and well type (i.e. hydrothermal, groundwater, saline water). Correlations were investigated using Spearman's method to calculate the rank-order correlation coefficient (ρ) between continuous variables. The values of ρ can range from

+1.0 (perfect positive correlation) to 0.0 (no correlation) to −1.0 (perfect negative correlation). The significance level (p) used to test hypotheses for this report was compared to a threshold value (α) of 5% ($\alpha = 0.05$) to evaluate whether the relation was statistically significant ($p < \alpha$).

2.1.1. Multivariate statistical analyses

PRIMER (v. 6.0) software (Clarke and Warwick, 2001) was used for multivariate statistical analyses of the measured constituents. Prior to analyses, data were 4th-root transformed and normalized to eliminate inappropriate weighting of some measurements. In the multivariate analyses of environmental data, normalization is usually essential because environmental data have different measurement scales or units (e.g. concentrations in μg/L, mg/L, temperatures in °C, etc.) (Clarke and Warwick, 2001). After normalization, the data are dimensionless, and can be compared by examining and interpreting relationships based on Euclidean distances (distance measure between vectors where squared differences between corresponding elements are summed, followed by taking the square root of this sum). These transformed and normalized data were then used to generate a resemblance matrix based on Euclidean distances between site pairs for subsequent multivariate statistical analyses.

Cluster Analyses take a similarity matrix as their starting point and successively fuse samples into groups, and the groups into larger clusters starting with the highest mutual similarities; then gradually decrease the similarity levels at which the groups are formed (Clarke and Warwick, 2001). The results of the hierarchical clustering are represented by a dendrogram, with the samples on the x-axis, and the y-axis defining the similarity levels (in Euclidean distances) at which samples or groups are considered to have fused.

The purpose of Multidimensional Scaling (MDS) is to construct a visual representation of a complex set of relationships using a “map” or ordination of the samples in a specified number of dimensions, which attempts to satisfy all of the conditions imposed by the rank similarity matrix (Clarke and Warwick, 2001). An MDS map represents an optimal configuration of the sample points in two-dimensional space, where proximity indicates similarity.

Principal components analysis (PCA) is a recognition technique used to explain the variance of a large set of intercorrelated variables with a smaller set of independent variables, or principal components. PCA is the longest-established method of approximating high-dimensional information in low-dimensional plots, and is particularly useful for multivariate analyses of environmental data (Clarke and Warwick, 2001). PCA is an ordination in which samples are regarded as points in high-dimensional variable space, and are projected onto a best fitting plane, or other low dimensional solution. The principal components (PC) are simply a rotation of the original axis, and thus a linear combination of the input variables. The purpose of the principal components is to capture as much of the variability in the original space as possible, and the extent to which the first few PC’s allow an accurate representation of the true relationship between the samples in the original dataset is summarized by the % variation explained.

Analysis of Similarities (ANOSIM) tests operate on a resemblance matrix, and carry out an approximate analog of the standard 1- and 2-way ANOVA (Analysis of Variance) tests. ANOSIM provides a test of the null hypothesis that there are no assemblage differences between groups of samples (Clarke and Gorley, 2006).

Similarity Percentage Analyses (SIMPER) are useful in interpreting differences between groups of samples when they have been shown to exist. SIMPER examines the roles of individual constituents contributing to the separation between two groups of samples or the “closeness” of samples within a group. SIMPER operates on the dissimilarities in their high-dimensional relationships, and not on the approximations represented by a 2-D ordination (MDS) (Clarke and Gorley, 2006).

2.2. Multivariate Mixing and Mass-balance model (M3)

The M3 method uses multivariate analyses to aid in understanding groundwater compositions by assuming that the groundwater chemistry is a result of mixing between reference waters and water/rock interaction (Laaksoharju et al., 1999). In evaluating the chemical evolution of groundwater, the M3 model differs from many geochemical models which are based primarily on water–rock interaction, rather than groundwater mixing. M3 modeling of groundwater mixing involves (1) a standard Principal Component Analysis (PCA) of the data, (2) a mixing evaluation, and (3) a mass-balance calculation. M3 modeling uses the PCA method to analyze similarities in groundwater compositions in order to identify components that can be used to investigate mixing and chemical reactions within the groundwater, and to quantify component proportions. The method quantifies hydrochemical variations in δD, δ^{18}O, and chemical species due to mixing of groundwater masses by comparing groundwater compositions to reference waters. The mixing calculations (i.e., mixing portions as a percentage of a selected reference water) determine how much of the observed groundwater composition is due to mixing between the selected reference waters (Laaksoharju et al., 1999).

The following wells were selected as reference waters for M3 analyses: HOT-05, the hottest well (97.5 °C) as the reference hydrothermal fluids from Calistoga (HC); WG-03T as the reference well for groundwater (GW); and VP-36 as the reference well for saline water (SW) (Kulongoski et al., 2010). While none of these wells should be regarded as “pure end-members”, they are considered to be the most representative of each of the water types relevant to this study. Well VP-44 was not used in the M3 modeling because ^{3}He/^{4}He data were not available for this well, and the constituents Zn, Cu, Al, Fe, Pb, Ni, V, and Co were not used in the PCA analyses within the M3 model because they provided insignificant contributions to variance in the principal components.

3. Results

Concentrations of trace elements (in μg/L) are presented in Table 1. Values for temperature, pH, isotope ratios, concentrations of major and minor elements, and Total Dissolved Solids (TDS) are presented in Table 2. Table 3 presents mean and median values for some important constituents: water temperature, ^{3}He/^{4}He, B, Cl, F, Mg, As, Li, δD, and δ^{18}O, and the ratios of Cl/B arranged according to water types.

3.1. Statistical results

3.1.1. Spearman’s correlations

The constituents with significant ($p \leqslant 0.05$) positive correlation to water temperature were Li, W, Si, F, K, Na, Mo, Hg, Be, B, ^{3}He/^{4}He, pH and As. The constituents with significant negative correlation to water temperature were Sr, Mg, Ca, δD, Ni, δ^{18}O and Co. The constituents significantly positively correlated to ^{3}He/^{4}He were Hg, Al, Li, Be, W, water temperature, Si, pH and B; while Co and Mg were significantly negatively correlated with ^{3}He/^{4}He (Table 4).

Initial classifications of the wells in terms of water types were based on Spearman’s Correlation results, which identified constituents significantly correlated with water temperature and high ^{3}He/^{4}He ratios, known indicators of hydrothermal fluids (Murray, 1996; California Division of Mines and Geology, 1984; Farrar

Table 1

Trace elements for all 44 wells used in statistical analyses (in μg/L). Well type classifications are defined as follows: HC: hydrothermal fluids from Calistoga; HSON: hydrothermal fluids from Sonoma; MW: mixed hydrothermal/meteoric water; SW: saline water; GW: non-hydrothermal groundwater.

GAMA identification no.	Well type	Water temperature (°C)	Aluminum, dissolved (μg/L)	Arsenic, dissolved (μg/L)	Boron, dissolved (μg/L)	Barium, dissolved (μg/L)	Cobalt, dissolved (μg/L)	Copper, dissolved (μg/L)	Iron, dissolved (μg/L)	Lithium, dissolved (μg/L)	Molybdenum, dissolved (μg/L))	Manganese, dissolved (μg/L)	Nickel, dissolved (μg/L))	Lead, dissolved (μg/L)	Selenium, dissolved (μg/L)	Strontium, dissolved (μg/L)	Vanadium, dissolved (μg/L)	Tungsten, dissolved (μg/L)	Zinc, dissolved (μg/L)
VP-10	GW	16.5	0.01	0.3	323	100	0.141	0.4	117	3.5	0.5	203	3.02	0.05	0.01	195	0.9	0.01	2.5
VP-19	GW	22.9	0.01	0.4	331	100	0.072	1.8	10	3.4	0.6	42.2	1.76	2.07	0.01	214	1.2	0.01	9.2
VP-26	GW	22	0.01	1.5	124	247	0.113	1	21	14.6	4.5	241	0.39	0.09	0.01	264	2.7	0.01	5
VP-29	GW	16.7	0.01	0.1	258	172	0.127	2.3	0.1	6.5	0.2	0.1	2.48	1.02	0.01	352	1.4	0.01	49.7
VP-30	GW	18	1.4	0.5	1350	203	0.155	0.6	13	18.8	0.7	36.8	2.23	0.32	0.7	379	1.9	0.01	6.3
VP-33	GW	21.2	0.01	3.8	29	31	0.139	0.01	1090	14.1	0.5	139	1.03	7.97	0.01	127	0.001	0.01	19
VP-34	GW	19.4	0.01	7.1	120	35	0.072	0.4	999	4.7	1.8	434	0.26	0.001	0.01	30.9	1.3	0.01	20.1
VP-35	GW	19.3	1.4	8	171	35	0.067	0.7	17	27.5	5.1	3.6	0.69	0.38	1.6	384	7.4	0.01	10.1
VP-37	GW	19.3	0.01	3.4	73	64	0.056	3.4	6	25.6	0.6	6.4	1.49	0.37	0.7	134	11.2	0.01	5.6
VP-39	GW	20	0.8	6	293	27	0.096	0.3	43	68.9	1.8	78.1	0.34	2.68	0.01	39.8	3.7	0.9	9.4
VP-41	GW	20.4	0.01	2.6	52	148	0.331	13.1	0.1	33.5	1.6	928	0.62	1.77	0.01	262	3.1	0.01	5.8
VP-46	GW	18.1	0.9	0.01	70	106	0.079	0.5	22	6.2	0.6	1.6	2	0.35	1	278	1.4	0.01	18.8
VP-47	GW	18.1	3.7	0.2	627	19	0.052	14.8	0.1	12.3	0.4	0.4	0.4	15.3	0.01	98.9	0.6	0.01	34.3
VP-48	GW	19.8	1	4.9	380	194	0.061	0.3	714	17.2	0.7	181	5.21	0.08	0.7	213	0.7	0.8	0.7
VP-50	GW	18.6	0.01	7.7	109	71	0.205	0.01	57	2.9	5.7	687	0.62	0.07	0.01	131	6.4	0.01	8.9
VPFP-01	GW	28	1	1	53	9	0.08	0.4	36	20.5	1.1	66.4	0.05	0.58	0.01	86.2	0.001	0.01	0.6
VPFP-02	GW	18	0.01	2.2	93	223	0.138	0.5	0.1	10.8	1.1	355	0.3	0.27	0.01	276	10	0.01	1.9
VPFP-03	GW	18.2	0.01	1.3	825	70	0.509	2.5	4	4.8	1.3	938	5.58	0.29	1.7	167	1.4	0.01	2.2
VPFP-05T	GW	20	1.1	0.38	345	108	0.03	3.6	4	3	0.4	0.4	0.72	2.19	0.05	241	0.9	0.01	0.86
VPFP-07T	GW	18.5	5.3	1.4	80	121	0.03	1.4	0.1	10	0.9	2.2	0.39	0.37	0.33	214	6.6	0.01	4
VOL-01	GW	23.5	0.01	1.4	28	8	0.048	2.8	80	10.3	0.2	4.8	0.46	0.73	0.01	61.9	19.6	0.01	3.5
VOL-14	GW	21.5	0.9	7.1	18	6	0.132	3.5	0.1	11.2	1.5	10.7	0.76	2.79	0.01	38	15.4	0.01	8.2
VOL-19	GW	24.8	1	1.3	42	49	0.436	0.6	235	23.2	0.6	127	3.72	0.35	0.01	33.5	1.5	0.01	63.8
VOL-20	GW	25.2	0.01	5.6	71	9	0.019	0.4	4	42.3	1.9	0.3	0.25	0.63	0.01	29.8	11.6	0.6	15
WG-03T	GW	17	2.2	1.6	7	3	0.45	0.01	2420	8.5	0.6	76	8.8	0.23	0.01	176	0.06	0.01	3.9
WG-08	GW	19	1.1	13	16	1	0.17	0.9	40	12.4	1.2	12.9	1.08	0.11	0.3	198	1.1	0.01	1.7
WGFP-01	GW	19.5	0.01	4.8	22	4	0.14	0.4	29	10.6	0.7	16	0.43	0.59	0.01	188	1.7	0.01	4.2
WGFP-02T	GW	19	5.3	3.1	10	5	0.001	0.61	4	6	0.3	1.3	0.13	0.25	0.36	178	3.6	0.01	0.68
HOT-01	HC	81	17.3	70.5	9590	0.78	0.017	0.6	45	1920	6.5	12.4	0.27	0.06	0.8	110	0.6	112	0.6
HOT-02	HC	60	6.2	50.2	11,000	0.594	0.012	0.5	28	1450	6.2	17.2	0.17	0.001	0.01	39.6	0.001	138	0.01
HOT-04	HC	63	5.7	64.6	11,200	0.936	0.018	1	272	1440	3.4	88.8	0.57	0.04	0.7	34.5	0.1	234	26.8
HOT-05	HC	97.7	126	128	11,100	1	0.016	0.6	14	1720	10.3	2.8	0.2	0.11	0.01	54.9	0.7	152	15.7
HOT-07	HSON	41.5	4.7	3	15,700	20	0.023	0.3	41	1650	13.2	11.9	0.21	0.19	1.4	21.8	0.001	54.2	0.01
HOT-06	MW	34.3	2.4	12.4	3570	14	0.094	1.2	302	703	2.8	373	0.5	0.04	0.4	161	0.2	7.6	8.8
HOT-03	MW	29.5	1	30.9	2530	25	0.144	1.3	4	224	3.4	559	1.21	0.001	0.7	102	9.3	2	1.4
VP-32	MW	35.4	0.8	8.8	283	69	0.258	4.5	5	52.5	1.5	129	0.24	4.12	0.2	124	1.5	0.9	4.2
VP-38	MW	25.7	1.2	17.2	3830	69	0.031	3.3	12	61.3	13.7	14.5	0.25	0.73	0.5	83.9	1	0.01	5.9
VP-40	MW	33	1.2	0.01	474	13	0.067	0.01	331	80.9	1.8	40.1	1.23	0.001	0.01	127	0.6	0.7	15.6
VP-45	MW	28	0.01	32.8	496	56	0.033	0.2	135	72	8.4	50.1	0.68	0.77	0.01	84.6	2	2.3	13.2
VP-49	MW	19.6	1.8	24.6	316	115	0.216	0.3	362	72.9	20.3	435	0.66	0.24	0.01	142	0.9	0.01	4.3
VPFP-06T	MW	28	1.3	1.5	482	40	0.02	0.22	154	67	2.2	59.7	0.12	0.34	0.01	79.2	0.04	0.74	1.1
VOL-18T	MW	28.5	1	0.88	463	112	0.05	0.01	332	48.5	1.8	54.4	0.16	0.001	0.01	97.2	1	0.66	2
VP-36	SW	18.6	0.01	5.7	136	492	0.141	0.3	671	33.8	1	1220	0.65	1.07	2	440	0.4	0.01	49.8
VP-44	SW	21	1.1	3.3	819	121	0.092	1.4	310	68.3	20	135	0.63	0.001	0.3	384	0.4	0.01	29.6

Table 2

Values for temperature, pH, isotope ratios, concentrations of major and minor elements, and Total Dissolved Solids (TDSs) for all 44 wells used in statistical analyses (in mg/L). Well type classifications are defined as follows: HC: hydrothermal fluids from Calistoga; HSON: hydrothermal fluids from Sonoma; MW: mixed hydrothermal/meteoric water; SW: saline water; GW: non-hydrothermal groundwater.

GAMA identification no.	Well type	Water temperature (°C)	pH (standard units)	Deuterium/ protium (‰)	Oxygen-18/ Oxygen-16 (‰)	Helium-3/Helium4 (isotope ratio) × 10^{-6}	Total dissolved solids (mg/L)	Sodium, dissolved (mg/L)	Chloride, dissolved (mg/L)	Fluoride, dissolved (mg/L)	Silica, dissolved (mg/L)	Sulfate, dissolved (mg/L)	Magnesium, dissolved (mg/L)	Potassium, dissolved (mg/L)	Calcium, dissolved (mg/L)	Bromide, dissolved (mg/L)	Iodide, dissolved (mg/L)
VP-10	GW	16.5	6.5	−39.90	−6.28	0.83	213	10.9	6.73	0.37	29.6	11.3	26.3	0.94	23.2	0.06	0.005
VP-19	GW	22.9	7.4	−51.00	−7.41	0.96	162	8.39	5.19	0.37	22.5	9.63	18.7	1.25	22.8	0.03	0.004
VP-26	GW	22	7.5	−44.10	−6.92	0.58	381	76.7	13.2	0.3	38.1	21.7	19.2	1.3	34.5	0.11	0.069
VP-29	GW	16.7	7.5	−43.70	−6.98	1.02	231	9.06	5.94	0.001	24.9	26.2	24.8	0.9	35.7	0.17	0.001
VP-30	GW	18	6.6	−46.30	−7.04	0.81	254	13.3	9.82	0.001	24.8	19.1	24.5	0.99	38.4	0.07	0.007
VP-33	GW	21.2	7.2	−40.20	−6.40	0.90	270	19.6	8.52	0.16	94.4	19.3	15.6	3.48	26	0.14	0.011
VP-34	GW	19.4	7.7	−46.50	−7.12	2.85	185	23.4	10.6	0.34	85.3	2.58	6.26	4.7	9.14	0.07	0.02
VP-35	GW	19.3	7.3	−39.10	−5.89	1.04	318	58.8	30.8	0.22	74.6	21.2	12.6	2.2	25.1	0.07	0.003
VP-37	GW	19.3	7.6	−39.00	−6.00	1.34	255	28.5	23	0.16	67.8	14.8	14.7	1.32	25.1	0.12	0.002
VP-39	GW	20	7.6	−46.80	−7.11	1.17	267	42.3	7	0.37	105	5.46	7.88	6.79	13.9	0.09	0.077
VP-41	GW	20.4	7.3	−40.30	−5.99	0.98	313	39.2	42.4	0.24	50.2	23.6	20.9	2.09	33	0.19	0.015
VP-46	GW	18.1	7.2	−42.60	−6.43	2.95	249	21.5	9.88	0.13	29.7	43.7	22.4	0.59	32.7	0.71	0.003
VP-47	GW	18.1	7.3	−39.70	−6.06	1.22	194	25.4	21.3	0.56	33.1	37.9	7.47	0.55	22.9	0.14	0.005
VP-48	GW	19.8	7.7	−40.90	−6.35	0.39	464	113	62.9	0.16	47.6	14.9	16.2	13.2	18.6	0.11	0.179
VP-50	GW	18.6	7	−48.50	−7.15	0.63	261	27.1	5.1	0.26	69.5	3.84	22.7	8.19	18.9	0.07	0.038
VPFP-01	GW	28	8.1	−38.90	−6.25	0.61	179	30.2	13.2	0.1	54.4	4.7	3.64	1.52	17.5	0.05	0.006
VPFP-02	GW	18	6.9	−38.70	−5.93	0.97	364	40	47.2	0.001	38	15.4	29.4	1.89	44.2	0.13	0.024
VPFP-03	GW	18.2	7.2	−41.20	−6.33	0.38	346	31	41.7	0.26	52.9	32.7	40.1	2.67	28.4	0.25	0.011
VPFP-05T	GW	20	7	−49.50	−7.33	1.01	155	8.3	4.72	0.09	15.2	10.1	12	1.03	23.8	0.02	0.002
VPFP-07T	GW	18.5	7.3	−38.70	−5.94	1.06	315	28.6	23.8	0.17	38.7	14.7	30.2	1.79	37.2	0.14	0.005
VOL-01	GW	23.5	7	−41.00	−6.46	0.91	214	12.9	5.55	0.35	98.5	1.42	10.8	1.74	19.2	0.03	0.001
VOL-14	GW	21.5	7.2	−39.90	−6.12	1.03	185	13.4	7.79	0.15	87	7.4	7.57	2.18	12.8	0.03	0.004
VOL-19	GW	24.8	6.8	−47.00	−7.39	0.90	184	15.9	5.34	0.27	97.9	5.94	8.1	4.41	7.34	0.04	0.002
VOL-20	GW	25.2	7.7	−45.00	−6.98	0.86	197	31.3	4.62	0.34	87	4.56	4.09	4.34	8.96	0.03	0.001
WG-03T	GW	17	6.5	−37.20	−6.06	0.96	202	22.5	27.2	0.08	62.3	32.9	6.11	1.65	13.5	0.11	0.009
WG-08	GW	19	7.2	−37.70	−6.12	1.01	263	18.2	13.2	0.07	42.2	20.3	6.38	1.24	53.6	0.06	0.004
WGFP-01	GW	19.5	7.8	−37.70	−6.14	0.99	242	22	15.4	0.1	37.9	14	3.77	1.33	50	0.08	0.007
WGFP-02T	GW	19	7.8	−38.30	−6.14	1.06	237	18.4	16.1	0.001	45.5	7.79	2.94	1.13	44.6	0.08	0.002
HOT-01	HC	81	7.8	−50.90	−7.84	4.93	646	186	183	10.2	108	30.8	0.134	7.54	7.63	0.8	0.299
HOT-02	HC	60	7.7	−53.20	−8.28	4.95	685	185	190	9.56	165	0.97	0.274	8.37	5.04	0.73	0.334
HOT-04	HC	63	7.5	−53.90	−8.18	4.90	670	174	194	10.6	164	27.4	0.883	7.9	4.31	0.9	0.351
HOT-05	HC	97.7	8.1	−53.00	−8.25	5.01	648	173	187	11.3	151	10.8	0.015	7.91	6.32	0.75	0.226
HOT-07	HSON	41.5	8.3	−52.00	−7.64	2.53	1230	381	578	8.47	99.5	40.5	0.078	20.2	9.01	2.44	1.61
HOT-06	MW	34.3	7.3	−41.70	−6.09	2.69	673	208	255	2.01	85.6	14	6.33	17.5	39.7	0.37	0.19
HOT-03	MW	29.5	7.2	−40.70	−6.23	1.68	539	138	51.4	1.9	84.1	58.3	12.9	8.93	22.3	0.16	0.01
VP-32	MW	35.4	7.5	−44.20	−6.80	1.57	375	40.2	9.34	0.27	134	32.1	14.7	9.93	29.6	0.1	0.012
VP-38	MW	25.7	8.2	−50.40	−7.75	1.15	503	177	25.5	0.42	52.9	1.72	1.51	2.29	6.26	0.1	0.138
VP-40	MW	33	7.7	−47.10	−6.98	3.58	428	57.8	99.1	0.38	110	3.17	21	5.68	34.5	0.37	0.047
VP-45	MW	28	6.7	−52.30	−7.82	0.21	399	77.9	6.83	0.68	126	19.9	5.95	19.7	15.5	0.06	0.009
VP-49	MW	19.6	7.3	−50.60	−7.48	4.62	417	104	42.5	0.24	64.3	7.12	11	9.02	20	0.16	0.077
VPFP-06T	MW	28	7.7	−42.10	−6.61	3.40	336	49.6	17.2	0.26	86.8	0.11	17.3	7.3	27.3	0.07	0.057
VOL-18T	MW	28.5	7.2	−48.60	−7.22	1.23	321	42.5	12.6	0.35	120	3.76	17.9	6.48	14.5	0.06	0.024
VP-36	SW	18.6	7.6	−52.90	−7.72	0.32	685	148	250	0.16	46.8	12.4	50.8	7.14	51.6	1.44	0.261
VP-44	SW	21	7.9	−47.80	−7.18		1000	270	191	0.1	27.9	239	21.6	3.03	36.5	0.99	1.14

Table 3
Mean (first row) and median (second row in bold text) values of constituents important to the classifications of the wells from this study. Well type classifications are defined as follows: HC: hydrothermal fluids from Calistoga; HSON: hydrothermal fluids from Sonoma; MW: mixed hydrothermal/meteoric water; SW: saline water; GW: non-hydrothermal groundwater.

Well type	Water temperature (°C)	Helium-3/Helium4 (isotope ratio) × 10^{-6}	Boron, dissolved (μg/L)	Chloride, dissolved (mg/L)	Chloride/Boron	Fluoride, dissolved (mg/L)	Magnesium, dissolved (mg/L)	Arsenic, dissolved (μg/L)	Lithium, dissolved (μg/L)	Deuterium/protium (‰)	Oxygen-18/Oxygen-16 (‰)
ALL	27.4	1.68	1771	63.2	317.5	1.42	13.8	12.39	229.5	−44.6	−6.8
	20.7	**1.03**	**293**	**17.2**	**88.3**	**0.26**	**12.3**	**3.40**	**23.2**	**−43.9**	**−6.9**
GW	20.1	1.05	211.4	17.4	401.4	0.20	15.2	3.24	15.5	−42.1	−6.5
	19.4	**0.97**	**86.5**	**11.9**	**153.3**	**0.17**	**13.7**	**1.90**	**11.0**	**−40.6**	**−6.3**
MW	29.1	2.36	1383	57.7	61.3	0.72	12.1	14.34	153.6	−46.4	−7
	28.5	**2.18**	**482**	**25.5**	**33.0**	**0.38**	**12.9**	**12.40**	**72.0**	**−47.1**	**−7**
HC	75.4	4.46	10723	188.5	17.6	10.0	0.33	63.26	1633	−52.8	−8.1
	72	**4.93**	**11050**	**188.5**	**17.3**	**10.2**	**0.2**	**64.60**	**1650**	**−53.1**	**−8.2**

et al., 2006; Kulongoski et al., 2010; Kulongoski and Hilton, 2011). Based on the Spearman correlation tests, the following constituents were considered to be the best indicators of hydrothermal fluids: elevated water temperature and $^3He/^4He$, along with relatively high concentrations of Li, B, F and As. Using these criteria, samples were initially classified as hydrothermal fluids from Calistoga (HC), which included HOT-01, HOT-02, HOT-04, and HOT-05; hydrothermal fluids from Sonoma (HSON): HOT-07; mixed hydrothermal/meteoric water (MW): HOT-06, HOT-03, VP-38, VP-45, VP-49, VP-40, VPFP-06T, VOL18T, and VP-32; saline water (SW): VP-36, VP-44 (Kulongoski et al., 2010); and non-hydrothermal groundwater (GW), which included the remaining samples listed in Tables 1 and 2.

The distributions of isotopes and trace elements in the groundwater samples show several interesting patterns that complicate the analyses and interpretations. Relative to the non-thermal groundwater samples, the hydrothermal fluids from this study are not shifted to heavier $\delta^{18}O$ values, which is generally the case in hydrothermal systems (Panichi and Gonfiantini, 1981). Although the HC wells, and most of the MW wells display higher water temperatures and elevated $^3He/^4He$ ratios, some cold GW wells, such as VP-46 and VP-34 also have anomalously high $^3He/^4He$ ratios, approaching 3.0 Ra (Table 2). High $^3He/^4He$ values are often associated with hydrothermal systems, faulting and volcanism. However, given the sensitivity (and mobility) of He isotope systematics, one can often observe variations in the $^3He/^4He$ ratios, with no change in groundwater chemistry. Magnesium concentration generally decreases with increasing fluid temperature (Fournier and Potter, 1979), and as a result, Mg is very low in oceanic hydrothermal systems (Scott, 1997). While the HC wells showed Mg depletion (mean [Mg] = 0.33 mg/L), the mean [Mg] in the MW wells (12.1) was more similar to the GW wells (mean = 15.2) (Table 3). Moreover, both SW wells and HC wells have elevated concentrations of Na and Cl, and some GW wells have relatively high SiO_2 (i.e. VP-39, VP-33, VOL-19, VOL-01) (Table 1). Therefore, the division of the samples into types is not straightforward on the basis of any commonly used criterion and multivariate techniques were required for these analyses and classifications.

3.1.2. Cluster Analyses

Fig. 2 is a dendrogram of the results of the hierarchical clustering analyses for the samples. The dendrogram reveals that HC samples cluster together. Interestingly, the higher temperature hydrothermal well from Sonoma (HOT-07) forms an isolated branch that clusters with the HC wells at a greater Euclidean distance, suggesting similar composition and/or source. Two wells that had been previously identified as hydrothermal wells (Kulongoski et al., 2010), HOT-06 and HOT-03 do not cluster closely with the HC or HSON samples. In fact, HOT-03 and HOT-06 form clusters with the MW wells.

The HOT-03 sample was collected from a well located within 50 m of the HOT-02 hydrothermal well, however, HOT-03 was drilled to only 14 m below land surface (mbls), while HOT-02 was drilled to 61 mbls. The lower measured temperature of the HOT-03 sample (29.5 °C) compared with the HOT-02 sample (60.0 °C) suggests that it contains a greater proportion of groundwater mixed with hydrothermal fluids. Similarly, HOT-06 had a lower measured temperature (34.3 °C) than HOT-07, the other hydrothermal well from Sonoma (41.5 °C). No information concerning the well depth of HOT-06 is available, however HOT-07 has a reported depth of 300 mbls. HOT-03 and HOT-06, lower temperature and relatively shallower wells, are thus more appropriately classified as mixed hydrothermal/meteoric water (MW), with a significant proportion of groundwater diluting the hydrothermal fluids. The fact that these shallow wells cluster with some of the purportedly "non-hydrothermal" wells also suggests that

Table 4
Results of non-parametric Spearman's method analysis of correlations between selected water-quality constituents and groundwater temperature and $^{3}He/^{4}He$ ratio. *r*: Spearman's correlation statistic; *r* values are shown for tests in which the variables were determined to be significantly correlated on the basis of *p* values (significance level of the Spearman's test) less than threshold value (a) of 0.05; ns: Spearman's test indicates no significant correlation between groups; TDS: Total Dissolved Solids.

Selected water-quality constituent	Groundwater temperature (°C) *p* Value	Groundwater temperature (°C) *r*: Spearman's correlation statistic	$^{3}He/^{4}He$ *p* Value	$^{3}He/^{4}He$ *r*: Spearman's correlation statistic
Aluminum	0.024	0.340	0.000	0.616
Arsenic	0.005	0.426	0.048	0.305
Boron	0.003	0.451	0.009	0.403
Barium	0.003	−0.456	0.007	−0.415
Beryllium	0.001	0.493	0.001	0.502
Bromide	>0.05	ns	0.003	0.456
Calcium	0.001	−0.503	>0.05	ns
Chloride	>0.05	ns	0.004	0.443
Cobalt	0.003	−0.451	0.005	−0.432
Copper	>0.05	ns	>0.05	ns
$\delta^{18}O$	0.002	−0.472	>0.05	ns
δD	0.001	−0.500	>0.05	ns
Fluoride	0.000	0.670	0.006	0.425
Iron	>0.05	ns	>0.05	ns
Mercury	0.005	0.523	0.001	0.663
Iodide	0.004	0.438	0.027	0.341
Potassium	0.000	0.613	>0.05	ns
Lithium	0.000	0.748	0.001	0.508
Magnesium	0.000	−0.563	0.010	−0.396
Manganese	>0.05	ns	>0.05	ns
Molybdenum	0.000	0.552	0.016	0.372
Sodium	0.000	0.580	0.010	0.395
Nickel	0.001	−0.489	0.015	−0.377
Lead	>0.05	ns	0.031	−0.334
pH	0.004	0.437	0.009	0.405
$^{3}He/^{4}He$	0.004	0.447	–	–
Antimony	0.003	0.453	0.025	0.347
Selenium	>0.05	ns	>0.05	ns
Silica	0.000	0.699	0.007	0.418
Sulfate	>0.05	ns	>0.05	ns
Strontium	0.000	−0.617	0.023	−0.351
TDS	0.003	0.456	0.022	0.354
temperature	–	–	0.004	0.447
well type	0.000	0.581	>0.05	ns
Uranium	0.010	−0.390	>0.05	ns
Vanadium	0.048	−0.299	>0.05	ns
Tungsten	0.000	0.746	0.001	0.498
Zinc	>0.05	ns	>0.05	ns

some mixing with hydrothermal fluids is occurring in the following wells: VP-49, VP-45, VP-40, VP-38, VPFP-06T, and VOL-18T. Therefore, in subsequent multivariate analyses, these wells will be considered MW. Although VP-32 does not appear to cluster with the other MW wells, it will be classified with the MW wells due to its very elevated temperature (35.4 °C), $^{3}He/^{4}He$ (1.57 Ra), and [As] (8.8 μg/L).

3.1.3. Multidimensional scaling

Fig. 3 is an MDS map of the 44 samples. The 2-D stress result of 0.13 indicates that the MDS is a useful representation of the data, and supports the results from the Cluster Analyses. The HC wells and the HSON well cluster together at a Euclidean distance of <7. All of the MW wells, cluster together with HOT-03 and HOT-06 at a Euclidean distance of <7. However, a subset of these MW wells also cluster together with the GW wells at a Euclidean distance of <7. Wells with known saline water content (SW): VP-44 and VP-36 (Kulongoski et al., 2010) form a separate and distant cluster.

3.1.4. Principal components analysis

The PCA analyses of the constituents from the samples (Fig. 4) show the same groupings that were apparent in the MDS and Cluster Analyses. The HC wells and the HSON well cluster together. The MW wells cluster together, while the non-hydrothermal GW wells also generally group together, and the SW wells form a separate and distant cluster.

The first five principal components account for 72.9% of the variation (Table 5). The first principal component, PC1, accounting for 40.8% of the variance in the entire dataset, was most strongly correlated with Li, W, F, temp, B, Na, TDS, I and Cl, while PC2 accounted for 12.8% of the variance, and was most strongly correlated with Mn, Sr, Ba, Co, SO_4, Ca, Ni, TDS and Br.

3.1.5. Analysis of similarities

An ANOSIM was performed to test the null hypothesis, that there are no differences between constituents (see Tables 1 and 2) in the hydrothermal wells and the non-hydrothermal public-supply groundwater wells. The results of the ANOSIM test gave an overall (Global R) statistic of 0.728, and a significance level of 0.1% ($p < 0.001$). Therefore, the ANOSIM test indicates that the hydrothermal wells are significantly different from the non-hydrothermal public-supply groundwater wells.

3.1.6. Similarity Percentage Analyses

SIMPER tests were performed to examine the differences between the HC wells and the GW wells. Table 6 lists the average values (after normalization and transformation) of the variables from the GW and HC wells, and the % contributions of each variable to the differences between the two groups. The results indicate that higher values of temp, W, Li, F, B, As, Al, $^{3}He/^{4}He$ and $\delta^{18}O$, along with lower values of Mg in the HC wells provide the greatest

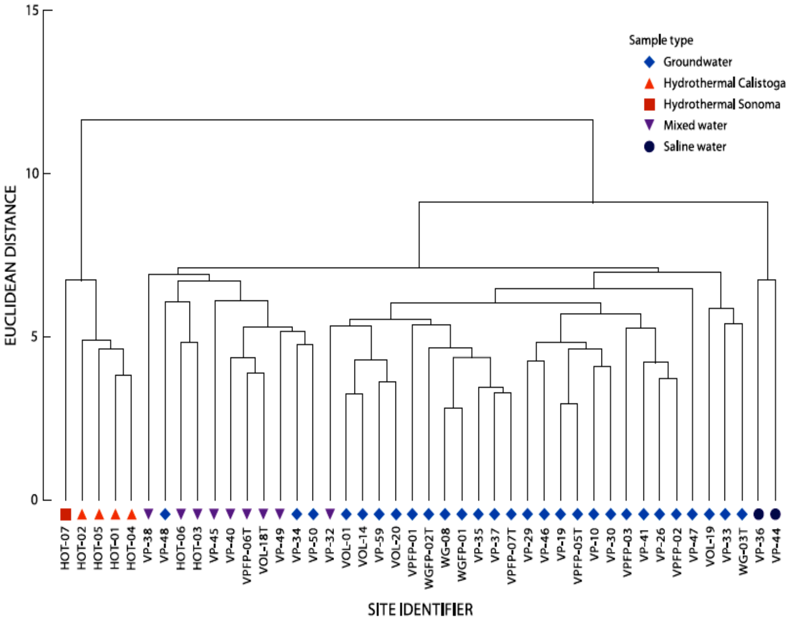

Fig. 2. Dendrogram of the results of hierarchical clustering analyses with the well samples on the *x*-axis, and the *y*-axis defining the similarity levels (in Euclidean distances) at which samples or groups are considered to have fused.

contributions to the differences between the HC wells and GW wells, and explains 52.6% of the difference (Table 6).

Table 7 lists the average values (after normalization and transformation) of the variables between GW wells and mixed MW wells, and the % contributions of each variable to the differences between the two groups. The results indicate that lower values of K, Mo, Si, $^3He/^4He$, Na, Fe, δD, SO_4 and As; and higher Cu, Pb and Ni in the GW wells provide the greatest contributions to the differences between the GW wells and the MW wells, and explains 50.5% of the difference (Table 7).

Table 8 lists the average values (after normalization and transformation) of the variables from the HC wells and MW wells, and the % contribution of each variable to the differences between the two groups. The results indicate that higher values of W, temp, Al, F, As, Li, B and lower values of Ca and Mg in the HC wells provide the greatest contributions to the differences between the HC wells and MW wells, and explains 51.8% of the difference (Table 8).

Table 9 lists the average values (after normalization and transformation) of the variables from the HC wells and the HSON well and the % contributions of each variable to the differences between the two groups. The results indicate that higher values of As, Zn, Al and temperature and lower values of Se, I and SO_4 in the HC wells, provide the greatest contributions to the differences between the HC wells and the HSON well, and explain 54.7% of the difference (Table 9).

3.2. M3 results

Results from the M3 mixing model, which compares all of the samples to the three reference-water components (HC, GW and SW), indicate that the proportion of hydrothermal fluids within the nine MW wells is between 14% and 30% (Fig. 5; Table 10). These results also corroborate the validity of the initial selections of the MW wells based on the Spearman's Correlation results, multivariate analyses, and trace element data from previous studies of hydrothermal systems in the area. The proportions derived from M3 for three hydrothermal wells from Calistoga, HOT-04, HOT-02 and HOT-01 indicate that they contain 81%, 77% and 76% mixtures of the reference hydrothermal well HOT-05. The lower temperature (29.5 °C) HOT-03 well contains only 18% hydrothermal fluids. The higher temperature (41.5 °C) hydrothermal well from Sonoma HOT-07 did not plot within the triangle connecting the reference waters (Fig. 5), while the lower temperature (34.3 °C) MW well from Sonoma HOT-06 appears to be a mix of groundwater (38%), saline water (33%), and hydrothermal fluids similar to those from Calistoga (29%) (Table 10).

4. Discussion

4.1. Constituents associated with hydrothermal wells

Major-ion data for wells with sufficient data were plotted on Piper diagrams (Fig. 6). Piper diagrams show the relative abundance of major cations and anions (on a charge equivalent basis) as a percentage of the total ion content of the water (Piper, 1944). In a majority of the GW wells, Ca plus Mg were the dominant cations, and HCO_3 generally accounted for a majority of the total anions, however some GW samples had more Na and K cations. In the MW wells, Na plus K were the dominant cations, and HCO_3 also accounted for a majority of the total anions. As such, the GW wells are described as Ca–Mg–HCO_3 type waters, while the MW wells are described as Na–K–HCO_3 type waters. The HC and HSON wells are described as Na–Cl waters.

In addition to elevated concentrations of Na and Cl, the multivariate statistical analyses employed indicate that the hydrothermal fluids from the HC wells also have higher values of water temperature, W, Li, F, B, As, Al and $^3He/^4He$, and are depleted in Mg when compared with GW wells. Of these constituents $^3He/^4He$, measured temperature, Cl, B and Li are the most likely to provide a means of evaluating the contribution of the hydrothermal system

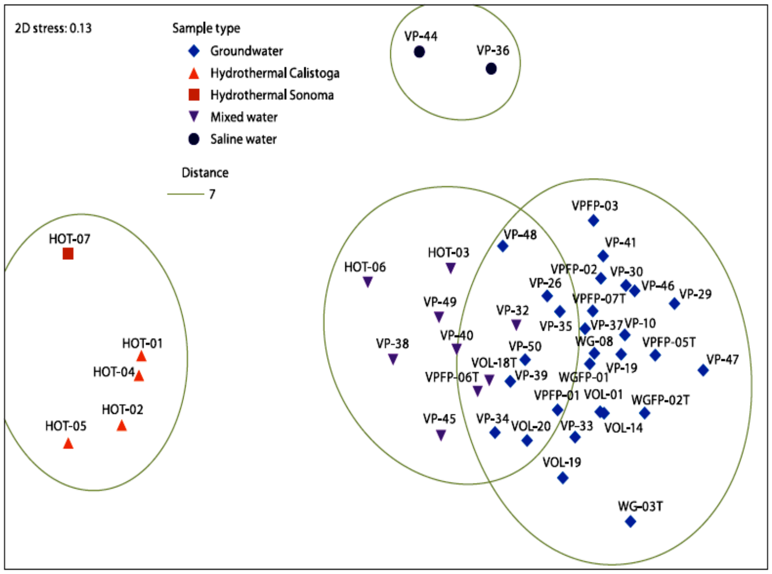

Fig. 3. Multidimensional Scaling (MDS) Map. The proximity of the well samples to each other indicates how similar they are to each other. The 2-D stress result of 0.13 indicates that the MDS is a useful representation of the data.

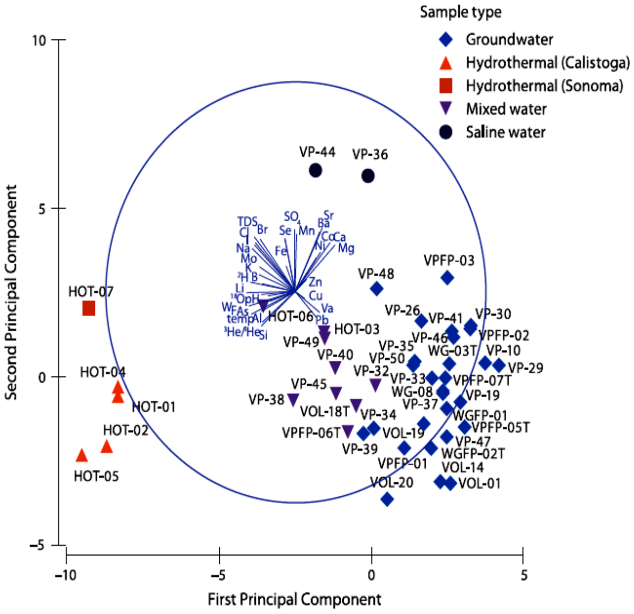

Fig. 4. Principal components analysis (PCA) ordination of well samples.

to drinking water wells because they generally behave conservatively, and their concentrations are not usually controlled by temperature- and pressure-dependent chemical reactions involving rock minerals (Ellis, 1970; Brondi et al., 1973; Henley and Ellis, 1983). Helium isotope ratios are extremely sensitive indicators of magma-related hydrothermal activity because the contrast in $^3He/^4He$ ratios between crustal (typical $^3He/^4He < 0.02Ra$, where Ra is the $^3He/^4He$ ratio of air = 1.4×10^{-6}) and magmatic systems (typical $^3He/^4He \sim 8Ra$) provides a means of identifying even small amounts of mantle input (Kulongoski and Hilton, 2011). Elevated B concentrations have also been attributed to hydrothermal contamination in multiple locations, including Mexico (Birkle and Merkel,

Table 5
Coefficients in the linear combinations of variables making up PC's (Eigenvectors), and % Variation explained by each PC.

PC	Eigenvalues	% Variation	Cum. % variation
Principal component analysis			
1.	13.5	40.8	40.8
2.	4.23	12.8	53.6
3.	2.89	8.8	62.4
4.	1.8	5.5	67.9
5.	1.65	5.0	72.9

Variable	PC1	PC2	PC3	PC4	PC5
Eigenvectors					
Temp	−0.243	0.109	−0.035	−0.031	−0.106
^{3}He/^{4}He	−0.176	0.142	−0.035	−0.149	−0.010
B	−0.240	−0.047	−0.098	0.049	−0.112
Li	−0.261	0.001	−0.060	−0.066	−0.084
As	−0.199	0.054	0.034	0.011	−0.126
Mo	−0.188	−0.118	0.046	0.201	0.087
Sr	0.137	−0.304	−0.171	−0.024	0.065
Na	−0.230	−0.205	−0.037	0.040	0.088
Cl	−0.210	−0.233	−0.111	−0.128	0.029
F	−0.245	0.060	−0.002	−0.037	−0.180
Si	−0.180	0.160	0.229	−0.080	−0.072
SO$_4$	−0.001	−0.295	−0.227	−0.096	−0.328
Mg	0.207	−0.227	0.088	0.089	0.038
K	−0.189	−0.081	0.249	0.035	0.052
Ca	0.167	−0.221	−0.171	−0.101	0.211
Br	−0.188	−0.255	−0.121	−0.043	−0.051
I	−0.217	−0.225	0.007	0.065	0.122
Al	−0.182	0.089	−0.181	−0.172	−0.077
W	−0.251	0.064	−0.032	−0.081	−0.125
TDS	−0.216	−0.263	−0.067	0.015	0.069
δ^{18}O	−0.182	0.010	0.167	0.386	−0.072
δD	−0.177	−0.040	0.158	0.430	−0.068
Ba	0.127	−0.289	0.045	0.357	0.086
Cu	0.047	0.032	−0.384	0.228	−0.293
Fe	−0.050	−0.128	0.424	−0.223	0.069
Mn	0.007	−0.281	0.306	0.006	−0.013
Pb	0.109	0.119	−0.144	0.233	−0.233
Ni	0.110	−0.166	0.137	−0.255	−0.413
Va	0.122	0.093	−0.098	0.154	−0.080
Zn	0.059	−0.067	0.109	0.280	−0.363
Co	0.130	−0.188	0.214	−0.104	−0.332
Se	−0.058	−0.260	−0.242	−0.122	−0.052
pH	−0.145	0.018	−0.203	0.146	0.333

2000), Ethiopia (Reimann et al., 2003), Argentina (Kasemann et al., 2004), Greece (Dotsika et al., 2006), Turkey (Dogdu and Bayari, 2005), and China (Guo et al., 2008).

Murray et al. (1985) reported concentrations of B and Cl from hydrothermal fluids from the upper Napa Valley near Calistoga that are similar to those from this study, and found a close grouping in the proportions of Cl and B. The present data indicate a similar grouping in the proportions of Cl and B from the HC samples, suggesting a single source aquifer. The Cl/B ratios of the HC samples from this study are fairly uniform, ranging from 16.8 to 19.1 (median = 17.3; Table 3); HOT-03, the MW well that was collected within 50 m of the HC well HOT-02, also has a very similar Cl/B ratio of 20.3. In contrast to these results, the Cl/B ratios from the HSON wells in this study (HOT-06 and HOT-07) are more variable (71.4 and 36.8, respectively), suggesting that these two wells in Sonoma may be derived from two distinct hydrothermal reservoirs (e.g. Goff et al., 1993), however additional data are needed. The Cl/B ratios in the MW wells range from 6.7 to 209 (median = 33.0), and Cl/B ratios in the GW samples are highly variable, ranging from 7.3 to 3885 (median = 153.3).

The elements F and As are also useful in determining whether hydrothermal contamination is occurring, despite the fact that they may be affected by water-rock interaction. Fluoride occurrence in groundwater is dependent upon the geology of the area, and is often associated with volcanic or marine deposits. Fluoride mobility in hydrothermal waters is temperature dependent; above ∼250 °C dissolved F concentrations increase as a result of the dissolution of F-bearing minerals, while at temperatures below ∼250 °C F concentrations decrease due to rock alteration and precipitation of F-bearing phases (Seyfried and Ding, 1995; Valentino and Stanzione, 2003). Hydrothermal fluids commonly exceed recommended water quality criteria for F (Ellis and Mahon, 1967), which has a US EPA MCL of 4.0 mg/L, and a secondary standard (SMCL) at 2.0 mg/L. This is important because skeletal fluorosis may develop after long-term intake of drinking water with a F concentration above 4 mg/L, and crippling fluorosis is observed at F concentrations >10 mg/L (Dissanayake, 1991). The incidence of dental and skeletal fluorosis is well documented within the Rift Valley in Ethiopia, and the geographical distribution suggests a hydrothermal origin for high F concentrations in drinking water wells (Reimann et al., 2003). Similarly, in the Choma District of Zambia, a study found that all schoolchildren who drank water from hot springs had moderate to severe fluorosis, while the majority of the pupils who drank water from other sources had no dental fluorosis (Shitumbanuma et al., 2007). All hydrothermal wells from this study exceed the MCL for F, and the median F concentration for the HC and HSON wells is 10.2 mg/L. The median F concentration for MW wells is 0.38 mg/L (Table 3), but HOT-06 has a F concentration of 2.01, and HOT-03 has a F concentration of 1.9 (Table 2). The median F concentration for GW wells is 0.17 mg/L (Table 3).

The concentration of As in rock is not determinative of its concentration in surrounding porewater (Ballantyne and Moore, 1988), as As mobility is controlled by the redox conditions in the aquifer (Kulongoski and Belitz, 2010). Along a groundwater flow path, As may become enriched in groundwater by rock leaching or depleted by precipitation, ion exchange, or surface complexation (Aiuppa et al., 2003; Kulongoski and Belitz, 2010). However, high temperatures will leach As from rock, which explains the high concentrations of As in many hydrothermal fluids. In the Cimino-Vico volcanic area in central Italy, As in groundwater is mainly associated with upflowing fluids from deep hydrothermal systems, and elevated As concentrations (from 20 to 100 μg/L) occur due to mixing between deep groundwater and water recharged by infiltration in the volcanic aquifer, indicating that hydrothermal areas and faulted zones in the units underlying the shallow aquifer represent lower quality drinking water (Angelone et al., 2009).

It appears that similar processes and pathways are occurring in Northern California, with potentially harmful concentrations of As occurring in hydrothermal wells, and public supply wells contaminated with geothermal fluids. Nearly all of the HC and HSON wells (except HOT-07), and five of the MW wells HOT-03, HOT-06, VP-45, VP-49, and VP-38 have As concentrations exceeding the MCL for drinking water of 10 μg/L. The As concentrations in the HC wells (HOT-01, HOT-02, HOT-04, HOT-05) range from 50.2 μg/L to 128 μg/L (median = 67.55 μg/L) (Table 3), and the shallow MW well HOT-03 collected within 50 m of the HC well HOT-02 also has a highly elevated As concentration (30.9 μg/L).

4.2. Utility of multivariate statistics

Due to the presence of three or more distinct water types and the occurrence of admixtures of hydrothermal fluids and groundwaters in this study, multivariate statistics were required to adequately identify and characterize the constituents associated with each well. Cluster Analyses (CA) and Multidimensional Scaling (MDS) provide excellent visual representations of the groupings of the wells, and a relative assessment of contamination levels indicated by the proximity of the mixed water wells to the hydrothermal wells. Principal Components Analyses offers more

Table 6
Similarity Percentage Analyses (SIMPER) of differences between ground water from public supply wells (Group GW) and hydrothermal wells from Calistoga (Group HC).

Variable	Group GW	Group HC				
	Av. value	Av. value	Av. sq. dist	Sq. dist/SD	Contrib %	Cum. %
Temp	−0.488	2.7	10.6	2.59	7.16	7.16
W	−0.457	2.74	10.3	5.20	7.00	14.16
Li	−0.542	2.42	8.88	5.34	6.02	20.18
F	−0.449	2.44	8.55	2.80	5.79	25.97
B	−0.515	2.19	7.5	3.32	5.08	31.06
Al	−0.326	2.02	7.5	0.93	5.08	36.14
As	−0.4	2.18	7.14	1.92	4.84	40.98
Mg	0.264	−2.07	6.04	1.68	4.09	45.08
^{3}He/^{4}He	−0.353	1.96	5.62	2.62	3.81	48.89
δ^{18}O	−0.439	1.78	5.49	1.72	3.72	52.61
Si	−0.401	1.57	4.68	1.28	3.17	55.78
Ca	0.206	−1.77	4.63	1.39	3.14	58.92
δD	−0.462	1.51	4.53	1.60	3.07	61.99
Cl	−0.485	1.57	4.41	2.58	2.99	64.98
Na	−0.589	1.42	4.29	2.30	2.91	67.89
Br	−0.43	1.53	4.16	2.21	2.82	70.71
TDS	−0.586	1.37	4.04	2.29	2.74	73.45
Ba	0.104	−1.65	3.91	1.24	2.65	76.10
I	−0.505	1.4	3.9	2.37	2.64	78.74
Co	0.216	−1.3	3.31	1.00	2.24	80.99
Zn	6.57E−2	−0.362	2.89	0.85	1.96	82.95
Mo	−0.503	1.03	2.85	1.41	1.93	84.88
Va	0.294	−0.927	2.83	0.86	1.92	86.80
Sr	0.173	−1.03	2.62	1.07	1.77	88.57
K	−0.527	0.86	2.43	1.57	1.65	90.22

Groups GW vs HC.
Average squared distance = 147.50.

Table 7
Similarity Percentage Analyses (SIMPER) of differences between ground water from public supply wells (Group GW) and mixed hydrothermal/meteoric wells (Group MW).

Variable	Group GW	Group MW				
	Av. value	Av. value	Av. sq. dist	Sq. dist/SD	Contrib %	Cum. %
K	−0.527	0.957	3.23	1.10	6.26	6.26
Mo	−0.503	0.685	2.6	0.78	5.04	11.30
Cu	0.121	−0.25	2.35	0.70	4.57	15.87
Pb	0.276	−0.341	2.29	0.68	4.44	20.30
Si	−0.401	0.668	2.12	0.88	4.11	24.42
Na	−0.589	0.575	2.02	0.98	3.91	28.33
^{3}He/^{4}He	−0.353	0.455	2.01	0.98	3.89	32.22
Ni	0.269	−0.385	1.96	0.66	3.80	36.03
Fe	−0.19	0.319	1.95	0.91	3.79	39.81
δD	−0.462	0.367	1.86	0.92	3.62	43.43
SO_4	−2.64E−2	−0.388	1.85	0.80	3.58	47.01
As	−0.4	0.302	1.81	0.98	3.52	50.53
pH	−0.27	5.43E−2	1.79	0.70	3.47	54.00
Mn	−0.115	0.364	1.77	0.87	3.44	57.44
Co	0.216	−4.88E−2	1.7	0.71	3.30	60.75
δ^{18}O	−0.439	0.252	1.69	0.89	3.29	64.03
Va	0.294	−0.162	1.66	0.76	3.22	67.25
TDS	−0.586	0.477	1.56	1.03	3.03	70.27
Se	−0.151	−9.49E−2	1.55	0.84	3.01	73.28
Ca	0.206	6.97E−3	1.33	0.73	2.58	75.86
B	−0.515	0.361	1.29	0.85	2.50	78.35
Cl	−0.485	0.101	1.23	0.63	2.39	80.74
Sr	0.173	−0.249	1.2	1.07	2.33	83.08
Zn	6.57E−2	−0.157	1.13	0.68	2.20	85.27
Li	−0.542	0.344	1.11	0.76	2.15	87.42
Ba	0.104	0.109	1.09	0.89	2.11	89.53
I	−0.505	0.163	0.967	0.91	1.88	91.41

Groups GW vs. MW.
Average squared distance = 51.55.

detailed information regarding the significance of specific constituents that are indicative of hydrothermal contamination. Similarity Percentage Analyses further refines these differences, and identifies the constituents providing the greatest contributions to the differences between each of the well types.

The Multivariate Mixing and Mass-balance model (M3) was particularly useful in determining the percentage of hydrothermal contamination occurring in the mixed water wells. However, this method is constrained by the necessity to identify individual wells that best represent end-members for each water type, which

Table 8
Similarity Percentage Analyses (SIMPER) of differences between hydrothermal wells from Calistoga (Group HC) and wells from mixed hydrothermal/meteoric wells (Group MW).

Variable	Group HC	Group MW				
	Av. value	Av. value	Av. sq. dist	Sq. dist/SD	Contrib %	Cum. %
W	2.74	0.107	7.16	2.76	8.07	8.07
Temp	2.7	0.301	6.22	1.79	7.02	15.09
Al	2.02	0.107	5.32	0.81	6.00	21.08
F	2.44	0.174	5.31	2.88	5.99	27.07
Mg	−2.07	8.03E−2	5.16	1.75	5.82	32.89
As	2.18	0.302	4.7	0.94	5.29	38.18
Li	2.42	0.344	4.6	2.58	5.19	43.36
Ca	−1.77	6.97E−3	3.84	1.43	4.33	47.69
B	2.19	0.361	3.68	1.91	4.15	51.84
Ba	−1.65	0.109	3.37	1.87	3.80	55.64
$^3He/^4He$	1.96	0.455	3.37	0.79	3.80	59.43
Br	1.53	−0.136	3.07	1.89	3.46	62.89
$\delta^{18}O$	1.78	0.252	3.06	1.08	3.45	66.33
Cl	1.57	0.101	2.84	1.44	3.20	69.54
Zn	−0.362	−0.157	2.32	1.09	2.61	72.15
SO_4	−9.36E−2	−0.388	2.3	0.82	2.60	74.74
Co	−1.3	−4.88E−2	2.23	0.96	2.52	77.26
δD	1.51	0.367	1.94	1.00	2.19	79.45
I	1.4	0.163	1.83	1.39	2.07	81.52
Se	0.196	−9.49E−2	1.73	0.97	1.95	83.47
pH	0.863	5.43E−2	1.71	0.77	1.93	85.40
Pb	−0.877	−0.341	1.48	0.70	1.66	87.06
Va	−0.927	−0.162	1.46	0.67	1.65	88.72
Mn	−0.502	0.364	1.42	0.83	1.60	90.31

Groups HC vs. MW.
Average squared distance = 88.71.

Table 9
Similarity Percentage Analyses (SIMPER) of differences between hydrothermal wells from Calistoga (Group HC) and the hydrothermal well from Sonoma (HSON).

Variable	Group HC	Group HSON				
	Av. value	Av. value	Av. sq. dist	Sq. dist/SD	Contrib %	Cum. %
As	2.18	−0.217	5.92	2.61	12.91	12.91
Zn	−0.362	−2.2	5.31	0.93	11.58	24.49
Se	0.196	1.7	3.26	0.94	7.11	31.60
I	1.4	3.09	2.86	5.27	6.25	37.84
temp	2.7	1.16	2.7	1.26	5.89	43.74
Al	2.02	0.926	2.68	0.56	5.85	49.59
SO_4	−9.36E−2	1.08	2.32	0.67	5.07	54.66
Br	1.53	3.05	2.3	7.09	5.02	59.68
TDS	1.37	2.86	2.24	11.99	4.88	64.56
Cl	1.57	2.95	1.91	26.18	4.17	68.73
K	0.86	2.15	1.66	12.38	3.61	72.34
pH	0.863	2	1.53	1.27	3.33	75.68
Ba	−1.65	−0.427	1.51	10.57	3.29	78.96
Va	−0.927	−1.88	1.28	1.12	2.80	81.76
$^3He/^4He$	1.96	0.895	1.14	33.40	2.48	84.24
Na	1.42	2.48	1.13	10.71	2.47	86.71
Sr	−1.03	−1.89	0.986	0.82	2.15	88.87
Mo	1.03	1.88	0.885	1.06	1.93	90.79

Groups HC vs. HSON.
Average squared distance = 45.85.

requires *a priori* information derived from statistical analyses. The consistency of the results from the M3 analyses with the similarities and proximities of the various well types indicated by the CA, MDS, and PCA confirms that the well classifications are robust.

4.3. Significance and implications of hydrothermal contamination

This work is the first study to utilize a broad suite of statistical methods to analyze and characterize mixing between relatively shallow groundwater and deeper hydrothermal fluids in the Napa and Sonoma Valleys in California. Traditional approaches were combined to evaluate the extent and nature of this hydrothermal contamination with more detailed information gleaned from multivariate statistical analyses, and the powerful Multivariate Mixing and Mass-balance model (M3) to determine the constituents associated with hydrothermal contamination, as well as proportions of hydrothermal fluids, saline water and fresh groundwater present in each sample. Constraining the composition and extent of hydrothermal contamination could allow agencies to confidently attribute contaminants (e.g., As anomalies) to hydrothermal sources instead of other potential origins, such as aresenical pesticides applied to grape vineyards, a major crop in the area. It will also allow agencies to isolate wells affected by hydrothermal contamination, seal well perforation intervals associated with the contaminated aquifers, and assure consumers that they are not served drinking water with constituents such as As, F, B and Li at potentially harmful levels.

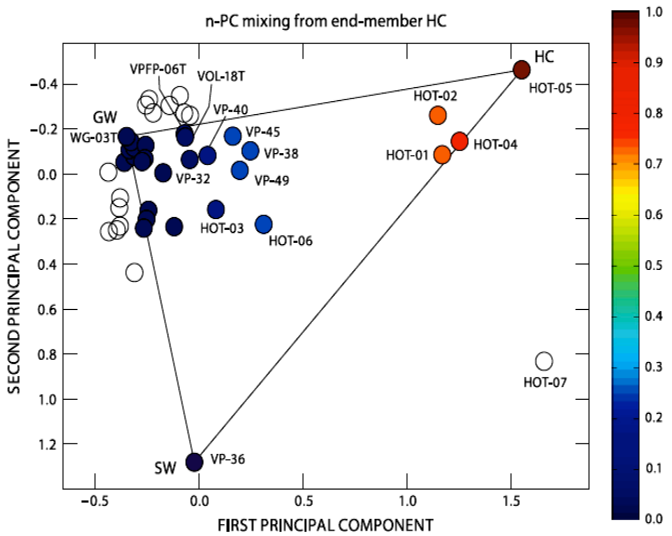

Fig. 5. Diagram showing mixing proportions from The Multivariate Mixing and Mass-balance model (M3) using the following reference waters: HOT-05 as the reference hydrothermal well from Calistoga (HC), WG03T as the reference well for groundwater (GW), and VP-36 as the reference well for saline water (SW).

Table 10
Mixing proportions from The Multivariate Mixing and Mass-balance model, M3 using the following reference waters: HOT-05 as the reference hydrothermal well from Calistoga (HC), WG03T as the reference well for groundwater (GW), and VP-36 as the reference well for saline water (SW).

Well ID	Well type	% Ground water	% Saline water	% Hydrothermal water
WG-03T	GW	1.00	0.00	0.00
VP-36	SW	0.00	1.00	0.00
HOT-05	HC	0.00	0.00	1.00
HOT-04	HC	0.01	0.18	0.81
HOT-02	HC	0.14	0.09	0.77
HOT-01	HC	0.02	0.21	0.76
VP-38	MW	0.60	0.10	0.30
HOT-06	MW	0.38	0.33	0.29
VP-45	MW	0.69	0.05	0.26
VP-49	MW	0.58	0.16	0.26
VP-40	MW	0.71	0.10	0.19
HOT-03	MW	0.56	0.26	0.18
VPFP-06T	MW	0.83	0.02	0.14
VOL-18T	MW	0.83	0.03	0.14
VP-32	MW	0.76	0.10	0.14
VP-50	GW	0.80	0.13	0.07
VP-48	GW	0.64	0.29	0.07
VP-33	GW	0.92	0.04	0.04
VP-19	GW	0.89	0.07	0.03
VP-37	GW	0.89	0.08	0.03
VP-35	GW	0.75	0.23	0.02
WGFP-02T	GW	0.95	0.04	0.01
VP-47	GW	0.98	0.02	0.01
VPFP-05T	GW	0.93	0.06	0.01
VP-26	GW	0.74	0.26	0.01
WGFP-01	GW	0.96	0.04	0.00
VP-46	GW	0.72	0.28	0.00
WG-08	GW	0.93	0.07	0.00

5. Conclusions

Due to the presence of at least two hydrothermal systems and a saline water component, traditional methods for investigating hydrothermal systems, such as plots involving a single species (e.g. Cl or SiO_2), or ratios of elements commonly associated with the hydrothermal systems (e.g. B vs. Cl) were insufficient to characterize wells from the Napa and Sonoma Valleys. Therefore, multivariate statistical analyses and M3 modeling techniques were essential to characterize the occurrence and extent of contamination by hydrothermal fluids.

The identification of mixing between hydrothermal fluids and meteoric waters is important to prevent contamination of drinking water. The statistical methodology implemented here accurately characterizes the chemical and isotopic differences between hydrothermal fluids and meteoric waters in the Napa and Sonoma Valleys, and provides powerful techniques to help identify mixing in wells. By recognizing mixing, and the potential for mixing, steps

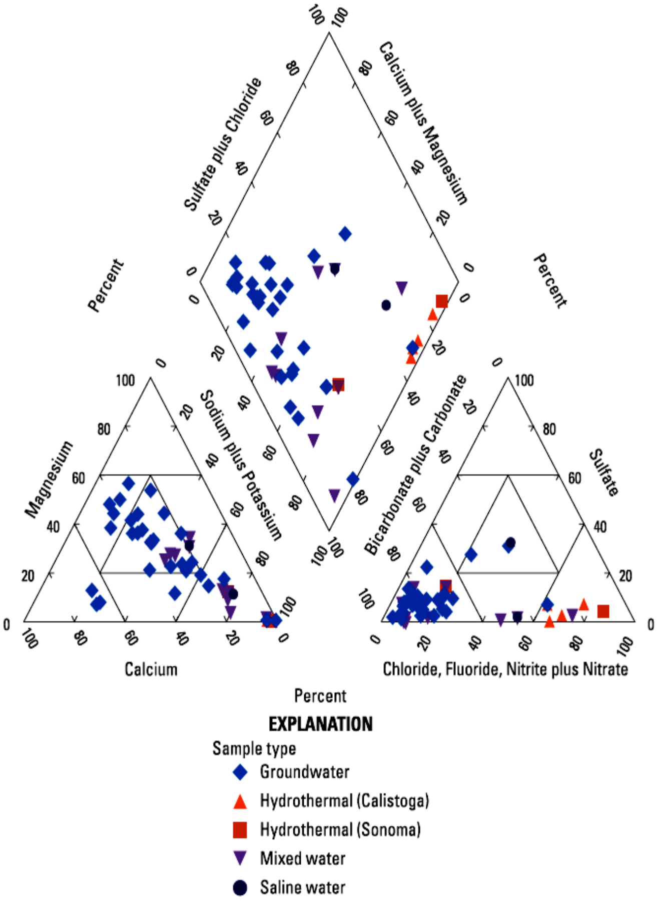

Fig. 6. Piper diagram of sampled wells. Symbols represent different well types: groundwater (GW) = Light Blue Diamonds; hydrothermal well from Calistoga (HC) = Orange Triangles; hydrothermal fluids from Sonoma (HSON) = Red Squares; mixed hydrothermal/meteoric water (MW) = Purple Upside Down Triangles; saline water (SW) = Dark Blue Circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can be taken to prevent harm to the valuable resources, both potable water and hydrothermal wells.

The multivariate statistics employed in this study allowed identification of the constituents most indicative of hydrothermal fluids: water temperature, $^3He/^4He$, Li, Cl, B, As, and F, and to distinguish the wells where mixing between hydrothermal fluids and groundwater is occurring. Of these constituents, $^3He/^4He$ and measured temperature, along with the elements Li, Cl and B are most useful in evaluating the contribution of the hydrothermal system to the wells in this study because they generally behave conservatively, and are not controlled by temperature- and pressure-dependent chemical exchange involving rock minerals. Although F and As do not necessarily behave conservatively, the hydrothermal and mixed hydrothermal/ground water wells from this study generally contained elevated concentrations of F and As, often in excess of the US EPA Maximum Contamination Levels.

Results from the Multivariate Mixing and Mass-balance model (M3) confirm the initial classifications of the mixed hydrothermal/meteoric wells (MW), and indicate that the nine MW wells contain between 14% and 30% hydrothermal fluids. The results from the M3 model are consistent with those from the other multivariate statistical analyses. M3 is a valuable tool for determining the proportions of mixing of selected reference waters present in waters from mixed origins such as in the Calistoga area. Used in tandem with multivariate statistical analyses, the M3 model provides a clear indication of the occurrence and extent of contamination of local groundwater used for drinking water by hydrothermal fluids. These methods could also be applied in other areas such as Mexico, Iceland, Japan, Turkey, Greece, Ethiopia and Italy, where the potential for mixing between ground and surface waters utilized for drinking water and crop irrigation and hydrothermal fluids also exists.

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CHAPTER 2: Nothing a Hot Bath Won't Cure: Infection Rates of Amphibian Chytrid Fungus Correlates Negatively with Water Temperature Under Natural Field Settings

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Nothing a Hot Bath Won't Cure: Infection Rates of Amphibian Chytrid Fungus Correlate Negatively with Water Temperature under Natural Field Settings

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Abstract

Dramatic declines and extinctions of amphibian populations throughout the world have been associated with chytridiomycosis, an infectious disease caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Previous studies indicated that *Bd* prevalence correlates with cooler temperatures in the field, and laboratory experiments have demonstrated that *Bd* ceases growth at temperatures above 28°C. Here we investigate how small-scale variations in water temperature correlate with *Bd* prevalence in the wild. We sampled 221 amphibians, including 201 lowland leopard frogs (*Rana* [*Lithobates*] *yavapaiensis*), from 12 sites in Arizona, USA, and tested them for *Bd*. Amphibians were encountered in microhabitats that exhibited a wide range of water temperatures (10–50°C), including several geothermal water sources. There was a strong inverse correlation between the water temperature in which lowland leopard frogs were captured and *Bd* prevalence, even after taking into account the influence of year, season, and host size. In locations where *Bd* was known to be present, the prevalence of *Bd* infections dropped from 75–100% in water <15°C, to less than 10% in water >30°C. A strong inverse correlation between *Bd* infection status and water temperature was also observed within sites. Our findings suggest that microhabitats where water temperatures exceed 30°C provide lowland leopard frogs with significant protection from *Bd*, which could have important implications for disease dynamics, as well as management applications. *There must be quite a few things a hot bath won't cure, but I don't know many of them* - Sylvia Plath, "The Bell Jar" (1963).

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Introduction

Chytridiomycosis, an infectious disease caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is a primary factor in worldwide amphibian declines and species extinctions [1], [2]. *Bd* belongs to a group of virulent multi-host pathogens that have had profound effects on entire communities and ecosystems [3]. In fact, *Bd* has been called "possibly the most deadly invasive species on the planet (excluding humans)" [4]. Although amphibian susceptibility to *Bd* and chytridiomycosis is species-specific, environmental conditions also appear to modify host-disease dynamics [2]. The prevalence of *Bd* (i.e. the proportion of infected animals) and the virulence of chytridiomycosis are particularly influenced by temperature [5]. Field studies conducted in disparate geographic regions show *Bd* infections are generally more severe in winter months, and when hosts are found in cooler temperatures [6]–[9].

In the laboratory *Bd* cultures grew and reproduced at temperatures between 4–25°C, with maximal growth at 17–25°C, but growth ceased at temperatures above 28°C [10]. Incubation of *Bd* cultures at 30°C for 8 days killed 50% of colonies [10], and 100% mortality occurred within 96 hours at 32°C and within 4 hours at 37°C [11]. Similarly, *Bd* does not persist in amphibian hosts above certain temperature thresholds. In laboratory experiments, short-term exposure to temperatures between 27 and 37°C successfully cleared *Bd* infections from five species of adult frogs with no reported side effects [7], [12]–[14]. Although *Bd* is susceptible to certain antifungal agents when tested in vitro, there are few proven methods for clearing infections in adult amphibians, and acute drug toxicity can be a problem for tadpoles and juveniles [15]–[17]. Therefore, heat treatments may be a superior alternative to currently available antifungal drugs for captive animals infected with *Bd* [14], [17]. However, whether amphibians in the wild can also be cleared of *Bd* by short-term exposure to elevated temperatures remains unknown.

Several species of leopard frogs naturally inhabit geothermal ecosystems in the southwestern United States, including endangered and threatened species. For example, all naturally occurring populations of the relict leopard frog (*Rana* [*Lithobates*] *onca*) are now associated with perennial geothermal springs in Nevada with source temperatures exceeding 30°C [18], and geothermal spring sites in New Mexico are particularly important breeding habitats for the threatened Chiricahua leopard frog (*Rana* [*Lithobates*] *chiricahuen-*

sis) [19]. Observations from a previous study indicated that *Bd* was less prevalent in frogs inhabiting geothermal waters in Arizona, USA [20]. Consequently, we tested whether *Bd* is negatively associated with water temperature by sampling amphibian populations from several geothermal ecosystems, as well as non-geothermal sites.

All seven native Arizona ranid species have experienced significant population declines and local extinctions [21], and chytridiomycosis appears to be an important contributory factor–particularly during the winter months [6], [22]. Lowland leopard frogs (*Rana* [*Lithobates*] *yavapaiensis*) have been extirpated from nearly half of their historic geographic range [21], and populations continue to decline or disappear from additional sites [23].

Identifying environments and climactic conditions that provide natural refuges from *Bd* will benefit imperiled amphibian populations [24], [25], and may provide some susceptible species with opportunities to evolve evolutionary responses to the pathogen [26]. Information on the environmental limitations of *Bd* in the wild is critical to the conservation of amphibians affected by this disease [27], yet a clear understanding of how temperature modulates host-disease dynamics in the field has remained elusive to date [28], [29]. Geothermal settings provide unique opportunities to examine the effects of a wide range of environmental temperature on chytridiomycosis host-disease dynamics in wild amphibian populations. To our knowledge, this study is the first to

Table 1. Sites sampled, Latitude and Longitude (Datum = WGS 84), dates sampled, range of water temperatures measured during sampling, species sampled, number of individuals, and *Bd* prevalence (percent infected) per species sampled.

Location	Dates Sampled	H_2O Temp (°C)	Species Sampled (n)	Species *Bd* Prevalence
Aravaipa Creek	Oct 2004	17.7	*Rana yavapaiensis* (11)	0.64
N32 52.725	May 2009	23.0		
W110 23.767	Feb 2010	10.5		
BHP Hotwell*	Feb 2010	24.3–28.1	*R. yavapaiensis* (7)	0.00
N32 37.961				
W110 33.537				
Dankworth Pond*	Mar 2009	34.1	*Rana catesbeiana* (1)	0.00
N32 43.279				
W109 42.089				
El Dorado Hot Spring*	May 2009	36.5–34.1	*R. yavapaiensis* (18)	0.00
N33 29.588			*Bufo alvarius* (1)	0.00
W112 56.442				
Essence of Tranquility*	Mar 2009	30.7	*R. catesbeiana* (1)	0.00
N32 45.480				
W109 43.510				
Hassayampa – TNC	Oct 2004	19.7	*R. yavapaiensis* (8)	0.63
N33 55.838			*R. catesbeiana* (2)	0.00
W112 41.520				
Hassayampa Preserve	Oct 2004	19.7	*R. yavapaiensis* (6)	0.00
N34 02.472				
W112 42.235				
San Pedro River	Mar 2009	16.0	*R. yavapaiensis* (1)	1.00
N32 55.511			*R. catesbeiana* (3)	0.66
W110 44.489				
Mammoth Hot Well*	Oct 2004	22.4–37.2	*R. yavapaiensis* (60)	0.21
N32 41.660	Mar 2009	18.4–36.2	*R. catesbeiana* (10)	0.00
W110 37.341	Feb 2010	15.1–33.4	*Bufo woodhousii* (1)	0.00
Markham Creek[1]	May 2009	22.4–24.0	*R. yavapaiensis* (29)	0.00
	Feb 2010	11.2–14.5	*Hyla arenicolor* (1)	0.00
Muleshoe Hot Spring*	Aug 2004	22.8–51.2	*R. yavapaiensis* (27)	0.19
N32 20.229	Mar 2009	22.0–27.0		
W110 14.331	Feb 2010	13.1–30.0		
Secret Spring*	Aug 2004	21.6–23.6	*R. yavapaiensis* (34)	0.56
N32 20.395	Mar 2009	13.9–38.7		
W110 14.613	Feb 2010	16.6–18.3		

*Indicates presence of a geothermal source at location.

[1] Coordinates excluded at the request of U.S. Bureau of Land Management (Safford, AZ).

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utilize steep natural temperature gradients in field settings, and measure water temperature at the precise time and place where each amphibian was captured, thereby providing valuable information collected at a fine spatial scale to help elucidate the thermal restrictions of *Bd* under natural field conditions.

Results

We sampled 221 post-metamorphic anurans belonging to five species, of which the lowland leopard frog (n = 201) was the most common (Table 1). The primary result was a significant negative association between *Bd* infection status of lowland leopard frogs and water temperature. Overall, the temperature of the water in which we captured *Bd*-positive (*Bd*+) individuals (mean = 19.8 ±0.67°C, n = 50) was significantly cooler than for *Bd*-negative (*Bd*−) individuals (mean = 25.8±0.50°C, n = 151; 2-sample t-test: df = 108, t = 7.1, $p<0.001$; Fig. 1A). The negative association between water temperature and *Bd* infection status was apparent in the univariate test, but also after accounting for the influence of other significant factors such as Distance to Solstice (i.e., seasonality) and Year (Table 2).

To control for a possible absence of *Bd* from certain sites (Table 1) we repeated the binomial logistic regression analysis excluding samples from Markham Creek, Upper Hassyampa, El Dorado Hotspring and BHP Hotwell, where more than one individual was tested, and *Bd* had not been detected. The analysis on the restricted dataset (n = 148) yielded a qualitatively identical result for the binomial regression, with *Bd* prevalence declining

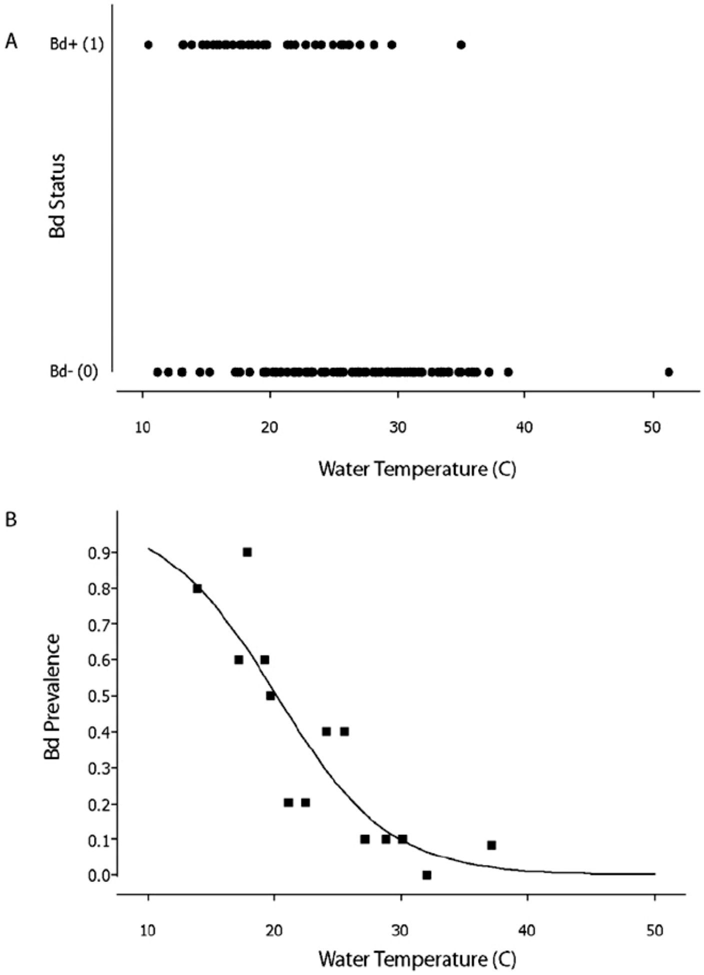

Figure 1. Occurrence of *Bd* in lowland leopard frogs as a function of water temperature. A) Presence (1) and absence (0) of *Batrachochytrium dendrobatidis* (*Bd*) in lowland leopard frogs *Rana* (*Lithobates*) *yavapaiensis* as a function of water temperature across all years and sites. N = 50 *Bd*+ individuals and N = 151 *Bd*−. B) Occurrence of *Bd* in lowland leopard frogs as a function of water temperature, excluding four sites where *Bd* was not detected (see Table 1 for details). Each point represents the fraction of frogs infected and mean water temperature of groups of 10–12 individuals (ranked by water temperature, then binned). Logistic equation: $y = 1/(1+e^{-z})$ where $z = 4.56 - temp*0.226$ (coefficients extracted from univariate analysis). Total sample size from sites with *Bd*, N = 148.
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Table 2. Logistic binary regression of infection status (0 = not infected; 1 = infected with *Batrachochytrium dendrobatidis*) of lowland leopard frogs *Rana* (*Lithobates*) *yavapaiensis* (N = 198; 3 missing SVL).

Predictor[1]	Coefficient	Standard Error Coefficient	Z	P	Odds Ratio	Lower 95% CI	Upper 95% CI
Constant	−1.02834	2.18103	−0.47	0.637			
Water Temp	−0.188474	0.0424268	−4.44	0.000	0.83	0.76	0.90
Dist Solstice	0.0648780	0.0155885	4.16	0.000	1.07	1.03	1.10
SVL (mm)	−0.0240867	0.0188217	−1.28	0.201	0.98	0.94	1.01
Year							
2009	0.310455	0.579158	0.54	0.592	1.36	0.44	4.24
2010	−3.80550	0.865731	−4.40	0.000	0.02	0.00	0.12

Log-Likelihood = −70.328.

Test that all slopes are zero: G = 83,12, DF = 5, P-Value = 0.000.

[1] Years 2009 and 2010 contrasted with 2004. Dist. Solstice = Distance to Solstice, the absolute difference in days between the sampling date and June 21st (used to capture seasonal variation). SVL = Snout-Vent Length.

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sharply from 100% at 10–15°C until 30°C, where it approaches zero (Fig. 1B). Our subsequent analyses include data from all sites because our samples sizes at any particular site were too small to be confident that *Bd* was truly absent (Table 1).

Distance to Solstice and Year were also significantly associated with *Bd* prevalence (Table 2). The positive association between *Bd* prevalence and Distance to Solstice illustrates that individuals were more likely to be *Bd+* during early spring and late fall sampling than during the summer, even after taking into account water temperatures (Table 2). *Bd* prevalence also varied between years, with the prevalence in 2009 being significantly lower than in 2004 and 2010 once the time of year was taken into account (Table 2). Because slightly different methods for assaying *Bd* were used in the 2004 study, we repeated all analyses using only the 2009 and 2010 data and found qualitatively similar results.

Size of host (SVL) was not significantly associated with *Bd* prevalence in the binomial regression (Table 2). Nevertheless, other results suggest that the relationship between *Bd* and host may change with size. We observed that seven of the eight *Bd+* individuals that were captured in waters warmer than 25°C were juveniles (≤50 mm SVL). Furthermore, the mean water temperature in which *Bd+* juveniles were captured (21.6±1.31°C, n = 20) was marginally higher than that of *Bd+* adults (18.7±0.64°C, n = 30; t-test: df = 28, t = 2.00, p = 0.056). Our results suggest that juveniles and adults may differ in their susceptibility to *Bd* or in their behavior once infected.

Bd prevalence clearly varies between sites (Table 1), and we wanted to ensure that the correlation between water temperature and *Bd* prevalence was not confounded by site characteristics. When Site was included in the binomial logistic model, Water Temperature remained a significant factor, but certain site-specific coefficients could not be estimated (likely because of small sample sizes). We therefore undertook an additional analysis in which we compared AICc values (Akaike information criterion corrected for finite sample size) of two logistic binomial models with Sex, SVL, Year and Site as factors, but where Water Temperature was present only in the first model. The much lower AICc value (139.1 vs. 158.3) associated with the first model, which included Water Temperature, strongly suggests a significant correlation between *Bd* prevalence and water temperature, even when accounting for variation in *Bd* between sites.

The negative relationship between *Bd* prevalence and water temperature was also apparent within the Secret Spring and Mammoth Hot Well sites, where sampling covered a wide range of water temperatures from the relatively constant, elevated temperatures at their sources to more variable, ambient temperatures in the distal portions of the ecosystems. *Bd* was detected at these sites, but more commonly in individuals located in cooler waters. At both sites, the prevalence of *Bd* was significantly lower in water temperatures of 30°C and above (Secret Spring: 19/30 Bd+ in water <30°C, and 0/4 Bd+ in water ≥30°C; two-tailed Fisher's exact test; p = 0.0294; Mammoth Hot Well: 13/45 Bd+ in water <30°C, and 1/26 Bd+ in water ≥30°C; two-tailed Fisher's exact test p = 0.0124). In 2009 we mapped the location of each individual captured at these two locations. Eight of the nine *Bd+* individuals (89%) at Mammoth Hot Well were found in waters cooler than 27°C (Fig. 2), and all 15 *Bd+* individuals (100%) at Secret Spring were found in waters cooler than 20°C (Fig. 3).

We also tested 20 individuals of four other species: 17 American bullfrogs (*Rana* [*Lithobates*] *catesbeiana*), 1 canyon tree frog (*Hyla arenicolor*), 1 Woodhouse's toad (*Bufo* [*Anaxyrus*] *woodhousii woodhousii*), and 1 Sonoran toad (*Bufo* [*Incilius*] *alvarius*) (Table 1). All were *Bd*−, with the exception of two American bullfrogs captured at 16°C.

Discussion

This study provides evidence that the probability that a lowland leopard frog is infected with *Bd* is significantly negatively correlated with water temperature at the point of capture (Fig. 1A and 1B). Previous field studies documented a correlation between *Bd* prevalence and seasonality, with higher infection rates during cool seasons [6]–[9], [28]. In this study, we documented that the inverse relationship between water temperature and *Bd* prevalence also holds true within seasons. Furthermore, the association between temperature and *Bd* prevalence was apparent even within bodies of water, with *Bd+* frogs largely absent from water warmer than 25°C (Figs. 2 and 3). This result appears surprising given the short distances involved, but the observed pattern suggests that these frogs may have relatively small home ranges, or that the detectability and status of the disease changes rapidly with environmental conditions. Although other factors such as water chemistry may potentially affect *Bd* infection rates, our results strongly suggest a functional link between water temperature and *Bd* infection status in frogs.

Previous laboratory work conducted on other species indicates that amphibian hosts are able to clear *Bd* if infected individuals experience temperatures between 27–37°C. Caging great barred frogs (*Mixophyes fasciolatus*) at 27°C (n = 8) cured 50% of the frogs,

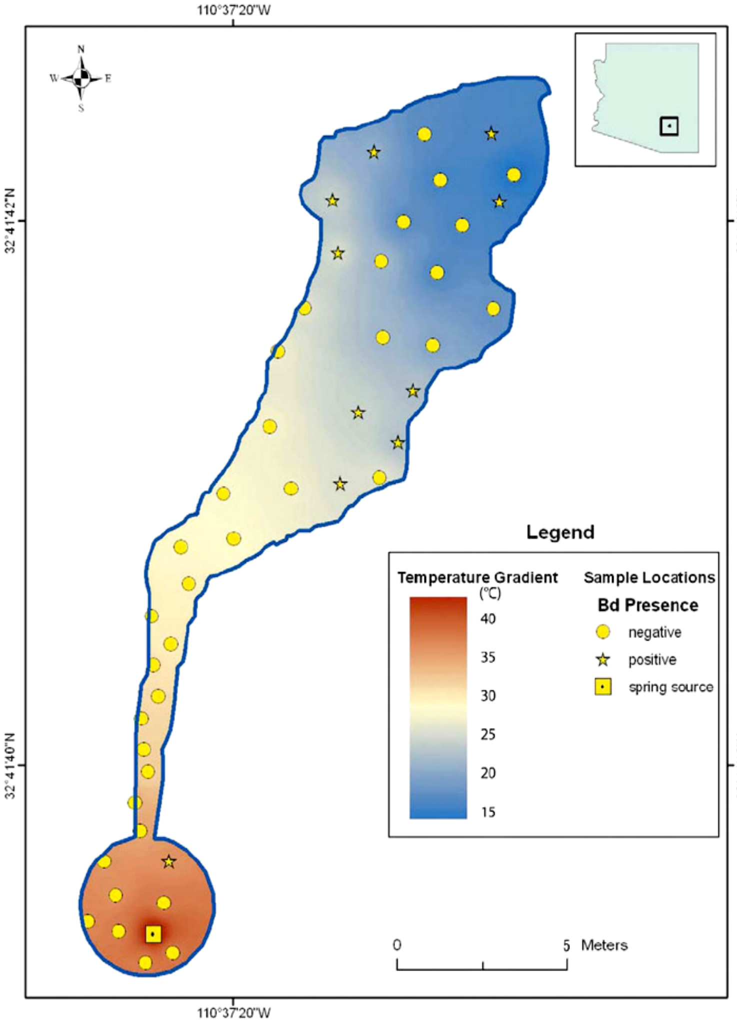

Figure 2. Schematic of Mammoth Hot Well showing approximate locations of amphibians and water temperatures. Symbols show approximate locations of lowland leopard frogs (redrawn from field notes) sampled in March 2009, their *Bd* infection status, and the range of measured water temperatures.
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which remained healthy, and tested negative for *Bd* when the experiment was terminated [7]. Orange-eyed treefrogs (*Litoria chloris*) cleared *Bd* within 16 hours after being caged at an environmental temperature of 37°C (n = 10) [12]. Western chorus frogs (*Pseudacris triserata*) collected in Arizona, cleared *Bd* following incubation at 32°C for 5 days (n = 6) [13]. American bullfrogs and northern cricket frogs (*Acris crepitans*) cleared *Bd* after being subjected to 30°C for 10 consecutive days, after which only one frog remained infected (n = 28) [14]. Heat treatments can also clear larval amphibians of *Bd*; 7 out of 8 tadpoles of the midwife toad (*Alytes obstetricans*) cleared *Bd* infection when exposed to temperatures higher than 26°C for 5 days [30]. Our results are consistent with these findings, despite having been conducted in uncontrolled environments. Indeed, the vast majority of *R. yavapaiensis* (51/52; 98%) captured in water warmer than 30°C were *Bd*− (Figs. 1A, 2, 3). Overall, our results are consistent with the hypothesis that warm waters exclude *Bd* from infecting *R. yavapaiensis* hosts, although we cannot rule out all hypothetical alternatives.

In addition to the well-documented negative effects of warm waters on *Bd*, temperature is also strongly linked to amphibian immune system responses. Declines in amphibian immune defenses as temperatures decrease are well documented [31]–[34], which may explain why amphibians are particular susceptible to pathogens such as *Bd* that survive and grows at low temperatures [7], [10], [35]. Conversely, at warmer temperatures amphibians may be less susceptible to *Bd* and chytridiomycosis due to greater effectiveness of the immune response [36], [37]. Thermal impacts on *Bd*, and on the amphibian immune system have important ramifications for the ecology of chytridiomycosis and its impacts on wild amphibian populations [38].

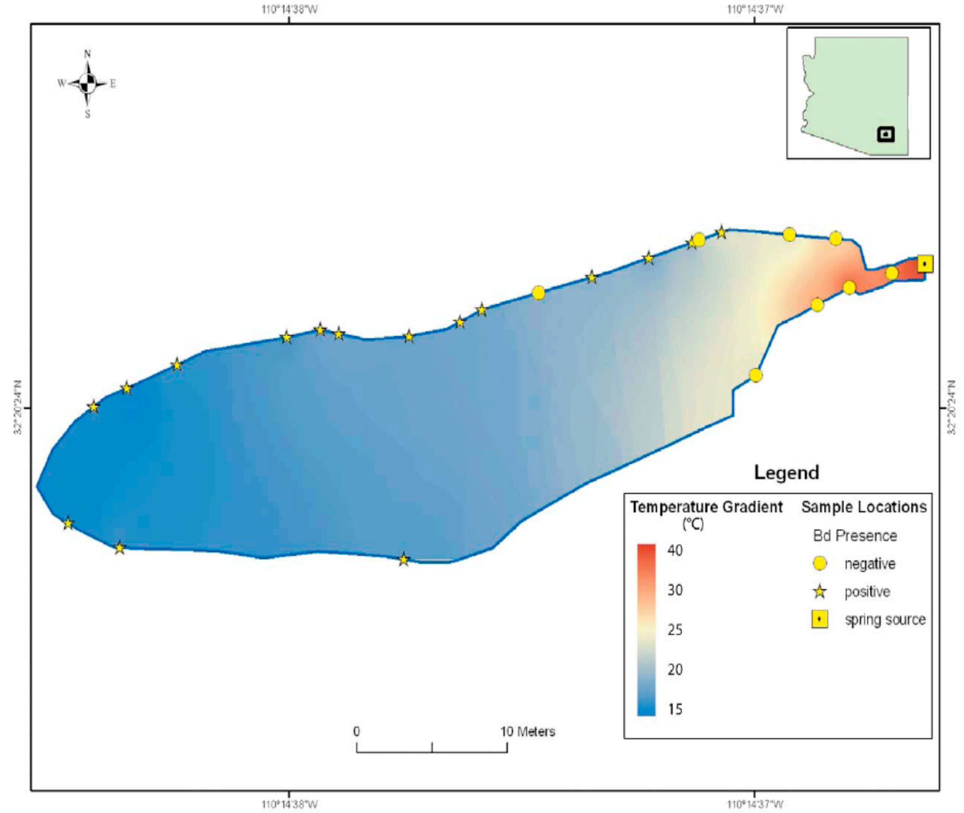

Figure 3. Schematic of Secret Spring showing approximate locations of amphibians and water temperatures. Symbols show approximate locations of lowland leopard frogs (redrawn from field notes) sampled in March 2009, their *Bd* infection status, and the range of measured water temperatures.
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We currently do not know if habitat choice is altered by a host's disease state, nor whether infected lowland leopard frogs deliberately seek out warmer sites to clear *Bd*. However, such speculation appears well supported, as diseased or parasitized amphibians and other ectotherms have been shown to actively seek temperatures above their thermal optima in order to generate 'behavioral fevers' that enhance host immune response or reduce pathogenic activity [12], [39], [40]; although see [41]. For example, green tree frogs (*Hyla cinerea*) behaviorally elevated their body temperature 2°C following inoculation with a pathogenic bacterium [42]. More directly, captive boreal toads (*Bufo* [*Anaxyrus*] *boreas*) with severe *Bd* infections shifted resting positions towards heat strips, suggesting a strategy to combat *Bd* infection [37]. Behavioral fever response to *Bd* has also been observed in wild populations. The average body temperature of a population of Panamanian golden frogs (*Atelopus zeteki*) increased 2.4°C following exposure to *Bd*, suggesting that the frogs exhibited a population-wide behavioral fever response during the epidemic [43]. The odds of *Bd* infection decreased with increasing body temperature, demonstrating that even slight environmental or behavioral changes have the potential to affect an individual's vulnerability to infection [43].

Since A.D. 79, when Pliny the Elder documented frogs inhabiting the hot springs of Pisa in his seminal work "Naturalis Historia", amphibian populations have been observed in geothermal ecosystems around the world, including in Algeria [44], Taiwan [45], China [46], and Chile [47]. There are hundreds of geothermal watersheds throughout western North America, Central America, and Eastern Africa [48], many of which are located within the historic ranges of vulnerable species of amphibians [49]. Although geothermal ecosystems make up only a small fraction of most landscapes, they may be demographically important if they provide amphibians with even partial protection from temperature-sensitive diseases such as chytridiomycosis during pandemic events. Models suggest that the key to long-term persistence with *Bd* is survival of at least some fraction of infected adults–if some individuals survive the initial epidemic, it is possible that the infected amphibian population will persist in a new endemic state [50], [51].

Geothermal ecosystems may confer disease-protection to other amphibian species besides lowland leopard frogs. For example, in our study we sampled 20 individuals of other species in water temperatures ranging from 16–36°C (Table 1). Only two (10%) of these individuals were *Bd*+, and both individuals were captured at 16°C. Furthermore, geothermal ecosystems in Yellowstone National Park appear to be protecting boreal toads from redleg, a potentially fatal bacterial disease [52]. Finally, the relict leopard frog *Rana* (*Lithobates*) *onca*, a close relative to the lowland leopard frog [53], [54], is a rare species whose survival may be contingent on geothermal watersheds. The relict leopard frog was once thought to be extinct, but several populations were rediscovered in the 1990's, and all naturally occurring *R. onca* populations are now

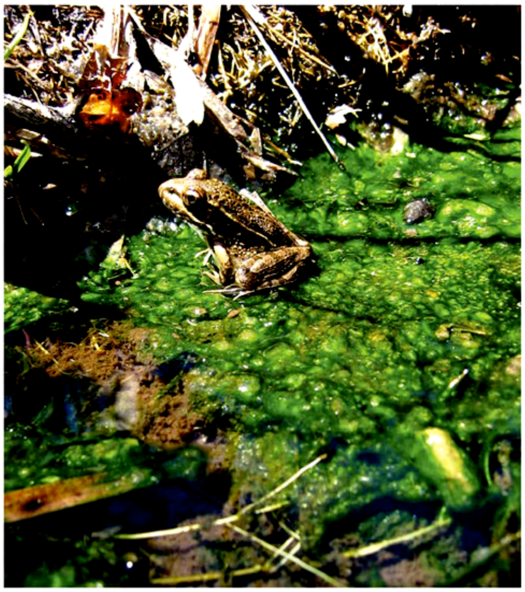

Figure 4. Lowland leopard frog in Muleshoe Hot Spring. Juvenile lowland leopard frog *Rana* (*Lithobates*) *yavapaiensis* inhabiting Muleshoe Hot Spring, a geothermal ecosystem near Willcox, Arizona. Frogs were repeatedly observed in waters 35–39°C during this study. (Photo credit: MA Schlaepfer).
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associated with perennial geothermal springs with source temperatures exceeding 30°C [18].

Elucidating the relationship between temperature and *Bd* prevalence has important implications for effective conservation, and reintroductions of threatened and endangered native amphibians [27]. No methods are currently available to treat amphibian populations against *Bd* in the wild; therefore susceptible species may persist only where conditions are not favorable for *Bd* or for chytridiomycosis outbreaks [24]–[26]. Our findings indicate that geothermal waters 25°C–37°C appear to provide amphibians with significant protection from *Bd* and, by extension, chytridiomycosis. While some species may not tolerate high temperatures, there is often a wide range of water temperatures present in geothermal ecosystems (Figs. 2 and 3). It may also be possible to experimentally augment temperatures in non-geothermal environments, thereby creating *Bd*-free microhabitats that can provide infected individuals with opportunities to clear themselves of the pathogen. Geothermal watersheds appear to represent habitats of exceptional conservation value for some amphibians, and emphasizing protection and restoration efforts, as well as native species translocations into suitable geothermal ecosystems could help recover threatened and endangered species.

Materials and Methods

The research presented here was conducted in accordance with State University of New York, College of Environmental Science and Forestry Institutional Animal Care and Use Committee (IACUC) permit 2009-4 (amended), with Dr. Martin Schlaepfer as the Principal Investigator. The IACUC at the University of California San Diego also approved our Animal Use Protocol (Protocol Number S11013) on 1/31/2011, with Dr. Gregory Rouse as Principal Investigator.

We sampled amphibians from twelve sites in Arizona in 2004, 2009, and 2010 (Table 1). Seven of the sites were influenced by geothermal springs or wells, while five were not. We searched for frogs at night (except at Markham Creek, which was sampled by day). At each site, we captured as many individuals as possible by hand, using a new pair of disposable Nitrile gloves to capture and handle each animal. Each individual was retained in a new, closable plastic bag (Ziploc®) until all sampling was completed to ensure that animals were only sampled once, and to prevent cross-contamination. In order to minimize storage times, frogs were processed in the order of capture, generally within 30–90 min. No individuals showed obvious signs of stress, and all animals swam or hopped away immediately upon release.

Bd is transmitted aquatically [38], [55], [56]; therefore we used the water temperature at the place and time of each capture as an independent variable. This approach was intended to capture microhabitat (spatial) variation in water temperature, rather than relying on daily or monthly air temperatures at sites. It also has the benefit of being measured at the same time as the frog was sampled for *Bd*. When the captured frogs were partially or fully immersed in water, temperature was measured at the point of capture using a digital thermometer (CDN® Model Q2-450; accuracy ±0.5°C). Six frogs were captured on the banks (within less than 2 m of water), in which case the temperature was measured at the nearest water point. Samples from 2004 represent a subset of records from a previous survey of *Bd* in Arizona [20] that also included water temperature at the point of capture. We determined the sex (male, female, or juvenile) of captured frogs, and measured snout-vent length (SVL) and mass before releasing at the point of capture. We defined juveniles as individuals shorter than 50 mm SVL; the approximate size at which ranids in this group of species ("*pipiens*") become sexually mature [57].

Methods for detecting the presence of *Bd* followed standard procedures. In 2009 and 2010 we used a Sterile Omni Swab (Whatman® WB100035) to sample skin cells from each animal's venter, flanks, and groin. We swabbed each amphibian a total of 25 times using the applicator, which was then ejected into in a 2-ml sterile tube filled with a buffer solution containing 50 mM Tris, pH 8, 50 mM EDTA, 25 mM Sucrose, 100 mM NaCl, and 1% SDS. In 2004, frogs were scraped 25 times in the same body locations using a wood applicator, which was then placed in 70% ethanol [20]. All samples were assayed within one month of being collected for the presence of *Bd* using Polymerase Chain Reaction amplification by a commercial lab (Pisces Molecular, Boulder, CO), following the methods from Annis et al [58] with modifications to increase sensitivity and specificity (J. Wood personal communication 2009). Experiments comparing skin scrapes versus skin swabs demonstrated that the ability to detect Bd-positive animals did not differ significantly between the methods [59]. Moreover, the Qiagen DNA spin column procedure that we used for DNA extraction is not inhibited by tannins or other compounds found in wood, unlike other DNA extraction methods (J. Wood, pers. comm., 2011).

We restricted our statistical analyses to the most common amphibian species, the lowland leopard frog (*Rana* [*Lithobates*] *yavapaiensis*) (Fig. 4). A binomial logistic regression using a logit link function tested for an association between the response variable (*Bd* presence/absence) and several predictor variables: Snout-Vent Length (SVL) of individual, Year, Distance to Solstice (absolute difference in days between the sampling date and June 21st used to capture seasonal variation), Site, and water temperature. Two-sample t-tests, Fisher's exact, and chi-square tests were used to test for differences in distributions. Analyses were conducted in Minitab (vers. 15), and results are reported as means and standard errors.

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Author Contributions

Conceived and designed the experiments: MF MS. Performed the experiments: MF MS. Analyzed the data: MS MF. Contributed reagents/materials/analysis tools: MF MS. Wrote the paper: MF MS.

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Chapter 3: First reports of amphibian chytrid fungus infections in the Ash Meadows National Wildlife Refuge

First reports of amphibian chytrid fungus infections in the Ash Meadows National Wildlife Refuge

Matthew J. Forrest and Jef R. Jaeger

Abstract

We tested amphibians within the Ash Meadows National Wildlife Refuge (AMNWR) for the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), an invasive pathogen that has been implicated in severe amphibian declines throughout the world. Fourteen toads tested positive for *Bd*, and infected animals were found at two different thermal spring ecosystems. Infections were restricted to western toads *Anaxyrus boreas* (= *Bufo boreas*), and one animal that appeared to be a hybrid of western and Woodhouse's toad *Anaxyrus woodhousii* (= *Bufo woodhousii*) based on visual inspections of morphological differences. No *Bd* infections were found in amphibians inhabiting the upper reaches of the thermal springs, where water temperatures above 28°C may provide refuge from the pathogen. These findings may have important conservation implications because chytridiomycosis (the disease caused by *Bd*) has been implicated in drastic declines in some populations of *A. boreas*. This is the first report of *Bd* infections within the AWNWR, a unique desert wetland ecosystem that harbors more endemic species than any other local area in the United States.

Introduction

Chytridiomycosis is an infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter referred to as *Bd*). This devastating pathogen has wreaked havoc on amphibian populations throughout the world, and *Bd* has been implicated as a primary cause of population declines and species extinctions (Stuart et al. 2004, Skerratt et al., 2007; Kilpatrick et al. 2010). According to the Global Amphibian Assessment, amphibians are one of the most threatened classes of vertebrate with approximately 41% of species considered threatened in 2012 (IUCN, 2012). In fact, *Bd* has been described as "possibly the most deadly invasive species on the planet (excluding humans)" (Rohr et al. 2008), because it has caused "the most spectacular loss of biodiversity due to disease in recorded history" (Skerratt et al. 2007).

Amphibian susceptibility to *Bd* and chytridiomycosis is species-specific (Searle et al., 2011); however, environmental conditions and infection intensities also influence virulence and host-disease dynamics (Vredenburg et al., 2010; Kilpatrick et al. 2010). Field studies show that *Bd* infections are generally more severe in winter months and when hosts are found in cooler temperatures (Bradley et al. 2002; Berger et al. 2004; Murray et al. 2009; Voordouw et al. 2010). Some species such as American bullfrogs *Lithobates catesbeianus* (= *Rana catesbeiana*) and Pacific treefrogs *Pseudacris regilla* appear to be minimally affected by *Bd* infections, and have been considered potential carriers of the disease (Daszak et al., 2004; Reeder et al., 2012). In contrast, lab studies demonstrated 100% mortality from *Bd* infections in *Anaxyrus boreas* (= *Bufo boreas*), and chytridiomycosis may reduce survival by 31–42% in wild *A. boreas* populations

(Carey et al., 2006; Pilliod et al., 2010). The Rocky Mountain populations of *A. boreas* in Colorado, Wyoming and the Yosemite area of the Sierra Nevada, California have undergone drastic declines since the 1970s (Carey 1993; Drost and Fellers 1996). *Bd* is suspected as the primary cause of the collapse of *A. boreas* populations in the southern Rocky Mountains, including a crash to near extinction in Rocky Mountain National Park in the mid-1990s (Muths et al. 2003).

Very little is known about the distribution of *Bd* and the repercussions of chytridiomycosis among native amphibian populations in Nevada. In April 2012, we tested amphibians for *Bd* within the Ash Meadows National Wildlife Refuge (AMNWR) in southern Nevada (Figure 1). AMNWR is a unique desert wetland ecosystem fed by more than 30 springs that harbors more endemic species than any other local area in the United States. Previous amphibian surveys at Ash Meadows identified western toads *A. boreas*, Woodhouse's toads *Anaxyrus woodhousii* (= *Bufo woodhousii*), *L. catesbeianus*, and *P. regilla*. Woodhouse's toads were the most common species observed, and hybrids between *A. woodhousii* and *A. boreas* have been reported from ANWR (Stebbins, 2003).

Methods

During April 2012, we sampled amphibians opportunistically for *Bd* within the Ash Meadows National Wildlife Refuge. We chose this time because air temperatures were warm enough to stimulate mating activities and high numbers of animals were present, but diurnal air temperatures were generally below the lethal limit for *Bd* (28°C). We searched for amphibians at five sites, but only encountered sufficient numbers of amphibians to sample within three thermal spring systems: Indian Springs, Crystal Springs Outflow, and Point of Rocks Springs (Fig. 1). Indian Spring has a source temperature of 31.7°C, and we sampled amphibians along the entire length of its accessible terrain, encountering toads in water temperatures ranging from 13.4 to 29.6°C. Point of Rocks Springs is a complex of seven thermal springs, six of which have source temperatures of 28.7°C and flow from a hillside before converging at the valley floor. The seventh spring has a source temperature of 32°C, and flows into King's Pool, a large pool on the valley floor. Within the Point of rocks springs complex, we encountered toads in water temperatures ranging from 27.2 to 31.5°C. Crystal Springs Outflow is concrete-lined and channelized, and most amphibians were observed clinging to the sides and dipping their water absorbing "seat patches" into the rapidly flowing water. Crystal Springs Outflow eventually overflows into a marshy meadow and then to a reservoir. We encountered amphibians in water temperatures ranging from 26.8 to 27.6°C. In total, we sampled *seven L. catesbeianus*, 44 *A. boreas*, 12 *A. woodhousii* and five toads that appeared to be hybrids of *A. Boreas*/*A. woodhousii* based on morphological difference (see below).

Anaxyrus woodhousii are very similar in appearance to *A. boreas* (Fig 2). Both toads are brownish or greenish in color and both have a cream-colored stripe down their backs. The major differences between the two species is the shape of the parotoid glands, and the presence of a cranial crest--a ridge that frames the inner rim of the upper eyelid--which is present on *A. woodhousii* but not on *A. boreas* (Figs. 2, 3). Parotoid glands of *A. woodhousii* are narrow, elongate, and spread apart by more than one (parotoid) length. Cranial crests run lengthwise from the front of the eyes to back edge of the eyes, then turn sharply towards the side of the toad and extend to the outside edges of the eyes. *A. woodhousii* also may grow larger than *A. boreas*, reaching sizes up to 125mm (SVL) for females and around 100mm for males (Stebbins, 2003).

The skin of adult *A. boreas* is covered with small round or oval warts on a background color that is usually green or brown; the warts may be reddish-brown and encircled by dark pigment. Parotoid glands are oval and larger than the eyes, and cranial crests are absent or indistinct (Figs. 2, 4). Usually there is a light stripe down the middle of the back, but this may be absent or inconspicuous in juveniles. Mature males have a dark patch on the inner surface of the innermost digit ("thumb") during breeding. Males lack a vocal sac, however, they may produce a repeated chirping sound (often referred to as a release call)—particularly when handled. Males rarely exceed 95 mm SVL, females rarely 110 mm (Stebbins, 2003).

Prior to sampling, boots and waders were scrubbed with a wire brush to remove all mud and dirt and all gear was sterilized by bathing or spraying it with a diluted (10%) bleach solution in hot water. This regime was also repeated before we moved to a new site. At each site, we captured individuals by hand, using a new pair of disposable Nitrile gloves to capture and handle each animal. Each individual was retained in a new, closable plastic bag (Ziploc®) that was left partially open to allow air exchange. Animals were restrained until all sampling was completed to ensure that animals were not resampled, and to prevent cross-contamination. To minimize stress, animals were processed in the order of capture (generally within 60 min), and were released at the point of capture. No individuals showed obvious signs of distress during sampling, and all animals swam or hopped away immediately upon release. When the toads and frogs were partially or fully immersed in water, temperature was measured at the point of capture using a Thermapen™ digital thermometer (accuracy ±0.4°C). Some amphibians were captured on the banks (within less than 10m of water), in which case the temperature was measured at the nearest water point, and body temperatures were obtained by placing the tip of the thermometer under the folded rear limb of the amphibian; the thermometer was sterilized with diluted bleach and rinsed between uses. We used a Sterile Omni Swab (Whatman® WB100035) to sample skin cells from each animal's venter, flanks, and groin. We swabbed each amphibian a total of 25 times using the applicator, which was then ejected into in a 2-ml sterile tube filled with70% ethanol.

All samples were assayed within one month of being collected for the presence of *Bd* by a commercial lab (Pisces Molecular, Boulder, CO). The sample DNA was extracted using the following protocol: the liquid in each of the skin swab samples was mixed by pipetting the liquid up and down repeatedly, with the entire volume of each sample then transferred into individual microfuge tubes. The tubes were spun in a microcentrifuge at ~16,000 x G for 3 minutes, and the supernatant drawn off and discarded. Lysis buffer was added and any pellet present was resuspended by vortexing. Carrier DNA (10 µg) was then added, and total DNA extracted using a spin-column DNA purification procedure.

The prepared DNAs were assayed for the presence of the *Bd* ribosomal RNA Internal Transcribed Spacer (ITS) region by quantitative real-time PCR (qPCR) using an assay developed at Pisces Molecular. PCR was conducted for 45 cycles on a Stratagene MX4000 Multiplex Quantitative PCR Cycler. The detection sensitivity of this assay was three target sequence molecules (approximately 0.02 zoospore equivalents). Each PCR run included a positive control—of prepared from a plasmid DNA constructed at Pisces Molecular containing the *B. dendrobatidis* ribosomal RNA Intervening Transcribed Sequence (ITS) region. Serial ten-fold dilutions of this plasmid DNA, from 3 x 106 to 3 x 100 molecules per reaction, were used to generate a standard curve. Negative controls consisted of sterile water in place of template DNA. The negative control reactions remained uncapped during addition of sample DNA during reaction set-up to detect potential carryover of DNA.

Results

Table 1 shows the results of the *Bd* surveys from the Ash Meadows National Wildlife Refuge during April 2012. In total, 14/68 (20.6%) amphibians were infected with *Bd* (= *Bd*+), including all species and locations sampled. At Indian Springs (Fig 1), 9/25 *A. boreas* were infected. Most of these infected toads (7/9) were captured out of water. For the two *Bd*+ toads caught in the water, water temperatures were 22.7, and 24.4°C. One toad was observed to be thin and listless, and had difficulty righting itself; this toad also proved to be *Bd*+ (644 zoospore equivalents). At Crystal Springs Outflow (Fig 1), 4/19 *A. boreas* were found to be infected, and for 3 *Bd*+ toads caught in water, the water temperatures were 27.6, 27.5, and 26.2°C. One *A. boreas* was observed to have a very red seat patch, and according to qPCR results this individual had the highest rate of infection of all amphibians sampled (15,700 zoospore equivalents). Of five potential hybrids of *A. boreas* / *A. woodhousii* sampled at Crystal Spring Outflow, one was *Bd*+. All seven *L. catesbeianus* caught at Crystal Springs tested negative (all caught in water, with temperatures 26.6, 26.8. 26.8. 27, 27.1, 27.5, 28.6°C). At Point of Rocks Springs (Fig 1), 12 *A. woodhousii* tested were negative for *Bd*, and all were encountered in water temperatures > 27.2°C.

Discussion

This is the first report of *Bd* infections within the Ash Meadows National Wildlife Refuge (AMNWR). AMNWR represents a watered island within the Mojave Desert, and the persistence of this water since the late Pliocene/early Pleistocene has provided essential habitat for 25 endemic species, including the critically endangered Devils Hole pupfish and 12 other endangered or threatened species (Sada, 1990). This is the greatest concentration of endemic species of any local area in the United States.

In this study, *Bd* infections were restricted to western toads *A. boreas*, and one animal that appeared to be a hybrid between this species and *A. woodhousii*. We sampled a total of five toads that appeared to be *A. boreas/A. woodhousi* hybrids based on visual inspection of morphological characteristics (See Materials and Methods section). These hybrids were restricted to one site, Crystal Springs Outflow. Interestingly, no *Bd* infections were found among *L. catesbeianus* (n=7) also inhabiting Crystal Springs Outflow, despite the fact that this species has been considered potential carrier and reservoir of the disease (Daszak et al., 2004). This site has also been invaded by exotic red swamp crayfish (*Procambarus clarkii*), which can become infected with *Bd* in nature and can transmit infections to amphibians (McMahon et al., 2012). Moreover, crayfish can maintain infections for months in the lab, and in the wild crayfish presence has been shown to be positively correlated with *Bd* infections in co-occurring amphibians (McMahon et al., 2012).

Our detection of *Bd* in *A. boreas* within AMNWR is particularly concerning because this species is classified as “endangered” by the states of Colorado and New Mexico, and has been listed as “Near Threatened” by the International Union for the Conservation of Nature (IUCN) because it is in significant decline due to diseases, including chytridiomycosis (Carey 1993; Drost and Fellers 1996; Muths et al. 2003; Hammerson et al., 2004).

Anaxyrus woodhousii are known to hybridize with many other amphibian species, including *A. boreas* at AMNWR (Stebbins, 2003). Hybridization can complicate recovery efforts for endangered and threatened species, therefore it is imperative to determine the extent and direction of hybridization and introgression, and whether hybrid zones are naturally occurring or human influenced (Allendorf et al. 2001; Schwaner and Sullivan, 2009). Several studies support the replacement hypothesis, in which *A. woodhousii* initially colonizes and then replaces native species in disturbed habitats (Sullivan, 1986; Sullivan and Lamb, 1988; Malmos et al. 2001). *A. woodhousii* have replaced native toad species in some locations in Arizona and Nevada—particularly in areas influenced by anthropogenic disturbance (Sullivan 1986, 1995; Malmos et al., 2001; Schwaner and Sullivan, 2009). For example, *Bufo microscaphus* was reportedly the only toad originally inhabiting lower elevation areas of the Las Vegas Valley prior to development in the early 1900s, but sometime between 1925 and 1976 *B. microscaphus* populations disappeared from the Las Vegas Valley and *A. woodhousii* appeared and became widespread (Bradford et al., 2005). *A. woodhousii* have been expanding their range in Nevada following the colonization of Las Vegas Valley, and may have been introduced directly by humans, along with bullfrogs and crayfish (Bradford et al., 2005). Although it is unknown when and how *A. woodhousii* arrived in Ash Meadows, it is likely that they have proliferated due to human disturbance of the wetlands before the establishment of AMNWR. Prior to the initiation of conservation activities, the springs were threatened by groundwater pumping, diversion of surface water for irrigation and impoundment, and impact from livestock and feral horses (Kodrik-Brown and Brown, 2007).

What remains unclear is whether *A. woodhousii* is less susceptible to *Bd* than *A. boreas*. Even more work will be necessary to determine the susceptibility of hybrid toads to *Bd*. *A. woodhousii* is reported to be more tolerant of higher temperatures than

A. boreas (Brattstrom, 1968), and are active at temperatures ranging from 22° C to 35° C; adult *A. woodhousii* can tolerate a maximum temperature of 40.5° C (Brattstrom 1968). Lillywhite et al. (1973) found that the preferred body temperature among *A. boreas* in the laboratory is approximately 26–27 ˚C, and adults can tolerate a maximum temperature of 38.1 ˚C (Brattstrom, 1968). Field studies conducted around the world show *Bd* infections are generally more severe in winter months, and when hosts are found in cooler temperatures (Bradley et al. 2002, Berger et al. 2004, Murray et al. 2009, Voordouw et al. 2010). In the laboratory *Bd* growth ceases at temperatures above 28°C, with 100% mortality occurring within 96 hours at 32°C and within 4 hours at 37°C (Piotrowski et al. 2004; Johnson and Speare, 2003). Similarly, *Bd* does not persist in amphibian hosts above certain temperature thresholds. In laboratory experiments, short-term exposure to temperatures between 27 and 37°C successfully cleared *Bd* infections from five different species of adult frogs (Berger et al. 2004; Woodhams et al. 2003; Retallick and Miera 2007; Chatfield and Richards-Zawacki, 2011). Temperature is also strongly linked to amphibian immune system responses, and declines in amphibian immune defenses as temperatures decrease are well documented (Green and Cohen 1977, Zapata et al. 1982, Maniero and Carey 1997, Rollins-Smith et al., 2002). Conversely, at warmer temperatures amphibians may be less susceptible to *Bd* due to greater effectiveness of the immune response (Andre et al., 2008; Murphy et al., 2011). Additionally, recent studies have demonstrated that resistance to *Bd* is stronger when amphibians experience constant temperatures rather than drastic fluctuations in temperatures (Raffel, 2012).

Identifying environmental and climactic conditions that provide natural refuges from *Bd* and chytridiomycosis will benefit imperiled amphibian populations (Puschendorf et al. 2009, Walker et al. 2010), and may provide some susceptible species with opportunities to evolve evolutionary responses to the pathogen (Tobler and Schmidt 2010). In lab studies, the outcome of *Bd* infections in *A. boreas* has been shown to be environmentally dependent. Periodic drying and moderate warming enhanced the survival of infected toads, and *A. boreas* with severe *Bd* infections shifted resting positions towards heat strips, suggesting a behavioral fever strategy to combat the disease (Murphy et al., 2011). Within the Greater Yellowstone Ecosystem, *A. boreas* breed predominantly in geothermal ecosystems, which also appear to be protecting them from redleg, a potentially fatal bacterial disease (Hawk, 2000). In fact, the only boreal toads that survived a mass mortality episode in Yellowstone National Park were those that hibernated near water warmed by thermal springs (Peterson, C unpub. data cited in Carey, 2000).

Studies in Arizona have demonstrated that thermal springs can provide amphibians with refugia from *Bd* (Schlaepfer et al., 2007; Forrest and Schlaepfer 2011; Chapter 2). At least 10 of the springs within the Warm Spring Complex at Ash Meadows have source temperatures exceeding 28°C, the upper thermal limit of *Bd* (Piotrowski et al., 2001), and we did not find *Bd* infections in amphibians inhabiting waters > 28°C. Therefore, it appears that the higher temperature portions of the warm springs at AMNWR may provide *A. boreas* with refuge from *Bd* and chytridiomycosis.

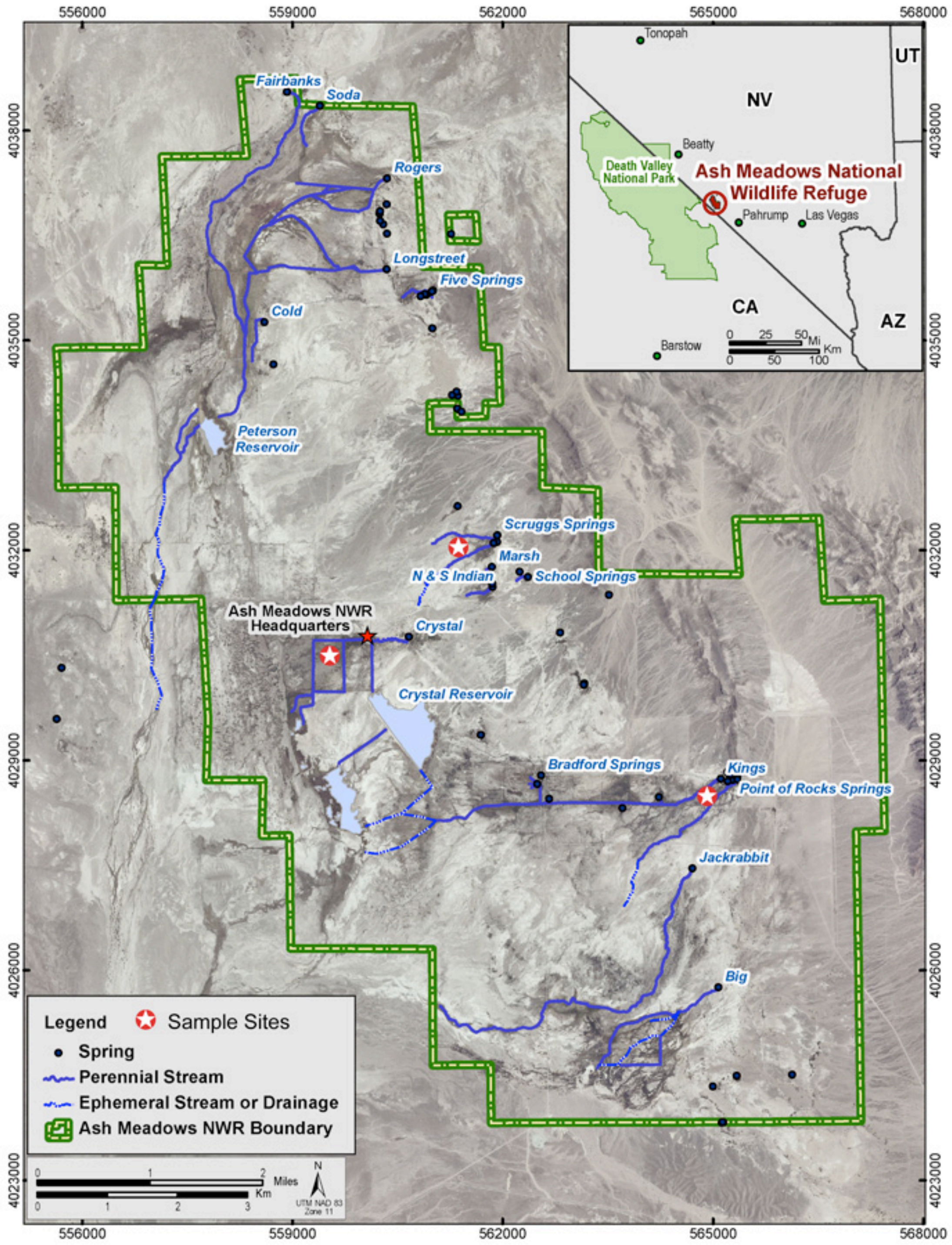

Figure 3-1: Map of Ash Meadows National Wildlife Reserve with locations of sites where amphibians were sampled for *Batrachochytrium dendrobatidis*.

Figure 3-2. Picture of a Woodhouse’s toad (*Anaxyrus woodhousii*) on the left and a western toad (*Anaxyrus boreas*) on the right. Note differences in coloration, size and shape. The most significant morphological differences are the shape of the parotoid glands and presence of a cranial crest in the Woodhouse’s toad (also see Figs 3,4).

Figure 3-3. Close-up picture of a Woodhouse's toad (*Anaxyrus woodhousii*) showing elongate parotoid glands and the presence of a cranial crest, a ridge that frames the inner rim of the upper eyelid.

Figure 3-4. Close-up picture of a western toad (*Anaxyrus boreas*) showing oval-shaped parotoid glands and the lack of a cranial crest.

Table 3-1. Locations, species and *Batrachochytrium dendrobatidis* prevalence of amphibians sampled in the Ash Meadows National Wildlife Refuge in April 2012.

Location	**Species (N)**	**Bd Prevalence**	**Range Intensity (Mean # Zoospores)**
Indian Springs	*Anaxyrus boreas* (25)	0.36	24 - 1840 (**634.8**)
Crystal Springs	*Anaxyrus boreas* (19)	0.21	35.7 - 15700 (**4091.9**)
Crystal Springs	*A. boreas x woodhousii* (5)	0.2	638
Crystal Springs	*Lithobates catesbeianus* (7)	0	
Point of Rocks	*Anaxyrus woodhousii* (12)	0	

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Chapter 4: Habitat Loss and the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* May Threaten the Dixie Valley toad, a Narrowly Distributed Endemic Species

Habitat loss and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* may threaten the Dixie Valley toad, a narrowly distributed endemic species

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Abstract

The Dixie Valley toad is a yet undescribed species with an extremely narrow distribution in northwestern Nevada, USA. Currently restricted to four spring-fed wetlands within Dixie Valley NV, this unique toad appears to be a relict member of the western toad *Anaxyrus* (=*Bufo*) *boreas* species group. Between 2009-2012, we surveyed populations of Dixie Valley toads and other nearby amphibians, including American bullfrogs (*Lithobates catesbeianus*) and a small population of western toads (*A. boreas*). In 2011 and 2012, we also tested individuals for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). None of the Dixie Valley toads or

western toads that we sampled tested positive for *Bd*. However, the prevalence of *Bd* infections among nearby populations of American bullfrogs increased significantly from 18% in 2011 to 75% in 2012. The high incidence of the amphibian chytrid fungus among invasive *L. catesbeiana*, which are known vectors of potentially fatal disease, may represent a serious threat to this yet undescribed species. Furthermore, one of the largest geothermal reservoirs in Nevada underlies the Dixie Valley, and plans to increase the exploitation of these geothermal resources may negatively impact the severely limited essential habitat available for this toad. Therefore we recommend continuous rigorous monitoring to identify and enact essential conservation measures, along with increased efforts to update the species status for the Dixie Valley toad.

Introduction

A unique species of toad, here referred to as the Dixie Valley toad (Fig. 1), is found only within the Dixie Valley in the northwestern part of the state of Nevada, USA (Fig. 2). Although the Dixie Valley toad has not yet been formally described, unpublished molecular phylogenetic analyses indicate that it is a valid species (R. Tracy 2012, pers. comm.), as does morphology (Figs. 1, 3). It appears to part of the western Toad *Anaxyrus* (=*Bufo*) *boreas* species complex (Noles, 2010), and may be a ‘relict species’ that had a far wider distribution during pluvial periods in the past when climatic conditions were wetter (Hovingh, 1997; Reheis et al., 2002). Today the entire extant population of the Dixie Valley toad is reliant on four spring-fed wetlands, which are the only perennial sources of water in their native habitat.

The Dixie Valley toad faces many threats, including climate change (USFWS, 2009), geothermal energy development (BLM, 2010; 2011), and invasive species--particularly American bullfrogs *Lithobates catesbeianus* (=*Rana catesbeiana*) and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Chytridiomycosis, an infectious disease caused by *Bd*, is considered a primary factor in amphibian species declines and extinctions throughout the world (Stuart et al., 2004; Kilpatrick et al., 2010). In fact, *Bd* has been described as "possibly the most deadly invasive species on the planet (excluding humans)" (Rohr et al. 2008), having caused "the most spectacular loss of biodiversity due to disease in recorded history" (Skerratt et al., 2007). Bullfrogs (*L. catesbeianus*) appear to be minimally affected by *Bd*, and therefore can act as efficient carriers or reservoir hosts spreading chytridiomycosis to more vulnerable native species (Daszak et al., 2004; Hanselmann et al. 2004).

Between 2009-2012 we surveyed populations of Dixie Valley toads (Fig. 1), and a small population of western toads *Anaxyrus* (=*Bufo*) *boreas* (Fig. 3) inhabiting McCoy Ranch approximately 75km to the northeast of Dixie Meadows (Fig. 2). In April 2011 and 2012 we also tested Dixie Valley toads and other nearby populations of *L. catesbeianus* and *A. boreas* for *Bd* in order to evaluate whether chytridiomycosis represents a significant threat to this unique species. Here we report the results from the *Bd* analyses and summarize observations from population surveys of Dixie Valley toads and western toads.

Materials and Methods

NDOW/USFWS surveys protocols

Prior observations indicated that Dixie Valley toad activity is highly correlated with weather conditions, and sufficient densities and abundances of toads for surveys were only regularly encountered during late March to early May on clear and calm days. During April and May 2009, we surveyed Dixie Valley toads by walking 17 1.3 km line transects in an attempt to quantify population abundance estimates. There were 13 locations within each transect line, and the distance was 100m between each point and 50m between each transect line (Fig. 4). However, we often encountered dense vegetation and poor habitat for Dixie Valley toads along these transects. Due to limited sampling success, and funding and resource constraints we discontinued the line transect surveys after 2009.

During 2010-2012, targeted surveys were conducted in high-density toad habitat within the northern part of Dixie Meadows. At McCoy Ranch, western toads were also surveyed opportunistically along the drainage of a thermal spring, and along agricultural irrigation ditches and canals. We avoided disturbing toads that were actively mating; other toads were collected by hand to determine sex, measure Snout-Vent Length (SVL), and scan for the presence of a Passive Integrated Transponder (PIT) Tag. In 2010 and 2011, if no tag was detected, a new PIT tag (Biomark HPT8

8.4mm length, 134.2kHz frequency) was inserted subcutaneously into a subset of the adult toads via a small incision made with sterilized surgical scissors to the side of the dorsal midline and anterior to the sacral hump. No new PIT tags were deployed in 2012.

Dixie Valley toads were also surveyed eight times between April and June 2011 in order to examine and record developmental stages. Developmental stages were recorded from each survey using the Gosner method (Gosner, 1960) until the first occurrence of metamorphosis. The Gosner method provides a simplified table for describing anuran larval development through 46 stages within three specific periods, i.e., pre-metamorphosis, pro-metamorphosis and metamorphosis climax (Gosner. 1960).

Bd sampling protocol:

In 2011 and 2012 we also tested amphibians for the presence of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). In 2011, we tested 39 Dixie Valley toads (DVT) from Dixie Meadows, and 11 American bullfrogs inhabiting Turley Pond (Fig. 2) *Bd*. In 2012, we sampled 56 DVT, 13 western toads at nearby McCoy Ranch, and 32 bullfrogs from Turley Pond. At each site we captured as many individuals as possible by hand, using a new pair of disposable Nitrile gloves to handle each animal. All sampling gear was thoroughly decontaminated using a 10% bleach solution immediately after sampling at a site to prevent spreading *Bd* and other invasive species or pathogens between sites. To minimize stress, animals were processed immediately and were released at the point of capture. No individuals showed obvious signs of distress during sampling, and all animals swam or hopped away immediately upon release. We used a Sterile Omni Swab (Whatman® WB100035) to sample skin cells from each animal's venter, flanks, and groin. We swabbed each amphibian a total of 25 times using the applicator, which was then ejected into in a 2-ml sterile tube filled with a buffer solution containing 70% ethanol. All samples were assayed within one month of being collected for the presence of *Bd* by a commercial lab, Pisces Molecular, Boulder, CO, following the methods from Annis et al (2004) with modifications to increase sensitivity and specificity (J. Wood personal communication 2012).

In 2012, we used skin swabs and a realtime quantitative PCR assay to quantify *Bd* prevalence and infection intensity for *L. catesbeiana*. The sample DNAs were assayed for the presence of the *Batrachochytrium dendrobatidis* ribosomal RNA Intervening Transcribed Sequence (ITS) region by 45 cycle PCR amplification using a qPCR assay developed at Pisces Molecular and a Stratagene MX4000 real-time PCR instrument. The detection sensitivity of this assay is three target sequence molecules (approximately 0.02 zoospore equivalents).

Results

The morphology of the Dixie Valley toad (DVT; Fig. 1) is quite distinct from that of western toads from McCoy Ranch (Fig. 3). Dixie Valley Toads are markedly smaller than western toads (Figs. 5, 6), and far more colorful. In 2009, we encountered 122 Dixie Valley toads in Dixie Meadows and 18 western toads at McCoy Ranch, and 100 DVT and 10 western toads were PIT tagged (Tables 1, 2). In 2010, we encountered 95 Dixie Valley toads in Dixie Meadows and 2 western toads at McCoy Ranch. In 2010 46 DVT and 2 western toads were PIT tagged, and 2 recaptures from prior PIT tagging efforts of DVT were recorded. In 2011, we encountered 71 Dixie Valley toads in Dixie Meadows and 10 western toads at McCoy Ranch. In 2011, 50 DVT and 9 western toads were PIT tagged, and 2 recaptures from prior tagging efforts of DVT were recorded. In 2012 we encountered 55 DVT in Dixie Meadows and 13 western toads at McCoy Ranch (Tables 1, 2). Figures 5 and 6 depict histograms of toad sizes recorded as Snout-Vent Lengths (SVL) for western toads at McCoy Ranch (Fig. 5) and DVT (Fig. 6). Because so few recaptures of PIT tagged animals were observed, we were unable to accurately estimate population parameters and abundances of Dixie Valley toads, therefore we discontinued PIT tagging efforts after 2011.

During surveys in March-May, DVT were actively mating and egg masses and tadpole aggregations were also often observed. Amplexus in DVT often occurs underwater (usually <1m) along the banks of the perennial wetland habitat in Dixie Meadows (Fig. 7). In 2011, developmental stages were investigated and recorded using the Gosner method (Gosner, 1960). Calling males were first detected on March 5th 2011, ovodeposition was first observed on April 5th 2011 and the first wave of completely metamorphosed toadlets was observed 10 weeks later on June 14th 2011

(Fig. 8). Newly metamorphosed toads (n=12) had mean SVL = 15.25 mm (Fig. 9) and a mean weight of 0.375 grams. 13 weeks later on September 14th 2011, we also weighed and measured metamorphed toads (n=14), mean SVL = 18.21 mm and mean weight 0.625 grams.

In 2011, we tested 39 DVT from Dixie Meadows, and 11 American bullfrogs inhabiting Turley Pond (Fig. 2) for *Batrachochytrium dendrobatidis* (*Bd*) the pathogenic fungus that causes the potentially deadly disease chytridiomycosis. All 39 DVT tested negative for Bd infection, however, two bullfrogs from Turley Pond in the Dixie Settlement Area did test positive for *Bd* (Table 3). In 2012, we sampled 56 DVT, 13 western toads at nearby McCoy Ranch, and 32 bullfrogs from Turley Pond. Again, none of the toads were *Bd*+. However, the prevalence of *Bd* infections in American bullfrogs in 2012 was 75% (24/32) (Table 3), significantly higher than in 2011 (1-tailed Fisher's exact test; $P < 0.00137$). The mean infection intensity in 2012 was 1341 zoospore equivalents (range 46.04-18500 zoospore equivalents); qPCR analyses were not performed in 2011.

Discussion

Despite the fact that the Dixie Valley toad has not been adequately studied, and is yet to be formally described as species, it has long been recognized as an important and potentially vulnerable species by the U.S. Fish and Wildlife Service, the Nevada Department of Wildlife (NDOW), and the Nevada Natural Heritage Program (NNHP).

In 2002, the NNHP listed the “Dixie Hot Springs toad *Anaxyrus* (*Bufo*) sp. nov. 'Dixie Hot Springs” as “critically imperiled due to extreme rarity, imminent threats, or and/or biological factors”. In 2008, a working group composed of the University of Reno-Nevada, the NNHP, the Fallon Naval Air Station (FNAS), the U.S. Fish and Wildlife Service (FWS), Bureau of Land Management (BLM), and NDOW began drafting a Candidate Conservation Agreement (CCA) due to concerns about activities on public/Federal lands that might affect the status the Dixie Valley toad, and to help determine whether it has the potential to become a candidate species under the Endangered Species Act (USFWS, 2009). This CCA is meant to address the conservation needs of the Dixie Valley toad, and intends to provide guidance and a framework for implementation of cooperative long-term conservation measures to benefit the species. The land in Dixie Valley is owned and managed by the FNAS, BLM, and private landowners, and ground and air training missions are run throughout the valley by FNAS. Although the Dixie Valley toad does not currently fall under any formal classification for federal protection, it is anticipated this will be addressed as a part of additional research and ongoing monitoring. Currently, the State of Nevada extends protection to the DVT through several statutes, and the cooperators signatory to the CCA support specific conservation actions geared towards identifying and reducing or eliminating threats to the species, and maintaining and enhancing properly functioning ecosystems for the Dixie Valley toad (USFWS, 2009).

The Dixie Valley toad appears to belong to the Western Toad Complex, and may represent a ‘relict species’ with broader distribution in the past when climatic conditions were wetter and large pluvial lakes covered much of North America's Great Basin region (Hovingh, 1997; Noles, 2010). Climate change at the end of the Pleistocene led to gradual desiccation of these paleolakes, and some basins such as Dixie Valley became hydrologically isolated (Reheis et al., 2002). The western toad *Anaxyrus* (=*Bufo*) *boreas* is currently distributed throughout much of the western United States (Stebbins, 1985). Information on the geologic history of the Eastern Great Basin in Nevada suggests that populations of *A. boreas* in this region may have separated from adjacent drainages during the Pleistocene (Hovingh, 1997; Noles 2010). These vicariance barriers resulted in allopatric speciation, and the *A. boreas* species group currently comprises four described species in western North America including the broadly distributed *A. boreas*, and three localized species, *Anaxyrus nelsoni*, *Anaxyrus exsul* and *Anaxyrus canorus* (Goebel et al. 2009). However, variation within the *A. boreas* species group is poorly studied and may mask a number of cryptic species (Goebel et al. 2009). In fact, unpublished molecular phylogenetic analyses suggest that the Dixie Valley toad is another unique species within *A. boreas* species group (R. Tracy, 2012 pers. comm.).

Due to extremely limited recapture rates of PIT-tagged DVT, accessibility issues, and insufficient resources, no accurate estimates of overall population abundance and structure are currently available. However, the most immediate current threat to the Dixie Valley toad appears to be the imminent expansion of the exploitation of nearby geothermal energy resources. Dixie Valley is the hottest (> 285°C at 3 km), and one of the largest geothermal systems in the Basin and Range province, and has supported a

63 MW power plant operating for over 20 years (Blackwell, 2007). In 2011, the Nevada BLM approved an Environmental Assessment analyzing the potential impacts associated with the proposed construction and testing of geothermal exploration wells, access roads, and ancillary facilities in Dixie Valley by Terra-Gen Power (TGP) Dixie Development Company (BLM, 2010). In 2012, the Nevada BLM also approved an Environmental Assessment analyzing the potential impacts associated with the proposed construction of up to 60 new geothermal exploration wells within Dixie Valley by Ormat Technologies (BLM, 2011). TGP and Ormat do not anticipate negative impacts on the Dixie Valley toads from the geothermal development and exploration projects. However, the presence of artesian hot springs in the Dixie Meadows lease area suggest that there may be a hydrologic connection between the geothermal aquifer and the aquifer that feeds surface water features (BLM, 2010; 2011).

Most springs in the Dixie Valley exhibit evidence of shallow groundwater mixing with thermal water before discharging at the ground surface, except artesian springs located along the eastern margins of Dixie Valley, which are associated primarily with the Buckbrush Fault System and bring cold, fresh water from aquifers at depth to the surface (Smith et al., 2001). A number of flowing wells with slightly anomalous temperatures of 21-24°C, are found in the central part of southern Dixie Valley approximately 10-12 Km south of Dixie Hot Springs. These wells may be related to the same thermal system active elsewhere along the west side of Dixie Valley (Blackwell, 2007). Turley Pond (Fig. 2), where bullfrogs were sampled for *Bd* in 2011 and 2012, is filled by one of these warm-water wells. The hot springs at McCoy Ranch have source temperatures of approximately 40°C, suggesting a mixture of shallow groundwater and thermal water. However, Dixie Hot Springs with source water temperature of approximately 72°C (Mariner, 1974), appear unaffected by shallow groundwater mixing, and, and are likely directly connected to the deeper geothermal system (Blackwell, 2007).

Permanent water sources that drain onto the Dixie Valley playa area include the Dixie Hot Springs and another cold spring to the south (Fig. 2). Although the volume of water discharged from these springs is not sufficient to completely inundate the valley floor, a topographically depressed area between the water sources and the playa intercepts some of the water, forming a perennial pond. Dixie Meadows (Fig. 2), the area adjacent to this consistently filled pond represents the only known breeding habitats for the Dixie Valley toad (BLM, 2011). If the hydrothermal resources are overexploited and the water table is lowered, these surficial springs may no longer flow. As an example, overexploitation of the Wairakei Geothermal Field in New Zealand resulted in the disappearance of hot springs and geysers in Geyser Valley, which were replaced by steam-heated pools and fumaroles (Allis, 2000). If essential breeding habitat is negatively impacted by the expansion of geothermal energy exploitation, it could lead to the extinction of the Dixie Valley toad. The small population of western toads at McCoy Ranch (Fig. 2) may also face threats from the planned expansion of geothermal energy exploitation in Dixie Valley. Water needed for construction and drilling operations is being trucked from McCoy Ranch several times each day. These disturbances may negatively affect the habitat of the western toad population, and could also result in the introduction of invasive American bullfrogs and *Bd* to McCoy Ranch.

None of the Dixie Valley toads or western toads that we sampled in 2011 and 2012 tested positive for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). However, infection rates among American bullfrogs increased significantly over one year (1-tailed Fisher's exact test; $P < 0.00137$). The prevalence of *Bd* among *L. catesbeianus* was 18% (2/11) in 2011, while 75% (24/32) were found to be infected in 2012 (Table 2). In 2012, the mean infection intensity was 1341 (qPCR analyses were not performed in 2011). Bullfrogs are known vectors of chytridiomycosis, and appear to be minimally affected by *Bd* infections (Daszak et al., 2004; Hanselmann et al. 2004). The highly alkaline soil between the ponds supporting bullfrogs and the main Dixie Meadows toad habitat may act as a barrier for the bullfrogs. However, it is feasible that these ponds could periodically become connected hydrologically with the toad habitat if heavy precipitation resulted in flooding. Moreover, bullfrogs and DVT are found together in the pond associated with the cold spring (Fig. 2), and these toads have not yet been tested for *Bd*.

The prevalence of *Bd* and the virulence of chytridiomycosis are particularly influenced by temperature (Woodhams et al., 2008). Field studies conducted around the world show *Bd* infections are generally more severe in winter months and when hosts are found in cooler temperatures (Bradley et al. 2002, Berger et al. 2004, Murray et al. 2009, Voordouw et al. 2010). In the laboratory, *Bd* growth ceased at temperatures above 28°C; 100% mortality of *Bd* occurred within 96 hours at 32°C, and within 4 hours at 37°C (Piotrowski et al. 2004; Johnson and Speare, 2003). Similarly, *Bd* does not persist in amphibian hosts above certain temperature thresholds. In laboratory experiments, short-term exposure to temperatures between 27 and 37°C successfully cleared *Bd* infections from five different species of adult frogs (Berger et al. 2004; Woodhams et al. 2003; Retallick and Miera 2007; Chatfield and Richards-Zawacki, 2011). Amphibians may also be less susceptible to *Bd* when they experience warmer temperatures due to greater effectiveness of the immune response (Andre et al., 2008; Murphy et al., 2011).

Studies in Arizona have demonstrated that thermal springs can provide amphibians with refugia from *Bd* (Schlaepfer et al., 2007; Forrest and Schlaepfer 2011). The relict leopard frog *Rana* (*Lithobates*) *onca* was once thought to be extinct, but several populations were rediscovered in the 1990's and all naturally occurring *R. onca* populations are now associated with perennial geothermal springs with source temperatures exceeding 30°C (Jaeger et al., 2001; Bradford et al., 2004). Within the Greater Yellowstone Ecosystem, *A. boreas* breed predominantly in geothermal ecosystems, which also appear to be protecting them from redleg, a potentially fatal bacterial disease (Hawk 2000). In fact, the only toads that survived a mass mortality episode in Yellowstone National Park were those that hibernated near water warmed by thermal springs (Peterson, C. unpubl. data cited in Carey, 2000). In lab studies, the outcome of *Bd* infections in *A. boreas* has been shown to be environmentally dependent--periodic drying and moderate warming enhanced survival and toads with severe *Bd* infections moved towards heat strips, suggesting a behavioral fever strategy to combat the disease (Murphy et al., 2011).

Thermal springs with water temperatures high enough to clear *Bd* infections from amphibians (>28°C) are present throughout the Dixie Valley. However, it is unclear if Dixie Valley toads inhabit or utilize the thermal water from Dixie Hot Springs. Although the source temperatures above 70°C would certainly be lethal to amphibians, the water cools quickly as it flows away from the source and waters of virtually any temperature would be available. The Dixie Valley toads that we sampled for *Bd* in 2011 and 2012 were captured in or near water temperatures ranging from 9 to 19.3°C. We have not observed toads within the warmer portions of the Dixie Hot Springs, however much of this habitat is overgrown and obfuscated by dense vegetation. Dixie Valley toads may also benefit from the constant temperatures available year-round in the thermal springs and nearby warm-water wells, as recent studies have demonstrated that resistance to *Bd* is stronger when amphibians experience constant temperatures rather than drastic fluctuations in temperatures (Raffel, 2013). The western toad habitat at McCoy Ranch is also associated with a thermal spring with source temperatures of approximately 40°C.

Chytridiomycosis has devastated many populations of amphibians throughout the world, and has been documented to lead to complete extinctions of particularly vulnerable species (Stuart et al., 2004; Kilpatrick et al., 2010). Therefore, it is imperative that we investigate the extent of *Bd* infections among nearby bullfrog populations, and continuously monitor the Dixie Valley toads for any signs of *Bd* infections and chytridiomycosis outbreaks. As exploitation of the geothermal resources in the Dixie Valley expands, any disturbances to the wetlands could lead to the disappearance of essential habitat for the Dixie Valley toad. Additionally, very little is known about the population dynamics and the life-history characteristics of the Dixie Valley toad. Consequently, we recommend enhanced monitoring efforts along with immediate efforts to update the species status for the Dixie Valley toad, lest we lose a unique species before it is even officially recognized as such.

Permit information: All amphibians were collected under the authority of the Scientific Collection permits issued by the Nevada Department of Wildlife to the Nevada Department of Wildlife, (permit number 34992) University of California, Davis (permit number 33983) and University of Nevada, Reno (permit number 35517.)

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Figure 4-1. Image of adult Dixie Valley Toad sampled in 2009 from Dixie Meadows (photo credit K. Urquhart).

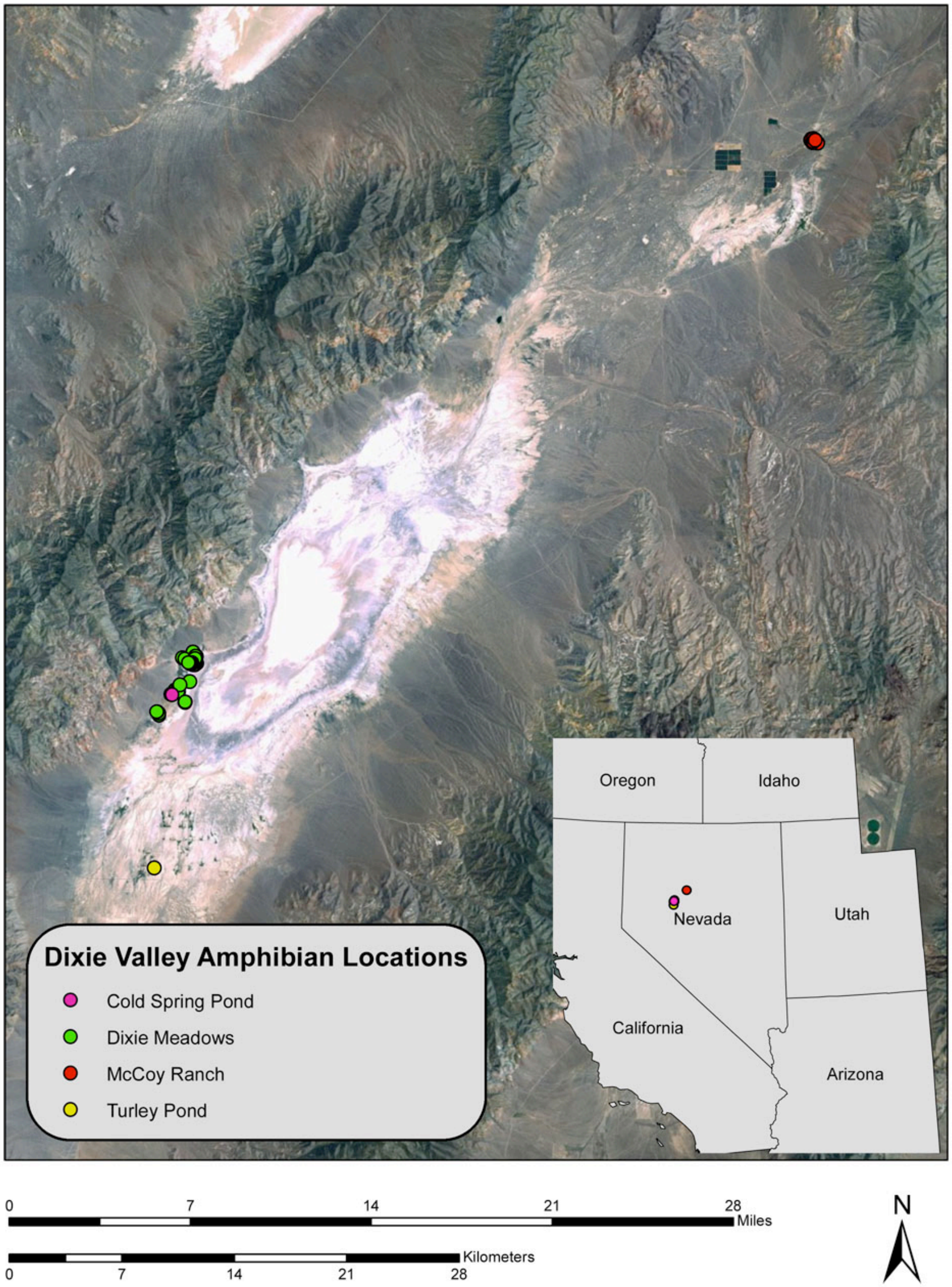

Figure 4-2. Map of the Dixie Valley, Nevada area showing locations of sampling sites for Dixie Valley toads, western toads *Anaxyrus* (=*Bufo*) *boreas*, and American bullfrogs *Lithobates catesbeianus*.

Figure 4-3. Image of adult western Toad *Anaxyrus* (=*Bufo*) *boreas* sampled in 2009 from McCoy Ranch (photo credit K. Urquhart).

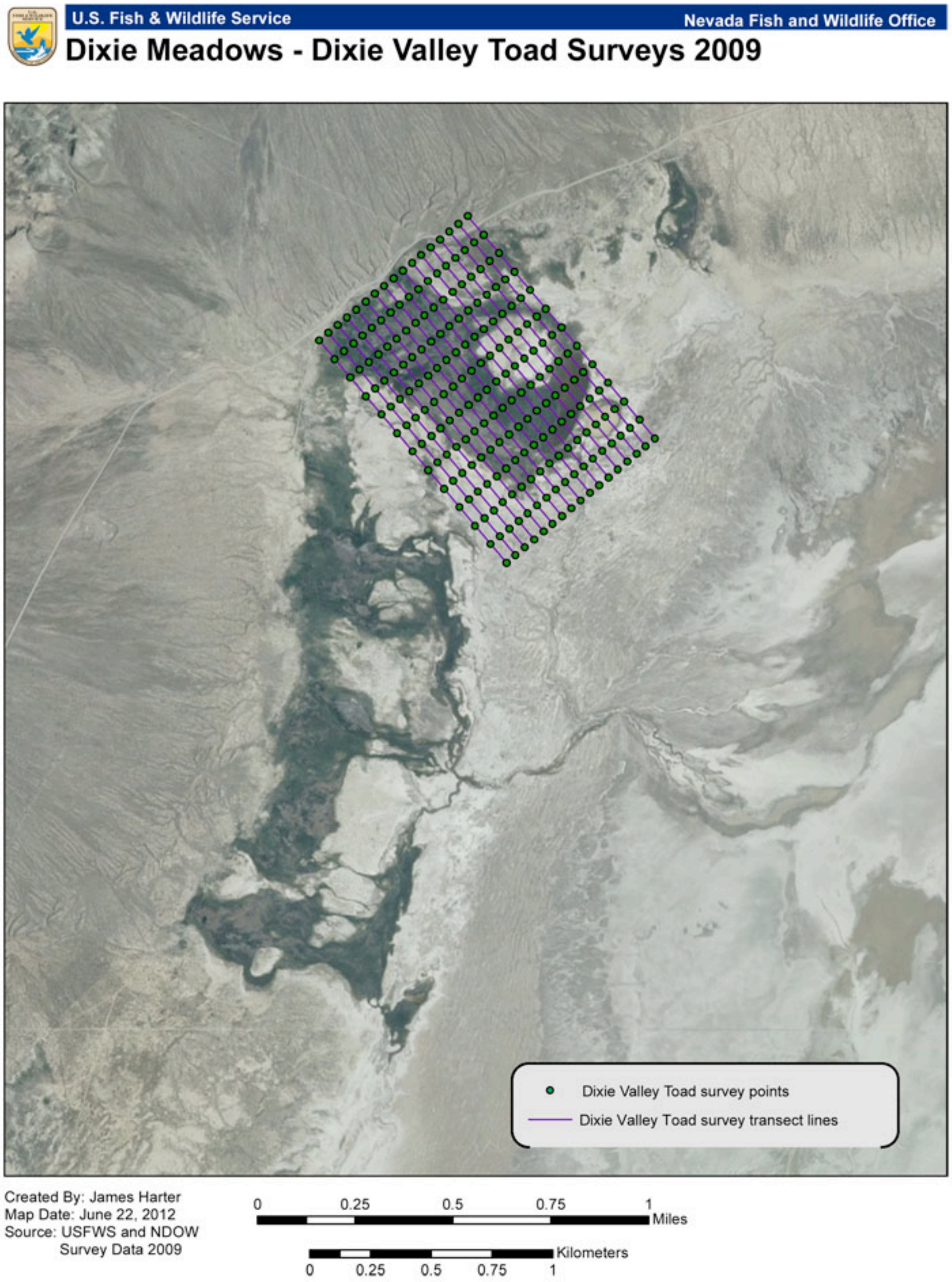

Figure 4-4. Map of line transect survey locations for Dixie Valley toads from 2009.

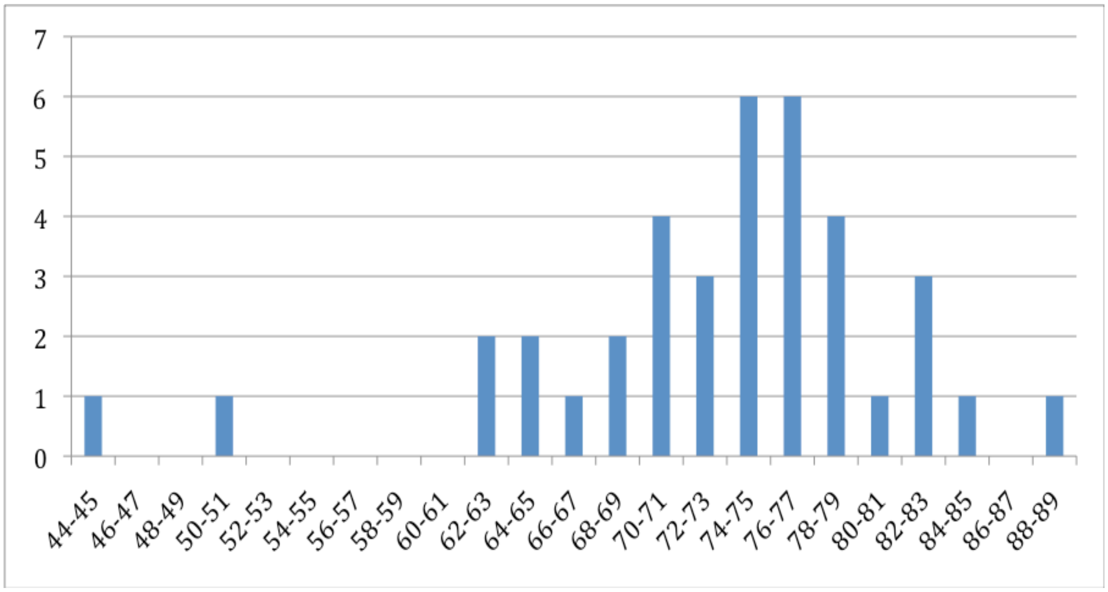

Figure 4-5. Distribution of SVL from western toads *Anaxyrus* (=*Bufo*) *boreas* sampled at McCoy Ranch from 2009-2012.

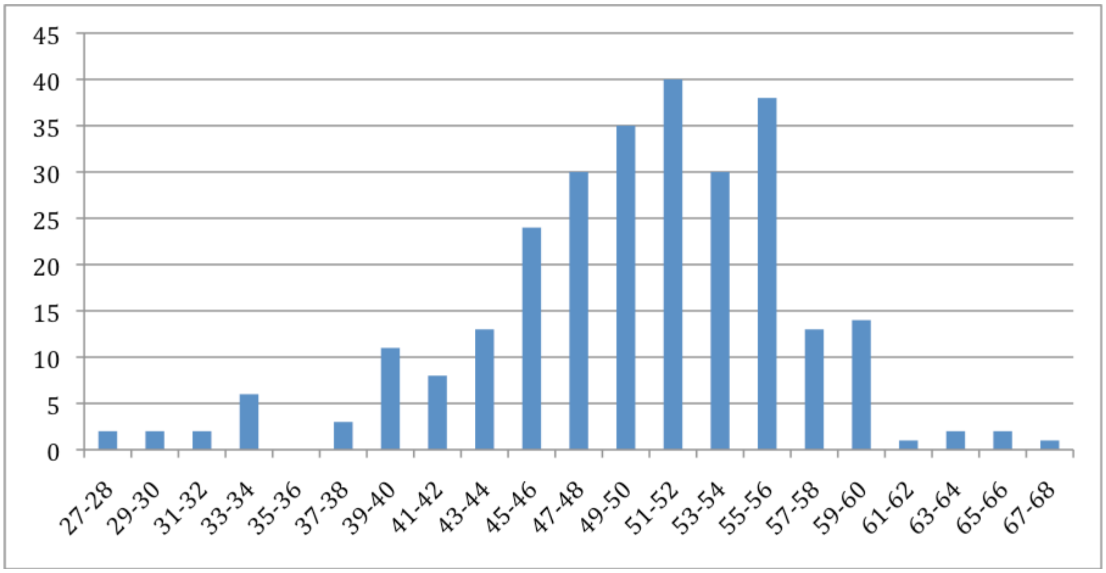

Figure 4-6. Distribution of SVL from Dixie Valley toads sampled in Dixie Meadows from 2009-2012.

Figure 4-7. Dixie Valley toads in amplexus underwater and egg masses in the perennial pond in Dixie Meadows (photo credit K. Urquhart).

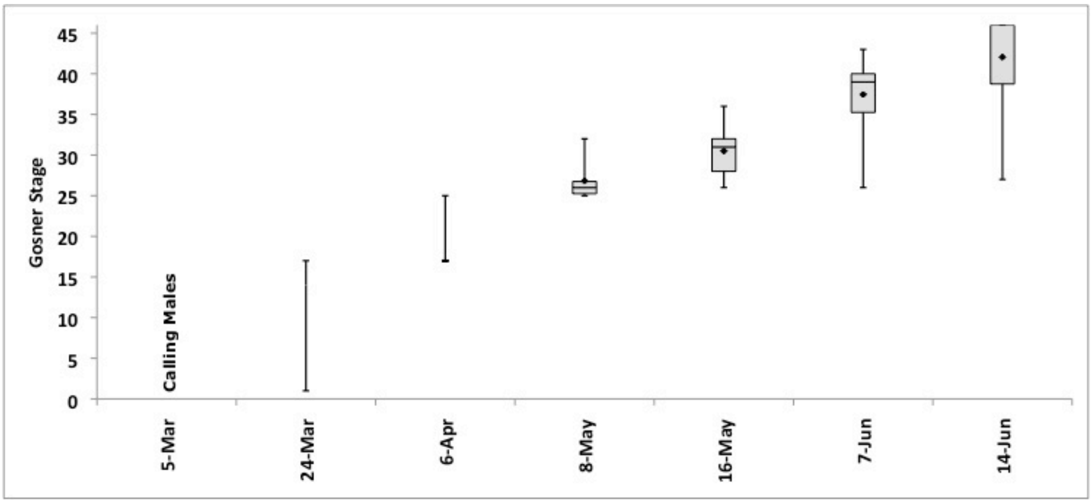

Figure 4-8. Developmental stages of Dixie Valley Toads recorded using the Gosner method (Gosner, 1960) during 2011. Calling males were first detected on March 5th 2011, ovodeposition was first observed on April 5th 2011 and the first wave of completely metamorphosed toadlets was observed 10 weeks later on June 14th 2011.

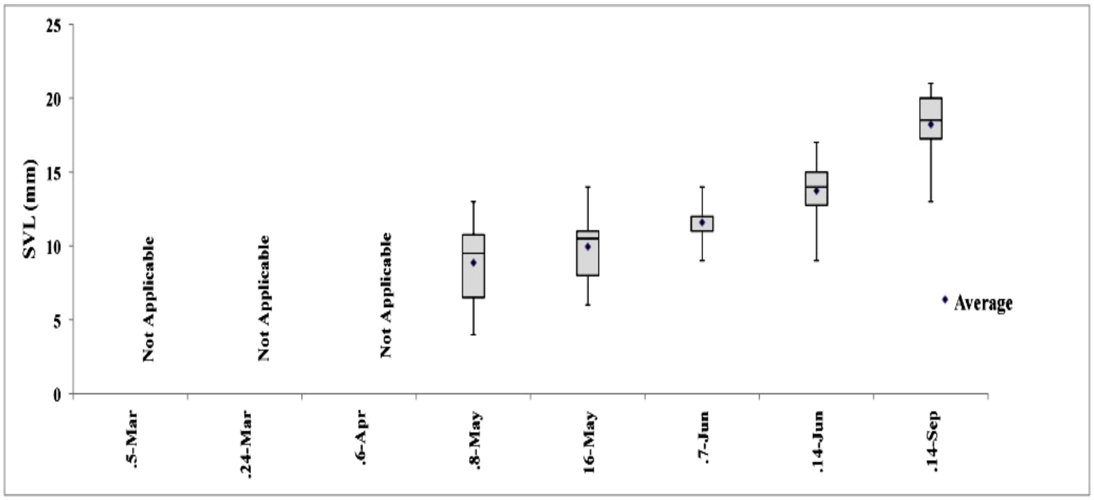

Figure 4-9. Snout-Vent Lengths (SVL) of Dixie Valley Toads recorded during investigations of developmental stages during 2011.

Table 4-1. Population survey data from 2009-2012 for western toads *Anaxyrus* (=*Bufo*) *boreas* at McCoy Ranch.

McCoy Ranch

Year	**PIT tagged**	**Recaptures**	**Total# Encountered**
2009	10	0	18
2010	2	0	2
2011	9	0	10
2012	0	0	13

Table 4-2. Population survey data from 2009-2012 for Dixie Valley Toads in Dixie Meadows.

Dixie Meadows

Year	**PIT tagged**	**Recaptures**	**Total# Encountered**
2009	86	0	122
2010	46	2	95
2011	50	2	71
2012	0	0	55

Table 4-3. Results from sampling for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) during 2011 and 2012.

Date	**Site**	**Species**	**n**	**Bd prevalence**
6-Apr-11	Dixie Meadows North	Dixie Valley toad	39	0.00
5-Apr-11	Turley Pond	*Lithobates catesbeianus*	11	0.18
10-Apr-12	Turley Pond	*Lithobates catesbeianus*	32	0.75
11-Apr-12	McCoy Ranch	*Anaxyrus boreas*	13	0.00
11-Apr-12	Dixie Meadows North	Dixie Valley toad	17	0.00
20-Apr-12	Dixie Meadows North	Dixie Valley toad	36	0.00

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Chapter 5: Biogeographic patterns of desert springs in the Great Basin with an emphasis on regional aquifer thermal springs as refugia for vulnerable species

Biogeographic patterns of desert springs in the Great Basin with an emphasis on regional aquifer thermal springs as refugia for vulnerable species

Abstract

The desert springs of the Great Basin in western North America provide ideal systems to study biogeographic and evolutionary patterns. In arid regions, springs are biodiversity hotspots because they often provide the sole source of water for the biota within and around them. These aquatic environments represent island ecosystems surrounded by seas of desert, and researchers have compiled large databases of their biota and chemistry. Consequently, desert springs are ideal systems for biogeographic studies and multivariate statistical analyses of relationships between the chemical and physical characteristics of the springs and the biological communities that they support. Within the Great Basin, springs provide critical habitat for diverse and extensive aquatic fauna comprising of over 125 endemic species. Communities with the highest diversity of endemic species, including fishes, mollusks and aquatic insects, are concentrated in regional carbonate aquifer thermal springs. The purpose of this study is to elucidate the relationships between the physicochemical characteristics of springs and the biota that they support using multivariate statistical analyses to characterize the differences between 947 cold (< 20 °C) local aquifer springs, 313 geothermal (>20 °C) local aquifer springs, and 66 regional aquifer thermal springs.

The analyses showed that regional aquifer thermal springs harbor a disproportionate number of vulnerable endemic species. However, regional aquifer thermal springs also contain a higher number of introduced species than cold and geothermal local aquifer springs.

Introduction

Spring environments in desert regions are rare, fragile, and endangered ecosystems that often support vulnerable endemic species (Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007). The desert springs of the Great Basin in western North America represent ideal systems to study biogeographic and evolutionary patterns because they are numerous, have abundant endemic species (species whose entire distributions are limited to one area) with histories that may reflect differential colonization, extinction and local adaptations, may preserve relict species by virtue of their isolation, and particularly in the case of regional aquifer thermal springs, because they have unusual physicochemical properties that may exclude competitors and pathogens. Springs are also among the most threatened ecosystems in the world (Hubbs et al., 1974; Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007; Deacon et al., 2007; Stevens and Meretsky 2008; Springer and Stevens, 2009). Primary anthropogenic impacts include groundwater depletion and pollution, alteration of source area geomorphology, flow diversions, and the introduction of exotic species (Hubbs et al., 1974; Sada and Vinyard, 2002;

Deacon et al., 2007; Springer and Stevens, 2009). This is important because springs have significant conservation value due to their roles as refugia for rare and vulnerable species.

The Great Basin is the most arid region in North America, containing only a few, widely dispersed permanent wetland habitats--yet there are hundreds of aquatic species that are endemic to the region (Sada and Vinyard, 2002). The springs of the Great Basin have also been relatively well studied, and researchers have compiled large databases of their biota and chemistry. Consequently, these systems lend themselves to biogeographic studies and multivariate statistical analyses of relationships between the chemical and physical characteristics of the springs and the biological communities present within them. Here I examine the physicochemical and biological characteristics of desert springs in the Great Basin specifically to investigate the correlates of spring physicochemical characteristics to their retention of endemic taxa. My study is motivated by a desire to elucidate the factors involved in thermal spring systems roles as refugia for vulnerable species, as discussed for amphibians in previous chapters (Chapters 2,3,4; Forrest and Schlaepfer, 2011).

Biogeography has a long and distinguished history that is inextricably linked with the development of evolutionary biology and ecology, as many of the foremost scientists of their eras--from Charles Darwin and Alfred Russel Wallace to Ernest Mayr, Robert MacArthur, and Edward O. Wilson--were biogeographers (Lomiolino et al., 2006). In the 1960’s, MacArthur and Wilson (1967) developed the Equilibrium

Theory of Island Biogeography, a model that attributed species richness on oceanic islands to an equilibrium between rates of recurrent extinctions and colonizations. Brown (1971) found that the mammalian faunas of the isolated mountains in the Great Basin do not represent equilibria between the rates of extinction and colonization because boreal mammals colonized these “islands” during the Pleistocene, and subsequently there have not been further colonizations--only extinctions. Therefore, Brown proposed that these extant populations are relicts, vicariant remnants of formerly widespread distributions during the Pleistocene, and he proposed a new model that he called “Nonequilibrium Insular Biogeography” (Brown, 1971). In arid regions, spring environments also represent ‘island’ ecosystems surrounded by ‘seas’ of deserts--often providing the sole source of water for the biota within and around them (Soltz and Naiman, 1978).

There are two broad categories of springs and aquifers commonly found within the Great Basin that will be examined and compared in this study: local aquifer springs and regional aquifer springs. Local aquifer springs are fed by modern precipitation from a larger nearby area (e.g., a local mountain range), and the springs that they supply are generally located between valley floors and the base of mountains (Sada and Pohlmann, 2006). Local aquifer springs are usually cool (> 10 °C and < 20°C), however geothermal springs (> 20°C) are also often supported by local aquifers. Geothermal springs occur where meteoric water (water recently derived from precipitation) circulates near magma or other residual heat sources and then ascends along fault or fracture zones to near surface depths (Waring, 1965). Water-rock interactions at depth can affect geochemistry, significantly increasing the concentrations and numbers of certain chemicals (i.e. Cl, Li, B, As, F) present in geothermal springs (Forrest et al., 2013; See Chapter 2). Discharge from springs fed by local aquifers may change seasonally and annually in response to precipitation, but typically these springs only go dry during extended droughts (Sada, 2006).

The carbonate-rock province of the Great Basin is an area where thick sequences of carbonate rocks of Paleozoic age predominate in bedrock outcrops (Mifflin, 1968). Extensional faulting within the Great Basin has created numerous topographic basins, many of which have closed surface-water drainage (Hershey, 2010). However, groundwater flows between the basins in locations where there is carbonate rock, and these interbasin flow systems delineate and define the regional carbonate aquifer of the Great Basin (Hershey, 2010; Heilweil and Brooks, 2011). The Great Basin regional carbonate and alluvial aquifer system covers an area of approximately 180,000 km^2 (Heilweil and Brooks, 2011), predominantly in eastern Nevada and western Utah. Water flow through these aquifers is complex, controlled by faults and fractures, and may extend beneath valleys and topographic divides (Thomas et al. 1996; Schaefer et al., 2005; Heilweil and Brooks, 2011). Therefore, the movement of water through these aquifers is slow compared to local aquifers, and discharge from regional springs is relatively constant over long periods of time—often thousands to millions of years (Hershler et al., 2007). Springs fed by regional aquifers are generally warm (>20°C), and are supplied from recharge extending over vast areas (Heilweil and Brooks, 2011). Water in regional aquifer springs may also contain elevated concentrations of chemicals and high levels of total dissolved solids (TDS) because the long residence times and elevated temperatures enhance the dissolution of rocks and minerals (Schaefer et al., 2005).

In the Pliocene to middle Pleistocene, climatic conditions were wetter and pluvial lakes in the western Great Basin repeatedly rose to very high levels, connecting basins that are presently isolated (USGS and Reheis, 1999; Lyle et al., 2012). During the last glacial maximum in the Pleistocene, the aquatic habitats in the Great Basin and Mojave deserts expanded, and as the glaciers retreated with the warming climate the extent of aquatic habitats shrank and became fragmented (Reheis et al., 2002) Today, the Great Basin hosts only the remnants of Lakes Bonneville and Lahontan—two large pluvial lakes that formed during wetter climatic conditions. Lake Bonneville formed approximately 32,000 years before present (yr B.P.) and lasted until about 16,800 yr B.P., once covering nearly all of Utah as well as portions of Idaho and Nevada (Benson et al., 1990; Reheis et al., 2002). Lake Lahontan was another pluvial lake that covered nearly all of northwestern Nevada, as well as parts of northeastern California and southern Oregon (approximately 22,000 km^2) during its last highstand ~ 12,500 yr B.P (Benson and Thompson, 1987). However, as the climate changed and less precipitation fell into these lake basins, they continued to desiccate and shrink (Reheis et al., 2002). The Great Salt Lake and the Bonneville Salt Flats are the largest extant remnants of Lake Bonneville (Benson et al., 1990), and the only remaining lakes in the Lahontan Basin are Pyramid and Walker Lakes in Nevada--the rest of Lake Lahontan's remnants consist of dry playas, rock formations, and in some locations paleo-tufa deposits delineating where the ancient shorelines once stood. These paleohydrological expansions and contractions may have allowed certain aquatic species to enter now-isolated basins in the distant past, where they subsequently became stranded in spring ecosystems as climatic conditions became progressively drier (Hubbs and Miller, 1948; Hubbs et al., 1974; Hovingh, 1997).

Springs have relatively constant chemical compositions, water velocities, and temperatures when compared with lakes, rivers, marine environments, and terrestrial communities (Odum, 1971). The waters emanating from springs may reflect hydrological and biogeochemical processes derived from a much larger catchment area, particularly within regional carbonate and alluvial aquifer systems in the Great Basin (Schaefer et al., 2005; Heilweil and Brooks, 2011). Organisms living in springs are strongly influenced by the quality and quantity of the water, and are adapted to relative environmental constancy (Odum 1971; Strohbach et al., 2009). Within a spring system, environmental and geochemical variation is typically much lower near the source than downstream--where variability in temperature, discharge, dissolved oxygen concentration, and other factors tends to be much greater (Sada, 2006). As a result, the compositions of communities residing near spring sources are often quite different than those found downstream, and many species that occupy the source area are absent from downstream habitats (Hershler 1998; O'Brien and Blinn 1999). Crenobiota (obligate spring-dwelling species) are unable to live outside of an aquatic environment for long periods; they are generally restricted to springs with good water quality, and they never inhabit springs that periodically dry (Sada and Pohlmann, 2006). Extant populations of crenobiotic species are restricted to springs that have persisted for long periods of geological time (Taylor 1985, Polhemus and Polhemus 2002; Hershler et al., 2007). Therefore, crenobiotic species within regional aquifer springs in the Great Basin may represent relict populations that have persisted in isolated habitats for millennia (Sada and Pohlmann, 2006).

The importance of springs as essential habitat for flora and fauna has become progressively better understood and appreciated (Hubbs et al., 1974; Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007), and springs are now recognized as biodiversity hotspots that support disproportionate numbers of the aquatic and riparian species that inhabit arid regions (Sada and Pohlmann, 1996). Springs within the Great Basin provide critical habitat for diverse and extensive aquatic fauna comprising of over 125 endemic species, including at least three amphibians, 28 fish, eight insects, one fairy shrimp, 85 mollusks, and two amphipods, as well as 45 subspecies--one aquatic insect and 44 fish (Hubbs and Miller, 1948; Hubbs et al., 1974; Minckley et al., 1986; Hershler and Sada, 1987; Sada and Vinyard, 2002; Whiteman and Sites, 2008). Communities with the highest diversity of endemic species, including fishes, mollusks and aquatic insects, are concentrated in regional aquifer thermal springs in Ash Meadows and Pahranagat Valley in Southern Nevada (Sada and Vinyard, 2002). The Ash Meadows National Wildlife Refuge alone contains at least 25 endemic animals and plants, which is the highest known endemicity of any local area in the continental United States (Beatley, 1977; Hershler and Sada, 1987; U.S. Fish and Wildlife Service, 1990). Ash Meadows represents a watered island amidst the expansive Mojave Desert, and the persistence of this water since at least the late Pliocene/early Pleistocene has preserved relict plants and animals that gained access to the region during pluvial climates (U.S. Fish and Wildlife Service, 1990). The subsequent isolation of these species within this harsh environment permitted their differentiation from related taxa and resulted in the distinctive character of many extant flora and fauna (Miller, 1946; U.S. Fish and Wildlife Service, 1990).

The two groups of animals that have been the focus of the vast majority of previous biogeographic studies on endemic species in the Great Basin are springsnails and desert fish—particularly pupfish (*Cyprinodon* spp.). Hubbs and Miller (1948) observed that pupfish in the Owens, Amargosa, Mojave, and Colorado River were morphologically similar, and theorized that the hydrologic isolation and initiation of pupfish diversification began at the end of the Pleistocene. The pioneering work concerning desert fishes in the Great Basin, and particularly on pupfishes within Death Valley System suggested that these fishes evolved over the last 2,000 to 20,000 years as ancestral pupfishes were isolated and became adapted to small, and often harsh, habitats (Miller, 1946; Hubbs et al., 1974; Soltz and Naiman, 1978). The isolated aquatic "islands" in the sea of desert in Death Valley inspired many studies of evolution, and even evoked comparisons to the observations made by Darwin on the Galapagos Islands (Soltz and Naiman, 1978). More recent mitochondrial DNA (mtDNA) work on morphologically similar pupfish in the Death Valley region show a common ancestry with divergence beginning 3-2Ma (Smith et al., 2002; Echelle et al, 2005; Echelle, 2008). Additionally, the geologic record does not support a connection of the Owens, Amargosa, or Mojave Rivers to the Colorado River in the last 3-2Ma, however an older flowing system may have permitted ancestral pupfish dispersal (Knott et al., 2008). Therefore, speciation rates were likely not nearly as rapid as previously believed. However, one aspect of the pattern that Hubbs and Miller observed in 1948 remains unchallenged: ancestral pupfish were isolated at some point, and now some species only survive in small, harsh habitats--usually thermal springs.

Springsnails (*Tyronia* spp. and *Pyrgulopsis* spp.) are tiny, gill-breathing species of gastropods that have been popular subjects of biogeographic studies due to their antiquity and close linkage with aquatic systems (Taylor, 1985; Hershler and Sada, 1987; Hershler, 1998; Hershler et al., 2007; Hershler and Liu, 2008). All springsnails are crenobiotic species, and consequently are thought to be poor dispersers capable of spread only within their local habitats (Taylor, 1985; however also see Hershler and Liu, 2008). There are over 80 species of springsnails of the genus *Pyrgulopsis* in the Great Basin, many of which are endemic to single springs, spring systems or drainage basins (Hershler and Sada, 1987). In contrast with pupfish and springsnails of the genus *Tryonia*, which live exclusively in regional aquifer thermal springs (Miller, 1946; Hershler and Sada, 1987; Hershler, 1998), *Pyrgulopsis* inhabit diverse spring-fed waters within the Great Basin, including cold and geothermal local aquifer springs (Hershler and Sada, 2002; Hershler et al., 2007; Hershler and Liu, 2008). Molecular phylogenetic studies of Tyronid gastropods have documented deeply divergent Death Valley system lineages consistent with ancient origins (Hershler et al., 1999), and estimates from molecular clocks based on cytochrome c oxidase subunit I (COI) gene indicated that six lineages of *Pyrgulopsis* springsnails endemic to the Death Valley system were at least Pliocene in age (Hershler and Liu, 2008). Therefore, the Death Valley system springsnail fauna roughly parallels regional pupfishes and Tryonia gastropods in containing endemic lineages that diverged concurrent with or prior to the formation of the modern regional landscape (Hershler and Liu, 2008). Molecular clocks also suggest that the springsnail fauna from four thermal springs in Soldier Meadow may be a product of a lengthy history of evolution (Hershler et al., 2007). However, three other *Pyrgulopsis* lineages occurring in the Death Valley region showed divergence events younger than 0.7 Ma, suggesting that regional springsnail biogeography has also been shaped in part by geologically recent (Pleistocene) dispersal, and in some cases may have occurred by means other than paleohydrological connectivity (Hershler and Liu, 2008).

Thermal springs are generally more persistent and long-lived than non-thermal springs—particularly regional aquifer thermal springs, where water may flow continuously for thousands to several million years (Hershler et al., 2007; Coolbaugh et al., 2005). Thermal springs may serve as refugia for some species, ‘armoring them’ from extinction because they do not freeze or dry up seasonally (Brock, 1967; Brown and Feldmeth, 1971), can offer consistently ideal temperatures for growth and reproduction, and may also exclude predators and competitors who cannot tolerate their high temperatures and unusual water chemistry (Brock, 1967). In fact, studies in Arizona have demonstrated that thermal springs can provide amphibians with refugia from the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which causes the potentially fatal disease chytridiomycosis (Schlaepfer et al., 2007; Forrest and Schlaepfer 2011; see Chapters 2, 3, and 4). Additionally, the relict leopard frog *Lithobates* (*Rana*) *onca* was once thought to be extinct, but several populations were rediscovered in the 1990's and all naturally occurring *L. onca* populations are now associated with regional aquifer thermal springs in Nevada (Jaeger et al., 2001; Bradford et al., 2004).

It is unclear whether high levels of endemicity observed in regional aquifer thermal springs are due solely to their roles as refugia for species that formerly had wider ranges. Some authors have also suggested that higher rates of speciation may occur within thermal springs. Miller (1950) found that high temperatures accelerated the rate of evolution in warm-spring populations of pupfish by increasing the mutation rate and the number of generations produced each year. Witt et al (2008) posited that the higher temperatures found in thermal springs within the Great Basin may contribute to elevated rates of molecular evolution in the amphipod *Hyalella azteca* species complex due to higher metabolic rates resulting in oxidative damage and faster generation times. However, it is likely that the high levels of endemicity and biogeographic patterns found in modern desert springs were shaped primarily by the paleohydrological regimes that influenced the region in the past.

Several studies have shown that regional aquifer thermal springs contain the majority of the endemic species that are found in springs throughout the Great Basin (Sada and Vinyard, 2002; Whiteman and Sites, 2008). However, I am unaware of any published studies that have attempted to elucidate the large-scale relationships between the physical and chemical attributes of springs in the Great Basin and the biota that they support. In this chapter, I will use a dataset graciously provided by Dr. Don Sada from the Desert Research Institute in Reno, Nevada. Dr. Sada and some of his colleagues have been collecting physicochemical and biological data from springs throughout the Great Basin since the late 1980's. The dataset that I am using for this study contains information from approximately 1400 springs located in Nevada, Utah, Oregon and California. Using multivariate statistical analyses, I will compare the physicochemical and biological patterns found in cold (< 20 °C) local aquifer springs, geothermal (>20 °C) local aquifer springs, and regional aquifer springs within the Great Basin.

Materials and Methods

Description of Data

Data were collected between 1991 and 2008 by D.W Sada and his colleagues (W. Pratt, R. Hershler, G. Vinyard, C. Rosamond, and A.Schwaneflugel). All information was compiled at the spring source, and included the upper 50 m of aquatic habitat (at larger springs). The data fall into five categories, which are: 1) recording survey date and spring location, 2) water chemistry parameters, 3) physical characteristics of the aquatic and riparian environment (e.g., spring brook length, discharge, water depth, vegetative cover, and substrate composition, etc.), 4) natural and anthropogenic factors that may be stressing the aquatic and riparian systems, and 5) the presence or absence of important animals and plants.

The physicochemical data that were used in the multivariate analyses include: spring aquifer type (local cold spring, local geothermal spring, regional aquifer spring) spring depth, width, temperature, conductivity, pH, amount of emergent cover, bank cover, silt, sand, gravel, cobble, and estimates of disturbances from diversion, equine, cattle, and recreation. Biological parameters used in analyses include: total number of species recorded, number of endemic species, number of vulnerable species, number of introduced species, number of springsnail species, number of amphibian species, and number of fish species. Endemic and introduced species were determined from the literature (Hershler 1998; Sada and Vinyard, 2002; Hershler et al., 2007). Species defined as "vulnerable" possessed one or more of the following traits: (1) occurs in only a few limited areas, (2) is particularly vulnerable to prevailing landscape changes, (3) has a small population size, (4) has large area requirements, or (5) there is a great deal of uncertainty about its distribution and abundance. Data were analyzed two different ways: 1) using only the physicochemical data, 2) using both the physicochemical and biological data.

Multivariate statistical analyses

Springs were divided into cold (< 20 °C) local aquifer springs, geothermal (>20 °C) local aquifer springs, and regional aquifer springs for comparisons using multivariate analyses. PRIMER (v. 6.0) software (Clarke and Gorley, 2006) was used for multivariate statistical analyses of the measured constituents. Prior to analyses, data were 4th-root transformed and normalized to eliminate inappropriate weighting factors. In the multivariate analyses of environmental data, normalization is usually essential because environmental data have different measurement scales or units (Clarke and Gorley, 2006). After normalization, the data have dimensionless, comparable scales, and can be compared by examining and interpreting relationships based on Euclidean distances. These transformed and normalized data were then used to generate a resemblance matrix based on Euclidean distances between site pairs for subsequent multivariate statistical analyses. I used multidimensional scaling (MDS), Principal components analysis (PCA), and Similarity Percentage Analyses (SIMPER) to characterize the differences between cold (< 20 °C) local aquifer springs, geothermal (>20 °C) local aquifer springs, and regional aquifer springs.

The purpose of multidimensional scaling (MDS) is to construct a visual representation of a complex set of relationships using a “map” or ordination of the samples in a specified number of dimensions, which attempts to satisfy all of the conditions imposed by the rank similarity matrix (Clarke and Gorley, 2006). On a MDS map, proximity indicates similarity. MDS stress reflects the robustness of the ordination. The MDS map is displayed as a two-dimensional object, and represents an optimal configuration of the sample points in two-dimensional space (Clarke and Gorley, 2006).

Principal components analysis (PCA) is a recognition technique used to explain the variance of a large set of intercorrelated variables with a smaller set of independent variables, or principal components. PCA is the longest-established method of approximating high-dimensional information in low-dimensional plots, and is particularly useful for multivariate analyses of environmental data (Clarke and Gorley, 2006). PCA is an ordination in which samples are regarded as points in high-dimensional variable space, and are projected onto a best fitting plane, or other low dimensional solution. The principal components (PC) are simply a rotation of the original axis, and thus a linear combination of the input variables. The purpose of the principal components is to capture as much of the variability in the original space as possible and the extent to which the first few PC's allow an accurate representation of the true relationship between the samples in the original dataset is summarized by the % variation explained.

Similarity Percentage Analyses (SIMPER) are useful in interpreting differences between groups of samples when they have been shown to exist. SIMPER looks at the roles of individual environmental variables in contributing to the separation between two groups of samples or the "closeness" of samples within a group. This is implemented by a similarity percentages routine that decomposes average Euclidean distances between all pairs of samples, one from each group (or decomposes the similarities among samples within a group) into % contributions from each environmental variable. SIMPER operates on the dissimilarities in their high-dimensional relationships, and not on the approximations represented by a 2-D ordination (MDS) (Clarke and Gorley, 2006).

Results

A total of 1328 springs were analyzed in this study: 66 regional aquifer thermal springs, 313 geothermal (>20 °C) local aquifer springs, and 947 cold (< 20 °C) local aquifer springs. Average values for all factors analyzed in the multivariate analyses broken down by spring types are presented in Tables 1-4.

The MDS plots (Figs. 3, 4) show interesting grouping patterns. When the physicochemical and biological data from springs (Fig. 3) were analyzed together, regional aquifer thermal springs (shown as light blue squares) generally form a group distinct from the cold (< 20 °C) local aquifer springs (dark blue upside-down triangles) and geothermal (>20 °C) local aquifer springs (red triangles). However, there is a great deal of overlap between the cold and geothermal local aquifer springs. Moreover, some of the geothermal springs appear to group with the regional aquifer thermal springs. This may be due to the similarities in the physicochemical data, as well as the fact that some endemic species are also found in geothermal springs (i.e. springsnails in Soldier Meadows (Hershler et al., 2007).

MDS analysis of the physicochemical data from springs alone (without biological data included) shows very different patterns from those seen when the physicochemical and biological data were analyzed together (Fig. 4). Groupings are far less distinct than in the combined physicochemical and biological analysis (Fig. 3) Although each spring type did form loose groups, the cold (< 20 °C) local aquifer springs (dark blue upside-down triangles) and geothermal (>20 °C) local aquifer springs (red triangles) were more scattered, and often grouped with the regional aquifer thermal springs (light blue squares). This indicates that the physicochemical data alone were insufficient to accurately distinguish spring types, as some cold and geothermal local aquifer springs shared certain physicochemical characteristics with the regional aquifer springs.

The PCA plots (Figs. 5, 6) show very similar patterns to the MDS plots (Figs. 3,4). When the physicochemical and biological data from springs (Fig. 5) were analyzed together, regional aquifer thermal springs (shown as light blue squares) generally formed a group distinct from the cold (< 20 °C) local aquifer springs (dark blue upside-down triangles) and geothermal (>20 °C) local aquifer springs (red triangles), but several of the regional aquifer springs grouped with the local aquifer springs. Again, there was a great deal of overlap between the cold and geothermal local aquifer springs. Moreover, some of the geothermal springs appeared to group with the regional aquifer thermal springs. As with the MDS analyses, this may be due to the similarities in the physicochemical data, as well as the fact that some endemic and vulnerable species are also present in geothermal springs (i.e. springsnails in Soldier Meadows).

As with the MDS analyses, PCA analysis of the physicochemical data from springs alone showed very different patterns (Fig. 6). Groupings were far less distinct than in the combined physicochemical and biological analysis (Fig. 5), although each spring type did form groups, the cold (< 20 °C) local aquifer springs (dark blue upside-down triangles) and geothermal (>20 °C) local aquifer springs (red triangles) were more scattered, and often grouped with the regional aquifer thermal springs (light blue squares). This suggests that the physicochemical data alone were insufficient to accurately distinguish spring types, because some cold and geothermal local aquifer springs shared certain physicochemical characteristics with the regional aquifer springs.

A close examination of the eigenvalues of the individual PC's from the combined physicochemical and biological data (Table 5) revealed that the first 10 PC's described 72.1% of the variance. The eigenvectors for each PC are presented in Table 6. The strongest drivers of PC1, which described 16.7% of the variance, were primarily biological factors: #_Vulnerable (total number of vulnerable species recorded), #_Endemics (total number of endemic species recorded), #_Fish (total number of fish species recorded), Total_spp (total number of species recorded), and #_Springsnails (total number of springsnail species recorded). The strongest drivers of PC2, which described 11.4% of the variance, were primarily physical factors related to substrate types: Gravel (% gravel substrate present, qualitatively estimated to the nearest 5%), Silt (% silt substrate present), and Cobble (% cobble substrate present). The strongest drivers of PC3, which described 8.8% of the variance, were physicochemical and biological factors: Bank_Cover (qualitatively estimated (to the nearest 10 %) as the percent that live vegetation that covers spring brook banks within the riparian zone), Conduct (measured conductivity, a proxy for TDS), Temp (measured water temperature at spring source), #_Springsnails, and Total_spp. The strongest drivers for PC4, which described 6.7% of the variance, were: Depth and Width of the springs, followed by EQUINE (qualitatively estimated levels of disturbance from equine sources--primarily feral horses and burros). The strongest drivers for PC5, which described 5.5% of the variance, were #_Amphib (total number of amphibian species recorded), CATTLE (qualitatively estimated levels of disturbance from cattle), and Bank_Cover (Table 6).

When the Individual PC's were examined from the physicochemical data alone (Table 7), the first 10 PC's described 82.8% of the variance. The eigenvectors for each PC are presented in Table 8. The strongest drivers of PC1, which described 16.5% of the variance, were factors related to substrate types: Silt, Cobble, and Sand (% sand substrate present). The strongest drivers of PC2, which described 12.1% of the variance, were: Temp, Conduct, Depth, and Emerg_Cover (qualitatively estimated (to the nearest 10 %) as the percent that riparian and instream vegetation, debris, or other material arising within the wetted perimeter and shades the substrate of the spring and its spring brook). The strongest drivers of PC3, which described 9.9% of the variance, were: Bank_Cover, EQUINE, CATTLE, Width and Diversion (qualitative estimate of the amount of disturbance from spring water diversions). The strongest drivers of PC4, which described 8.6% of the variance, were Width, CATTLE, Temp, pH, Diversion, and Depth. The strongest drivers of PC5 were estimates of disturbances, which described 7.0% of the variance were RECREAT (qualitative estimate of the amount of disturbance from recreational use), EQUINE, and DIVERSION (Table 8).

SIMPER analyses comparing spring types revealed which individual variables best explain the differences between the spring types. When both the physicochemical and biological data were considered, SIMPER analyses revealed that the variables that explain most of the differences between cold local aquifer springs and hot (geothermal) local aquifer springs in order of their % contributions were: Temp, Conduct, pH, Depth, #_Amphib, Bank_Cover, and Width (Table 9). The variables that explain most of the differences between hot (geothermal) local aquifer springs and regional aquifer thermal springs in order of their % contributions were: #_Introduced (total number of introduced species recorded), #_Vulnerable, #_Fish and #_Endemics (Table 10). The variables that explain most of the differences between cold local aquifer springs and regional aquifer thermal springs in order of their % contributions were: #_Introduced, #_Fish, #_Vulnerable, #_Endemics, and Temp (Table 11).

When only the physicochemical data were considered, SIMPER analyses revealed that the variables that explain most of the differences between cold local aquifer springs and hot (geothermal) local aquifer springs in order of their % contributions were: Temp, Conduct, pH, Depth, Bank_Cover, and Width (Table 12). The variables that explain most of the differences between hot (geothermal) local aquifer springs and regional aquifer thermal springs in order of their % contributions were: Depth, RECREAT, Emerg_Cov, and Bank_Cover. (Table 13). The variables that explain most of the differences between cold local aquifer springs and regional aquifer thermal springs in order of their % contributions were: Temp, Conduct, Depth, and CATTLE (Table 14).

Discussion

General Patterns from analyses:

As hypothesized, I found that regional aquifer thermal springs do in fact harbor a disproportionate number of endemic and vulnerable species. The MDS (Fig. 1) and PCA (Fig. 2) analyses of both the physicochemical and biological data showed fairly robust groupings of the different spring types, although some of the local aquifer geothermal springs appeared to group with the regional aquifer thermal springs, perhaps due to the fact the some geothermal systems such as those found in Dixie Valley and Soldier's Meadow also harbor endemic species (Hershler, 1998; Hershler et al., 2007). The SIMPER analyses (Tables 9-14) provided more tangible information regarding the main drivers of the differences between spring types, and revealed that the main differences between regional aquifer thermal springs and local aquifer cold and geothermal springs were primarily related to biological factors, with higher numbers of introduced species, vulnerable species, endemic species and fish species present in the regional aquifer thermal springs. When only the physicochemical data were considered, the differences between the spring types were generally fairly predictable. Geothermal local aquifer springs differed from cold local aquifer springs due to higher water temperatures, conductivity, and pH. However, greater average values for water depth and width, and lower average values for bank cover also played significant roles (Table 12). When comparing physicochemical differences between geothermal local aquifer springs and regional aquifer thermal springs, higher values for water depth and recreational disturbances and lower values for emergent cover in regional aquifer thermal springs appeared to account for much of the differences (Table 13). Finally, when comparing cold local aquifer springs and regional aquifer thermal springs, higher average values for water temperature, conductivity, depth and lower average values for cattle disturbance in regional aquifer thermal were the most significant drivers of the differences (Table 14).

These results were particularly interesting in that they revealed that despite obvious differences in physicochemical characteristics such as water temperatures and conductivity, the main parameters that describe most of the variation between spring systems were primarily biological variables such as the number of introduced taxa, endemic and vulnerable taxa, and disturbance by cattle and horses. Factors such as the establishment of bank vegetation (a possible indicator of relatively rare disturbance by major floods), and the amount of sand to boulders (another potential scale of physical disturbance level) also are important in differentiating between types of spring systems. Finally, we found that a large number of springs show some level of physical diversion and disturbances by humans. Notably, geothermal systems (also indicated by their conductivity and unusual chemistry) also are recognized as discrete systems in the analyses. Both regional aquifer thermal springs and geothermal tend to be more disturbed by recreational usage, as people throughout history have been known to enjoy soaking in hot springs. Cold local aquifer springs are more likely to be used to provide drinking water for cattle and horses. Because the Great Basin is so arid, any source of water is coveted, and virtually every spring in the region has a long history of anthropogenic exploitation and disturbance (Brues, 1928; Hubbs et al., 1974; Sada and Vinyard, 2002; Deacon et al., 2007).

The Importance of Introduced Taxa:

Perhaps the most interesting difference that these analyses revealed was that regional aquifer thermal springs not only support the majority of the endemic aquatic species, but also a disproportionate number of the introduced species as well. The reasons for these patterns may be similar—regional aquifers thermal springs may also serve as refugia for introduced species if these species can withstand or adapt to their unique physical and chemical compositions. The most common invasive species documented in the springs considered from this study were bullfrogs (*Lithobates catesbeianus*), western mosquito fish (*Gambusia affinis*), sailfin mollies (*Poecilla latipinna*) and other aquarium fish, redrim melania snail (*Melanoides tuberculata*), and red swamp crayfish (*Procambarus clarki*) (Hubbs et al., 1974; Hershler and Sada, 1987; Sada and Vinyard, 2002). Many of these species were likely introduced intentionally, and once these invasive species are able to establish themselves in the springs, it is very difficult to eradicate them without negatively impacting the local species. Therefore, in many springs with endemic species it may be preferable to pragmatically maintain and manage the systems by actively enhancing the available critical habitat for the endemics, but not necessarily to attempt to completely remove invasive species (e.g. Simberloff, 2002).

Invasions of springs by nonnative species are often facilitated and accelerated by anthropogenic changes in flow regimes and disturbance (Hubbs et al., 1974; Sada and Vinyard, 2002; Fleishman et al., 2006). Excessive groundwater pumping threatens the flows and biota of springs in Ash Meadows (Deacon et al., 2007), where most of the springs have been perturbed by mining, agriculture, and municipal development (Hershler and Sada, 1987; U.S. Fish and Wildlife Service, 1990). The introduction and persistence of a number of exotic species has caused further alteration of spring ecosystems (Hubbs et al., 1974; Hershler and Sada, 1987; Sada and Vinyard, 2002). These perturbations have resulted in population declines of virtually all of the endemic taxa within Ash Meadows (Hershler and Sada, 1987), and the primary obstacle to restoring Ash Meadows to its pre-anthropogenic undisturbed conditions is the presence of invasive species. It is also quite possible that endemic species were lost before ever being described (Scoppettone, 2013). Hubbs and other early researchers foresaw these threats and lamented the “peril that the fishes of the remnant waters have faced and continue to face” including habitat degradation, overuse of water and “water mining” --lowering the fossil ground water, as well as the introduction of exotic fishes and bullfrogs (Hubbs et al., 1974).

Impacts of disturbance regimes:

The statistical analysis of Great Basin springs shows that many have suffered from a variety of physical and biological disturbances ranging form washouts from flood events to disruption by cattle and horses and diversions by people. Native and introduced species can have distinct responses to disturbances and alterations of flow regimes. Environmental and geochemical conditions are typically much more stable near the spring source than downstream--where variability in temperature, discharge, dissolved oxygen concentration, vegetation, and other factors tends to be much greater (Sada and Pohlmann, 2006). As a result, the compositions of communities near spring sources are often quite different than those found downstream, and many species that occupy the source area are absent from downstream habitats (Hershler, 1998; O'Brien and Blinn, 1999; Scoppettone et al., 2013). Don Sada believes that the high degree of endemism occurring in regional aquifer thermal springs is due to their unique geochemical characteristics. He has observed that some endemics--particularly springsnails--appear to require the unique geochemistry present in "their" springs in order to survive, and that they will die if they are transplanted to other springs, or placed in aquaria containing water from other sources (D. Sada, pers. comm., 2009.

Role of Autochthonous Resources, Persistence, and Stability:

Smaller springs are generally autotrophic aquatic systems with little dependence on allochthonous (derived from outside sources) carbon sources (Minshall, 1978; Cushing and Wolf, 1984). In larger springs, energy may enter the system during periodic floods that flush carbon from the surrounding landscape. As a consequence, most spring environments are less variable than other aquatic habitats (e.g., streams, rivers, and lakes), resulting in lower variability in population size and assemblage structures (Minckley, 1963). Thermal springs are generally characterized by simple food webs that are highly reliant on autochthonous (derived from sources within the system) production (Naiman, 1976), but also may receive pulses of allochthonous carbon (Wilson and Blinn, 2007). Typically there are copious amounts of primary producers such as cyanobacteria and thermophilic diatoms in thermal springs, and these organisms may serve as the base of local food webs. In contrast, much of the energy available in non-thermal springs may derive from allochthonous sources, such as leaves, seeds, and other material derived from nearby terrestrial vegetation (Naiman, 1976). Because food webs in thermal springs are usually more reliant on autochthonous sources, they may provide more stable environments that are less likely to be influenced by stochastic events that disrupt the input of allochthonous sources.

Carbon and nitrogen stable isotopes may have great utility in determining the extent that autochthonous sources contribute to food webs in thermal springs. Rounick et al. (1982) showed that allochthonous terrestrial plant material and autochthonous algae were isotopically distinct in small New Zealand streams, thereby providing a basis for identifying food resources used by aquatic animals. Further work by Rounick and James (1984) showed that the $\delta^{13}C$ values of the invertebrates and their algal foods at thermal and cold springs were drastically different as a result of differences in their respective sources of inorganic carbon. In studies in the Jackrabbit Spring system in Ash Meadows gut analyses revealed that filamentous algae were the predominant food item consumed by pupfish inhabiting thermal waters, and the $\delta^{13}C$ signature of Ash Meadows pupfish also suggested an algae-dominated diet (Scoppettone et al., 2013). The upper reaches of the thermal springs were particularly favorable to pupfish because the warm waters promote year-round growth of filamentous algae (Kennedy and Hobbie, 2004; Kennedy et al., 2005). Although filamentous algae appear to be important food sources for pupfish (Naiman, 1975; Kennedy et al., 2005; Scoppettone et al., 2013), some authors have raised questions about its digestibility (Naiman, 1979). Small epiphytic and grazing organisms associated with filamentous algae may be rapidly digested, and therefore not readily detected in gut analysis; however, they may contribute significantly to pupfish diet. In fact, analyses of the trophic position of pupfish using $\delta^{15}N$ indicated that these primary consumers contributed substantially to their diet (Scoppettone et al., 2013).

Thermal springs may persist for hundreds of thousands of years (Brock, 1967; Hershler et al., 2007), and they represent constant environments that do not freeze or dry up (Brock, 1967; Brown and Feldmeth, 1971). According to carbon-14 analysis, the average age of water discharging from regional aquifer thermal springs ranges from 4,000 to 14,000 years old (Thomas et al., 1996; Gates and Kruer, 1981). Hershler et al. (2007) also found that molecular clocks based on cytochrome c oxidase subunit I (COI) gene suggest that the springsnail fauna from four thermal springs in Soldier Meadow may be a product of a lengthy history of evolution within the basin. This is also supported by evidence that the thermal spring habitats of these snails, which are associated with geologic faults that are potentially millions of years based on the amount of offset of spring deposits across faults having known slip rates (Coolbaugh et al., 2005). Thermal springs are also very stable environments because they do not freeze, and may also be less prone to washing out during storm and/or flood events due to the fact that they are typically located outside of areas that are subjected to heavy flooding such as canyon floors (D. Sada, pers. comm., 2009).

Conservation and management:

In addition to their role as refugia for vulnerable endemic taxa, springs are also among the most threatened ecosystems in the world (Hubbs et al., 1974; Shepard, 1993; Hubbs, 1995; Sada and Vinyard, 2002; Kodric-Brown, 2007; Deacon et al., 2007; Springer and Stevens, 2008; Stevens and Meretsky 2008). Primary anthropogenic impacts include groundwater depletion and pollution, alteration of source area geomorphology, flow diversions, and the introduction of exotic species (Hubbs et al., 1974; Sada and Vinyard, 2002; Deacon et al., 2007; Springer and Stevens, 2008). For example, Ash Meadows is one of the most important endangered aquatic ecosystems in western North America (Hershler and Sada, 1987). Excessive groundwater pumping threatens the flows and biota of springs in Ash Meadows (Hubbs et al., 1974; Deacon et al., 2007), and approximately 16% (4000 hectares) of the total area within the Ash Meadows National Wildlife Refuge has been perturbed by mining, agriculture, and municipal development--including most of the springs (Hershler and Sada, 1987; U.S. Fish and Wildlife Service, 1990). The introduction and persistence of exotic species has caused further alteration of spring ecosystems, with invading biota including bullfrogs (*Lithobates catesbeianus*), mosquito fish

(*Gambusia affinis*), sailfin mollies (*Poecilia latipinna*), redrim melania snail (*Melanoides tuberculata*), crayfish (*Procambarus clarki*), and salt cedar (*Tamarisk* sp.) (Hubbs et al., 1974; Hershler and Sada, 1987; Sada and Vinyard, 2002). These perturbations have resulted in population declines of virtually all of the endemic taxa within Ash Meadows (Hershler and Sada, 1987).

Effective management of invasive species such as American bullfrogs, mosquitofish, crayfish is an essential part of restoring spring systems, because under certain habitat conditions they can outcompete and all but replace native species (Scoppettone et al., 2013). For example, in the Jackrabbit Spring system in Ash Meadows, spring restorations allowing more rapid water flow sustained the minimum water temperatures required for native pupfish reproduction farther downstream, and also served to reduce marsh-like habitat favoring introduced sailfin molly, mosquitofish, and crayfish (Scoppettone et al, 2005). Minimum water temperature for Amargosa pupfish reproduction is ~25 ºC (Soltz and Naiman, 1978). Thermal springs generally cool as they flow downstream; water temperature in the spring discharge is constant, but water temperatures downstream can range well over 20 ºC (Scoppettone et al., 2005). Therefore, restoring and channelizing the upper portions of the springs increases flow rate--which in turn causes the water to carry its thermal load further downstream, thereby increasing essential habitat for pupfish, as well as reducing cooler marshy habitat that favors introduced species (Scoppettone et al., 2013).

Conclusions

The desert springs of the Great Basin in western North America provide ideal systems to study biogeographic and evolutionary patterns as well as relationships between the physicochemical characteristics of the springs and the biological communities that they support. Paleohydrological expansions and contractions may have allowed certain aquatic species to enter basins in the distant past, where they subsequently became stranded in spring ecosystems as climatic conditions became progressively drier. Within the Great Basin, regional aquifer thermal springs harbor a disproportionate number of vulnerable endemic species, which is likely due to a number of reasons related to persistence and stability. Thermal springs do not freeze nor dry up seasonally, may offer organisms consistently ideal temperatures for growth and reproduction, and may also exclude predators and competitors who cannot tolerate their high temperatures and unusual water chemistries. High temperatures in thermal springs may also afford amphibians protection from potentially deadly pathogens such as *Bd*, the amphibian chytrid fungus. Additionally, hydrothermal ecosystems may provide more stability for organisms because they tend to support more autochthonous production due to luxuriant growths of algal and microbial mats, and may be less reliant on inconsistent inputs of allochthonous food sources than cold springs. Therefore thermal springs may serve as refugia for some species, 'armoring them' from extinction.

Unfortunately, springs in the Great Basin and around the world are also threatened by anthropogenic impacts including groundwater depletion and pollution, alteration of flow regimes, and the introduction of exotic species. In fact, one somewhat surprising result of this study was that one of the major factors that distinguished regional aquifer thermal springs from cold and geothermal local aquifer springs was the higher number of introduced species found in regional aquifer springs. This may be due to the influence of the same physicochemical characteristics that allow regional aquifer springs to serve as refugia for endemic species. Species that are able to gain access to these environments and cope with their extreme physicochemical characteristics may be provided with refuge from extinction. The disproportionate number of endemic species found in regional aquifer thermal springs, as well as local aquifer geothermal springs within the Great Basin strongly suggests that these hydrothermal habitats represent important biodiversity hotspots, and efforts to conserve and restore these unique ecosystems should be a major priority to ensure that the valuable information that these relict species can provide is not lost forever.

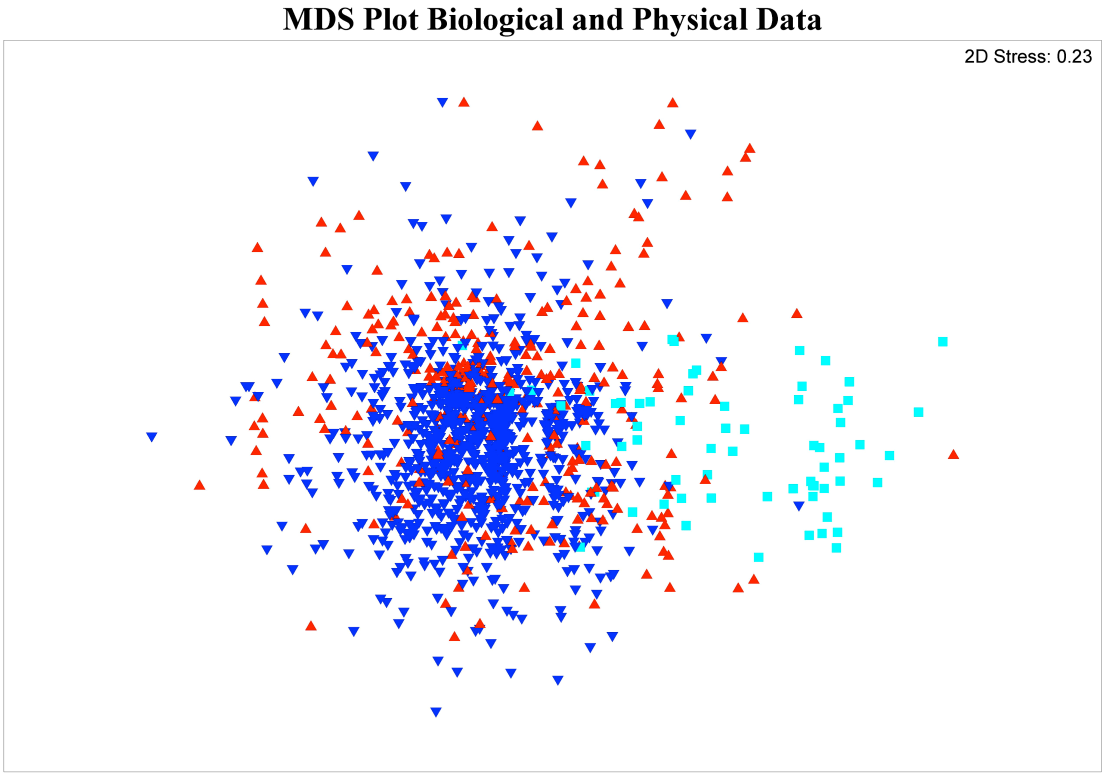

Figure 5-1: Multidimensional Scaling (MDS) plot of biological and physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles).

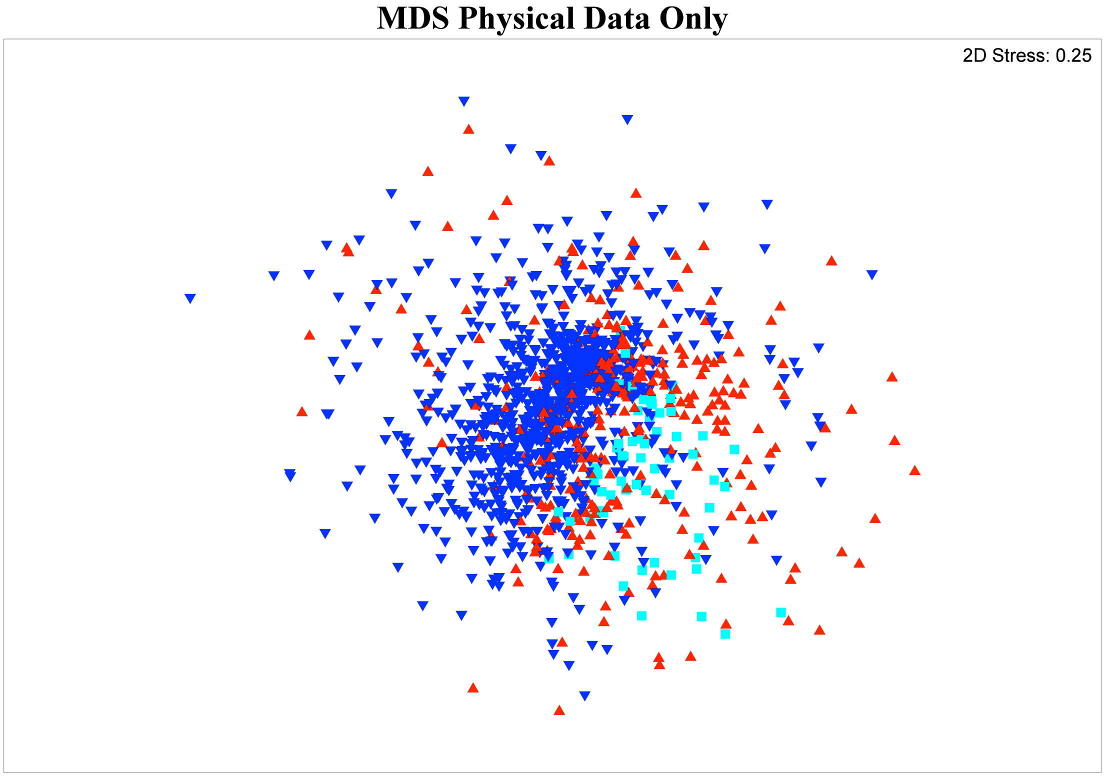

Figure 5-2: Multidimensional Scaling (MDS) plot of only physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles).

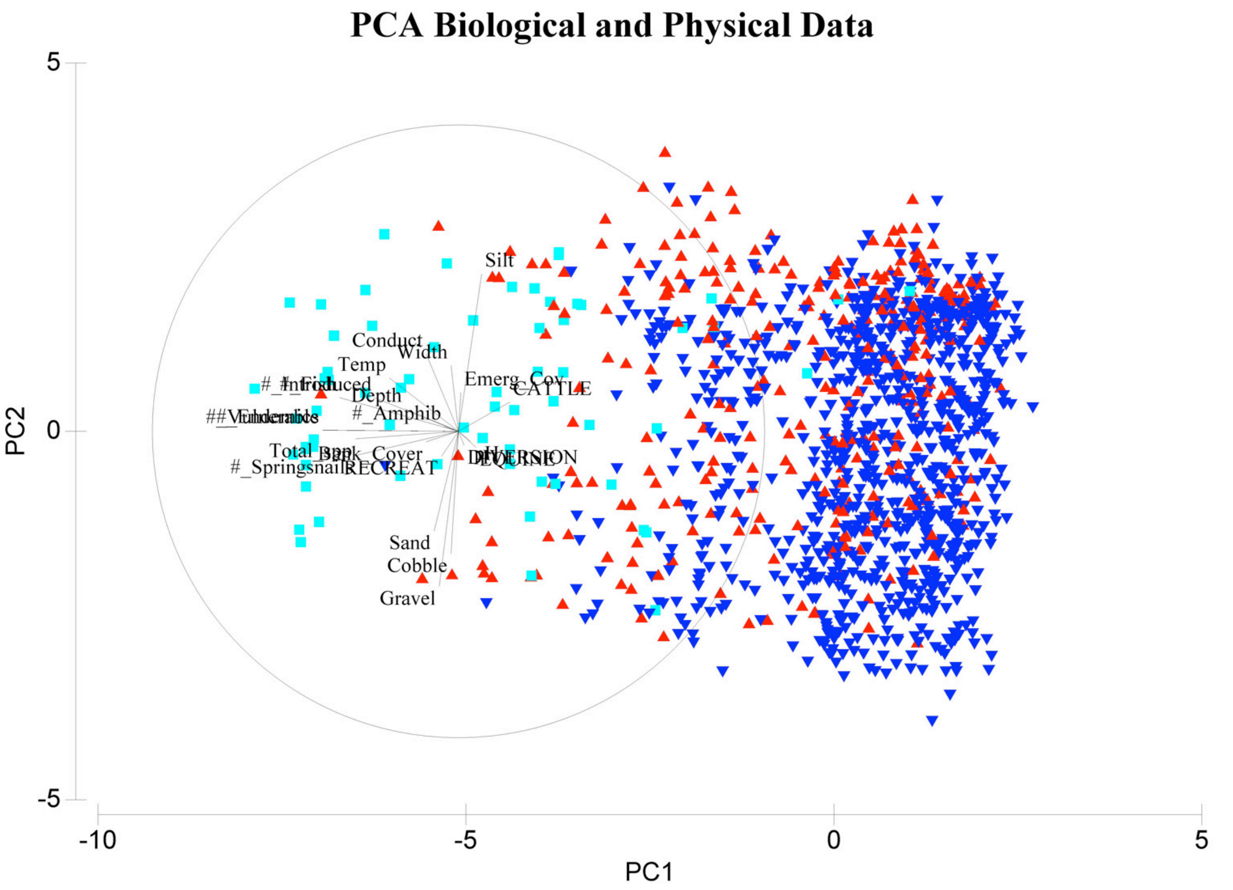

Figure 5-3: Principle Components Analysis (PCA) plot of biological and physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles).

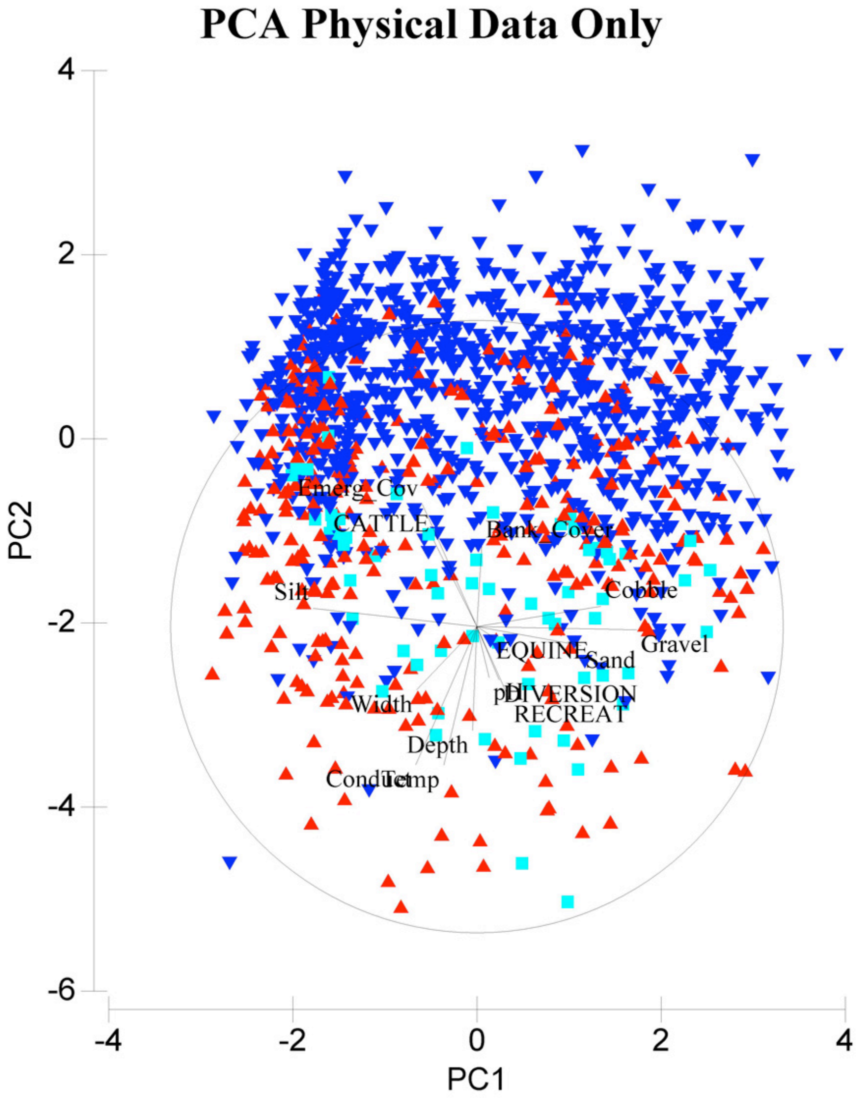

Figure 5-4: Principle Components Analysis (PCA) plot of only physicochemical data from regional aquifer thermal springs (represented by light blue squares), and local aquifer geothermal (red triangles) and cold springs (dark blue upside-down triangles).

Table 5-1: Mean values of certain physicochemical parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold).

Aquifer_Type	**N**	**Depth**	**Width**	**Temp**	**Conduct**	**pH**
Reg. Aquifer	66	22.8	205.4	30.8	1153.7	7.5
Local Hot	314	16.7	462.6	27.2	873.4	7.9
Local Cold	948	9.2	321.3	14.1	467.6	7.8

Table 5-2: Mean values of certain disturbance parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold).

Aquifer_Type	**DIVERSION**	**EQUINE**	**CATTLE**	**RECREAT**
Reg. Aquifer	1.8	1.0	1.3	1.4
Local Hot	1.8	1.2	2.2	1.4
Local Cold	1.7	1.2	2.5	1.3

Table 5-3: Mean values of certain habitat parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold).

Aquifer_Type	Emerg_Cov	Bank_Cover	%_Silt	%_Sand	%_Gravel	%_Cobble
Reg. Aquifer	31.5	72.3	36.8	24.1	26.1	8.0
Local Hot	45.5	68.2	58.3	19.8	16.1	4.4
Local Cold	56.0	68.1	50.1	18.4	20.7	9.1

Table 5-4: Mean values of certain biological parameters from regional aquifer thermal springs (Reg. Aquifer), and local aquifer geothermal (Local Hot) and cold springs (Local Cold).

Aquifer_Type	Total_spp	#_Endemics	#_Vulnerable	#_Introduced	#_Springsnails	#_Amphib	#_Fish
Reg. Aquifer	5.11	3.68	3.70	0.80	1.83	0.11	2.21
Local Hot	1.46	0.42	0.39	0.12	0.40	0.12	0.35
Local Cold	1.42	0.17	0.16	0.01	0.45	0.08	0.09

Table 5-5: Eigenvalues of the individual PC's from the combined physicochemical and biological data from springs in the Great Basin.

PC	Eigenvalues	%Variation	Cumulative %Variation
1	3.68	16.7	16.7
2	2.5	11.4	28.1
3	1.94	8.8	36.9
4	1.46	6.7	43.6
5	1.22	5.5	49.1
6	1.14	5.2	54.3
7	1.07	4.9	59.2
8	1.04	4.7	63.9
9	0.906	4.1	68.0
10	0.898	4.1	72.1

Table 5-6. The eigenvectors for each of the first 10 Principle Components (PCs) from the combined physicochemical and biological data from springs in the Great Basin.

Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**
Depth	-0.174	0.073	-0.213	-0.475	-0.110	-0.208	-0.118	0.066	-0.131	-0.214
Width	-0.024	0.215	-0.085	-0.470	-0.142	-0.172	0.068	0.358	-0.189	-0.213
Temp	-0.225	0.174	-0.291	0.153	0.130	0.197	0.081	-0.072	0.039	-0.093
Conduct	-0.105	0.252	-0.329	0.121	0.226	0.258	0.168	0.095	0.022	0.043
pH	0.040	-0.033	-0.154	-0.204	-0.240	0.253	0.448	0.167	0.536	0.362
Emerg_Cov	0.007	0.127	0.457	-0.013	0.162	-0.109	0.228	-0.203	-0.162	0.217
Bank_Cover	-0.107	-0.034	0.378	-0.291	0.290	0.144	-0.034	-0.150	-0.085	0.214
Silt	0.077	0.514	0.109	0.094	-0.115	-0.049	0.049	-0.091	-0.012	0.001
Sand	-0.079	-0.325	-0.090	0.236	0.057	-0.019	0.155	-0.151	-0.053	-0.419
Gravel	-0.063	-0.506	-0.092	0.006	0.041	-0.059	-0.057	-0.021	0.073	-0.089
Cobble	-0.025	-0.399	-0.006	-0.247	-0.088	-0.089	-0.085	0.058	0.055	0.328
DIVERSION	0.019	-0.046	-0.264	0.108	-0.248	-0.414	0.232	-0.321	-0.232	0.336
EQUINE	0.057	-0.053	-0.104	0.289	0.137	-0.269	0.191	0.534	-0.397	0.295
CATTLE	0.168	0.095	0.161	0.148	-0.372	-0.399	0.133	-0.129	0.353	-0.223
RECREAT	-0.055	-0.081	-0.167	-0.204	-0.025	0.174	0.516	-0.381	-0.332	-0.127
Total_spp	-0.337	-0.024	0.266	-0.090	0.104	-0.184	0.301	0.080	0.098	-0.139
#_Endemics	-0.444	0.005	0.080	0.210	-0.198	0.012	-0.072	0.095	0.001	0.058
#_Vulnerable	-0.445	0.004	0.068	0.204	-0.196	0.013	-0.078	0.098	-0.003	0.068
#_Introduced	-0.271	0.109	-0.198	-0.071	0.170	-0.204	-0.175	-0.299	0.134	0.228
#_Springsnail	-0.332	-0.077	0.275	0.031	-0.177	0.124	0.264	0.172	-0.052	-0.111
#_Amphib	-0.046	0.015	-0.068	-0.018	0.580	-0.413	0.210	0.106	0.364	-0.107
#_Fish	-0.385	0.111	-0.122	-0.066	-0.040	-0.129	-0.189	-0.149	0.066	0.121

Table 5-7: Eigenvalues of the individual PC's from only the physicochemical data from springs in the Great Basin.

PC	Eigenvalues	%Variation	Cumulative %Variation
1	2.48	16.5	16.5
2	1.81	12.1	28.6
3	1.48	9.9	38.5
4	1.29	8.6	47.1
5	1.04	7.0	54.1
6	0.994	6.6	60.7
7	0.951	6.3	67.0
8	0.87	5.8	72.8
9	0.812	5.4	78.3
10	0.688	4.6	82.8

Table 5-8. Eigenvectors for each of the first 10 Principle Components (PCs) from only the physicochemical data from springs in the Great Basin.

Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**
Depth	-0.014	-0.339	-0.414	-0.297	-0.112	-0.350	-0.093	-0.097	0.030	-0.030
Width	-0.197	-0.207	-0.324	-0.397	-0.268	-0.177	0.148	-0.273	0.169	0.127
Temp	-0.108	-0.452	0.012	0.359	0.104	-0.080	-0.064	-0.211	-0.127	-0.584
Conduct	-0.199	-0.451	0.088	0.289	-0.027	0.150	0.108	-0.048	-0.139	0.038
pH	0.040	-0.166	-0.038	-0.304	0.271	0.700	0.257	-0.407	-0.102	0.098
Emerg_Cov	-0.177	0.398	-0.176	0.169	0.258	-0.200	0.398	-0.205	-0.126	0.101
Bank_Cover	0.016	0.263	-0.535	0.267	0.115	-0.044	0.236	-0.135	-0.111	-0.241
Silt	-0.534	0.059	0.098	-0.051	0.115	-0.040	-0.033	0.067	0.043	-0.068
Sand	0.341	-0.062	0.172	0.230	0.169	-0.271	-0.055	-0.545	0.272	0.221
Gravel	0.522	-0.011	0.018	0.025	-0.041	-0.060	-0.084	-0.122	-0.040	-0.005
Cobble	0.404	0.066	-0.169	-0.235	-0.081	0.118	0.106	0.201	-0.190	-0.462
DIVERSION	0.072	-0.174	0.293	-0.296	0.338	-0.421	0.245	0.098	-0.594	0.115
EQUINE	0.048	-0.031	0.358	0.011	-0.408	-0.084	0.717	0.020	0.289	-0.196
CATTLE	-0.141	0.282	0.311	-0.384	0.197	-0.086	-0.214	-0.333	0.190	-0.491
RECREAT	0.107	-0.239	-0.134	-0.051	0.618	-0.039	0.177	0.407	0.558	-0.036

Table 5-9: SIMPER analyses of the differences between biological and physicochemical parameters from geothermal local aquifer (Local Hot) springs and cold local aquifer (Local Cold) springs.

	Local Hot	**Local Cold**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
Temp	1.16	-0.492	3.65	0.88	7.79	7.79
Conduct	0.431	-0.222	2.44	0.56	5.20	12.98
pH	0.173	-1.47E-2	2.27	0.50	4.83	17.82
Depth	0.17	-9.28E-2	2.25	0.50	4.80	22.62
#_Amphib	0.118	-4.07E-2	2.23	0.47	4.76	27.38
Bank_Cover	-8.37E-2	2.16E-2	2.22	0.54	4.75	32.12
Width	0.138	-2.59E-2	2.19	0.48	4.67	36.80
RECREAT	8.08E-2	-3.89E-2	2.13	0.50	4.55	41.34
Emerg_Cov	-0.171	8.88E-2	2.1	0.74	4.48	45.82
EQUINE	7.84E-4	1.95E-2	2.06	0.37	4.40	50.23
#_Fish	0.262	-0.233	2.06	0.63	4.39	54.62
Sand	-4.39E-2	-1.21E-2	2.05	0.95	4.36	58.98
CATTLE	-0.121	0.107	2.04	0.94	4.36	63.34
DIVERSION	2.83E-2	-1.44E-2	2.04	0.81	4.35	67.70
Silt	9.73E-2	-2.95E-2	2.03	0.80	4.34	72.03
Total_spp	-8.67E-2	-4.28E-2	1.99	0.89	4.24	76.28
Gravel	-0.172	3.99E-2	1.99	0.94	4.24	80.51
#_Springsnails	-0.154	-2.78E-2	1.89	0.95	4.03	84.54
#_Introduced	0.149	-0.193	1.85	0.32	3.95	88.49
Cobble	-0.246	7.95E-2	1.85	0.70	3.95	92.44

Table 5-10: SIMPER analyses of the differences between biological and physicochemical parameters from geothermal local aquifer (Local Hot) springs and regional aquifer thermal (Regional Aquifer) springs.

	Local Hot	**Regional Aquifer**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
#_Introduced	0.149	2.06	10.3	0.97	15.33	15.33
#_Vulnerable	6.8E-2	2.2	6.41	1.37	9.50	24.82
#_Fish	0.262	2.09	6.23	1.23	9.22	34.04
#_Endemics	9.24E-2	2.15	6.12	1.33	9.06	43.10
#_Springsnails	-0.154	1.13	3.22	1.18	4.77	47.87
Depth	0.17	0.523	3.01	0.60	4.45	52.32
RECREAT	8.08E-2	0.174	2.67	0.56	3.95	56.27
Total_spp	-8.67E-2	1.03	2.6	0.81	3.85	60.12
#_Amphib	0.118	2.06E-2	2.43	0.48	3.60	63.72
Emerg_Cov	-0.171	-0.463	2.34	0.83	3.46	67.18
Bank_Cover	-8.37E-2	8.73E-2	2.2	0.53	3.26	70.44
pH	0.173	-0.611	2.17	0.58	3.22	73.66
Width	0.138	-0.284	2.17	0.49	3.22	76.88
CATTLE	-0.121	-0.959	2.13	0.93	3.15	80.02
Gravel	-0.172	0.245	2.12	0.99	3.14	83.17
Conduct	0.431	1.14	2.09	0.85	3.09	86.26
Sand	-4.39E-2	0.383	1.99	0.97	2.95	89.21
DIVERSION	2.83E-2	7.17E-2	1.95	0.84	2.88	92.09

Table 5-11: SIMPER analyses of the differences between biological and physicochemical parameters from cold local aquifer (Local Cold) springs and regional aquifer thermal (Regional Aquifer) springs.

	Local Cold	**Regional Aquifer**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
#_Introduced	-0.193	2.06	10.4	0.97	14.40	14.40
#_Fish	-0.233	2.09	7.4	1.51	10.22	24.63
#_Vulnerable	-0.176	2.2	7.06	1.58	9.76	34.39
#_Endemics	-0.18	2.15	6.84	1.56	9.46	43.85
Temp	-0.492	1.57	4.72	1.56	6.53	50.38
Conduct	-0.222	1.14	2.95	0.97	4.07	54.45
#_Springsnails	-2.78E-2	1.13	2.94	1.08	4.07	58.52
Depth	-9.28E-2	0.523	2.69	0.57	3.71	62.23
CATTLE	0.107	-0.959	2.47	1.09	3.41	65.64
Total_spp	-4.28E-2	1.03	2.43	0.77	3.36	69.00
Emerg_Cov	8.88E-2	-0.463	2.36	0.81	3.27	72.27
RECREAT	-3.89E-2	0.174	2.36	0.53	3.26	75.53
Gravel	3.99E-2	0.245	2.06	0.96	2.84	78.37
Cobble	7.95E-2	2.97E-2	2.05	0.75	2.83	81.21
#_Amphib	-4.07E-2	2.06E-2	1.97	0.42	2.73	83.93
Sand	-1.21E-2	0.383	1.89	0.94	2.62	86.55
DIVERSION	-1.44E-2	7.17E-2	1.89	0.83	2.61	89.16
Bank_Cover	2.16E-2	8.73E-2	1.77	0.48	2.44	91.61

Table 5-12: SIMPER analyses of the differences between physicochemical parameters from geothermal local aquifer (Local Hot) springs and cold local aquifer (Local Cold) springs.

Variable	Local Hot Av.Value	Local Cold Av.Value	Av.Sq.Dist	Sq.Dist/SD	Contrib%	Cum.%
Temp	1.16	-0.492	3.65	0.88	10.96	10.96
Conduct	0.431	-0.222	2.44	0.56	7.32	18.27
pH	0.173	-1.47E-2	2.27	0.50	6.80	25.07
Depth	0.17	-9.28E-2	2.25	0.50	6.75	31.82
Bank_Cover	-8.37E-2	2.16E-2	2.22	0.54	6.68	38.50
Width	0.138	-2.59E-2	2.19	0.48	6.58	45.08
RECREAT	8.08E-2	-3.89E-2	2.13	0.50	6.40	51.48
Emerg_Cov	-0.171	8.88E-2	2.1	0.74	6.30	57.78
EQUINE	7.84E-4	1.95E-2	2.06	0.37	6.20	63.98
Sand	-4.39E-2	-1.21E-2	2.05	0.95	6.14	70.12
CATTLE	-0.121	0.107	2.04	0.94	6.13	76.25
DIVERSION	2.83E-2	-1.44E-2	2.04	0.81	6.13	82.38
Silt	9.73E-2	-2.95E-2	2.03	0.80	6.10	88.48
Gravel	-0.172	3.99E-2	1.99	0.94	5.96	94.44

Table 5-13: SIMPER analyses of the differences between physicochemical parameters from geothermal local aquifer (Local Hot) springs and regional aquifer thermal (Regional Aquifer) springs.

	Local Hot	**Regional Aquifer**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
Depth	0.17	0.523	3.01	0.60	9.97	9.97
RECREAT	8.08E-2	0.174	2.67	0.56	8.84	18.81
Emerg_Cov	-0.171	-0.463	2.34	0.83	7.75	26.56
Bank_Cover	-8.37E-2	8.73E-2	2.2	0.53	7.30	33.85
pH	0.173	-0.611	2.17	0.58	7.21	41.06
Width	0.138	-0.284	2.17	0.49	7.20	48.26
CATTLE	-0.121	-0.959	2.13	0.93	7.04	55.30
Gravel	-0.172	0.245	2.12	0.99	7.03	62.33
Conduct	0.431	1.14	2.09	0.85	6.92	69.25
Sand	-4.39E-2	0.383	1.99	0.97	6.60	75.85
DIVERSION	2.83E-2	7.17E-2	1.95	0.84	6.45	82.30
Cobble	-0.246	2.97E-2	1.69	0.66	5.60	87.90
Silt	9.73E-2	-3.95E-2	1.66	0.77	5.51	93.41

Table 5-14: SIMPER analyses of the differences between biological and physicochemical parameters from cold local aquifer (Local Cold) springs and regional aquifer thermal (Regional Aquifer) springs.

	Local Cold	**Regional Aquifer**				
Variable	**Av.Value**	**Av.Value**	**Av.Sq.Dist**	**Sq.Dist/SD**	**Contrib%**	**Cum.%**
Temp	-0.492	1.57	4.72	1.56	14.19	14.19
Conduct	-0.222	1.14	2.95	0.97	8.86	23.05
Depth	-9.28E-2	0.523	2.69	0.57	8.07	31.13
CATTLE	0.107	-0.959	2.47	1.09	7.41	38.54
Emerg_Cov	8.88E-2	-0.463	2.36	0.81	7.10	45.64
RECREAT	-3.89E-2	0.174	2.36	0.53	7.10	52.73
Gravel	3.99E-2	0.245	2.06	0.96	6.18	58.91
Cobble	7.95E-2	2.97E-2	2.05	0.75	6.16	65.07
Sand	-1.21E-2	0.383	1.89	0.94	5.69	70.76
DIVERSION	-1.44E-2	7.17E-2	1.89	0.83	5.68	76.44
Bank_Cover	2.16E-2	8.73E-2	1.77	0.48	5.31	81.75
Width	-2.59E-2	-0.284	1.74	0.51	5.24	87.00
Silt	-2.95E-2	-3.95E-2	1.67	0.79	5.01	92.01

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