University of South Dakota
USD RED

Honors Thesis | Theses, Dissertations, and Student Projects

Spring 2019

Surveillance of Ranavirus and Bacterial Microbiome Characterization of False Map Turtles (Graptemys pseudogeographica) Along the Lower Missouri River, USA

Madeline McClaine Butterfield
University of South Dakota

Follow this and additional works at: https://red.library.usd.edu/honors-thesis

Recommended Citation

Butterfield, Madeline McClaine, "Surveillance of Ranavirus and Bacterial Microbiome Characterization of False Map Turtles (Graptemys pseudogeographica) Along the Lower Missouri River, USA" (2019). *Honors Thesis*. 41.
https://red.library.usd.edu/honors-thesis/41

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, and Student Projects at USD RED. It has been accepted for inclusion in Honors Thesis by an authorized administrator of USD RED. For more information, please contact dloftus@usd.edu.

Surveillance of *Ranavirus* and Bacterial Microbiome Characterization of False Map Turtles (*Graptemys pseudogeographica*) Along the Lower Missouri River, USA

by
Madeline McClaine Butterfield

A Thesis Submitted for the Partial Fulfillment
of the Requirements of the
University Honors Program

Department of Biology
University of South Dakota
May 4th, 2019

The members of the Honors Thesis Committee appointed

to examine the thesis of Maddie Butterfield

find it satisfactory and recommend that it be accepted.

Jacob Kerby, Ph.D.
Associate Professor of Biology
Director of the Committee

Bernie Wone, Ph.D.
Assistant Professor of Biology

Scott Breuninger, Ph.D.
Associate Professor of History

ABSTRACT

Surveillance of *Ranavirus* and Bacterial Microbiome Characterization of False Map Turtles (*Graptemys pseudogeographica*) Along the Lower Missouri River, USA

Maddie Butterfield

Director: Dr. Jake Kerby, Ph.D.

Graptemys pseudographica, or the False Map Turtle, is a state-threatened species in South Dakota. The False Map Turtle, a river-dwelling species, is susceptible to the viral pathogen *Ranavirus*, leading to the deadly ranavirosis, which is a systemic infection transmitted through the water that can cause severe epizootics in turtles (Johnson et al. 2008). We trapped for False Map Turtles in July of 2017 at three different spots along the Missouri River between Yankton, SD and Vermillion, SD and describe the *Ranavirus* infection status of all 79 False Map Turtles trapped in this area. Additionally, being a river-dwelling species, the bacterial microbiome within the cloaca of False Map Turtles is presumed to vary along different geographic locations within the river and is largely understudied. The bacterial microbiome within the cloaca of an animal has been shown to have significant effects on the overall health of the animal (Ringo et al. 2010). From the 79 individuals sampled this summer, 21 were analyzed for the bacterial genera present within the bacterial community of their gut microbiome by 16S rRNA gene sequencing and the major bacterial genera were identified. Community structure based on taxonomic relationships between bacterial genera based on similarity of 16S rRNA

gene sequence is also presented. We conclude that there is no *Ranavirus* present in any individuals sampled this summer and that bacterial microbiome composition among the cloaca of False Map Turtles differs by geographic location, and that urination during cloacal swabbing does not significantly impact the efficacy of the bacterial community sample.

KEYWORDS: False Map Turtle, Ranavirus, Microbiome, Cloacal Microbiome, MNRR, Missouri River

TABLE OF CONTENTS

LIST OF FIGURES:

LIST OF TABLES

PREFACE

Literature Review of *Graptemys pseudogeographica*, the False Map Turtle

INTRODUCTION

All taxa of animals have been experiencing population declines in recent years. In 2018, nearly 28% of over 96,900 described species of animals from taxa including vertebrates, invertebrates, plants, fungi, and protists were considered to be threatened (Numbers 2018). While amphibian population declines have been widely publicized, lesser-covered reptiles like chelonians have been experiencing extreme population declines of their own (E.O. Moll and D. Moll 2000). Chelonians are aquatic reptiles, comprised of two suborders and thirteen families of aquatic tortoises and turtles. Based on the 2018 IUCN Red List of Threatened Species, 56.2% of 332 turtle and tortoise species are threatened and 85.7% of chelonian species are threatened (Rhodin et al. 2018). Additionally, testudines are the second-most threatened vertebrate group (56.2% of species considered threatened), bested only by primates (64.3%) and followed closely by caudates (ie. salamanders, 55.4%) (Rhodin et al. 2018). Causes of reptilian population declines are similar to those threatening amphibian population, including habitat loss and degradation, global climate change, invasive species, and environmental pollution. Anthropogenic causes aside, infectious diseases and parasitism are also cited as major sources of population decline in reptiles (Gibbons et al. 2000). Reptiles on whole are

susceptible to infection from a wide range of infectious agents, including bacteria, parasites, viruses, and fungi (Jacobson 1993); though a comprehensive list of pathogens that are infective to reptiles has not been made. Among important pathogens affecting chelonians, aerobic bacteria (*Mycoplasma*) have caused population reductions in desert tortoises (*Gopherus agassizii*) and gopher tortoises (*Gopherus polyphemus*, Jacobson 1993) and tortoise herpesvirus infection has become increasingly prevalent in captive chelonian populations and pet trade (Origgi et al. 2004, Johnson et al. 2005).

Graptemys pseudogeographica, the False Map Turtle, is a riverine turtle of the family *Emydidae* among the order *Chelonia* that is endemic to North America, more specifically to Missouri-Mississippi river system and the accompanying river tributaries (Fig. 1). False Map Turtles inhabit along the river system spanning from as northern as North Dakota to the southern parts of Louisiana and Texas (Conant et al. 2016). The species itself can be split into two subspecies, differentiated by habitat location and some morphological markings: *Graptemys pseudogeographica pseudogeographica,* or the Northern False Map Turtle, and *Graptemys pseudogeographica kohnii*, or the Mississippi Map Turtle (Fig. 2). The Northern False Map Turtle is native to the northern Missouri River system predominantly through the states of Minnesota, Wisconsin, North and South Dakota, Iowa, Illinois, Missouri, and Kansas (Fig. 3); thus is the focus of this thesis.

False Map Turtles are relatively small for river turtles, with significant sexual dimorphism. Females are usually between 15-27 cm and males are usually between 9-15 cm in carapace length (Conant et al. 2016). The size difference between male and female False Map Turtles is one of the most dramatic cases of sexual dimorphism in vertebrate

tetrapods, with males usually measuring half the carapace length and width as their female counterparts and usually one-tenth the weight (Lindeman 2013). Being overall smaller than other river turtles or members of *Emydidae* may be an evolutionary advantage since areas inhabited by False Map Turtles are usually smaller or less turbulent river systems than those that their larger counterparts may inhabit (E.O. Moll and D. Moll 2000). False Map Turtle reproduction follows that of most riverine turtles, considered Type I by E.O. Moll (1979) where females lay multiple relatively large clutches of small eggs in ancestral nesting locations during a definitive nesting season (E. O. Moll 1979). Clutch size varies significantly among riverine species, ranging from 178 or 150 eggs in the *Chitra* spp. and *Podocnemis expansa,* the giant South American river turtle, respectively; to less than 20 eggs in larger species including *Bagatur borneoensis*, the painted terrapin (E.O Moll and D. Moll 2000).

In terms of diet, False Map Turtles are largely opportunistic omnivores as well as scavengers: an adaptation to accommodate highly varying levels of animal and plant materials available during a given season, location, or ecosystem (E.O Moll and D. Moll 2000). Their diet is largely dependent on available resources but consistently includes aquatic vegetation, mollusks, and insects as both adults and larvae (Vogt 1981). They feed both underwater and on the surface of water. Scavenging provides False Map Turtles a stable protein source, since hunting for live prey is not an adaptation lent to the False Map Turtle. Additionally, the False Map Turtle has a unique feeding method called "benthic bulldozing", where turtles will indiscriminately eat river bottom detritus and the prey therein (D. Moll 1976).

Freshwater turtles such as the False Map Turtle play integral roles in aquatic ecosystems and are threatened with alteration due to human intervention, namely agricultural expansion and habitat loss (Quesnelle et al. 2013). A threatened species in the state of South Dakota (Ashton and Dowd 2008), the False Map Turtle plays a critical role in maintaining a healthy riverine ecosystem in the Missouri and Mississippi rivers as an endemic species. Understanding the disease load on threatened populations and understanding how external factors such as water quality impacts organism health is imperative to maintaining the health of both the species and their ecosystem on whole. In this thesis, we examine two facets of conservation research associated with the False Map Turtle population of the lower Missouri River: surveillance of deadly viral pathogen *Ranavirus* and characterization of cloacal bacterial microbiome with relation to geographic location and water quality.

FIG. 1*:* Adult male *Graptemys pseudogeographica pseudogeographica* from the Missouri River in southeast South Dakota. Catalogued and photographed by Drew R. Davis.

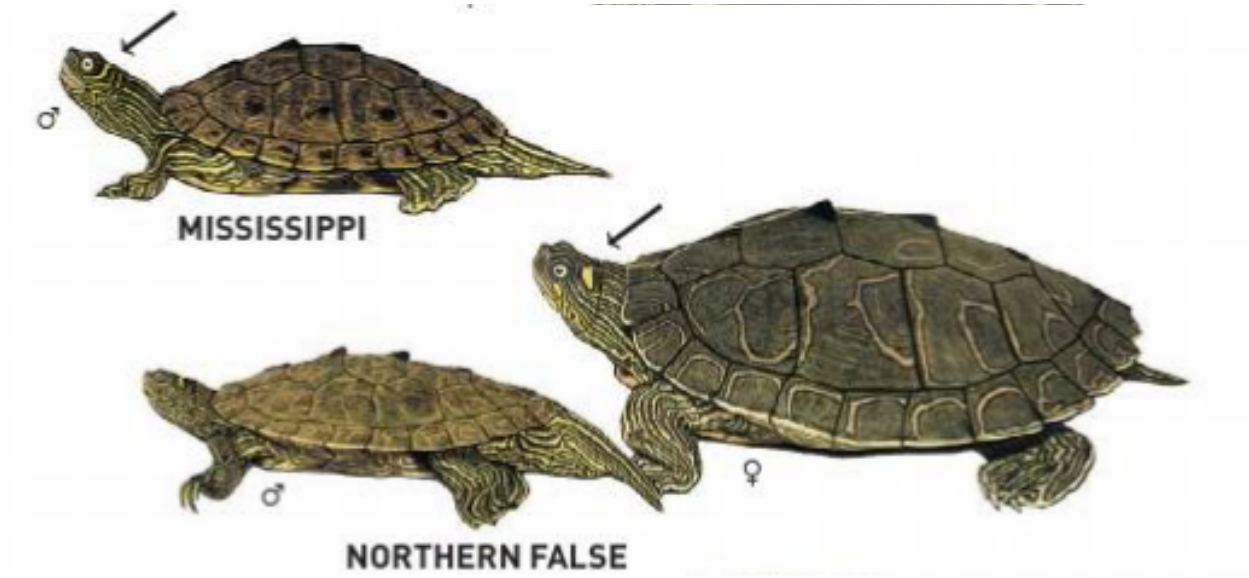


FIG. 2: Sample specimen of both subspecies of *Graptemys pseudogeographica:* the Mississippi Map Turtle (*G. p. kohnii*, no female shown) and the Northern False Map Turtle (*G. p. pseudogeographica*). From Conant et al. 2016, p. 179.

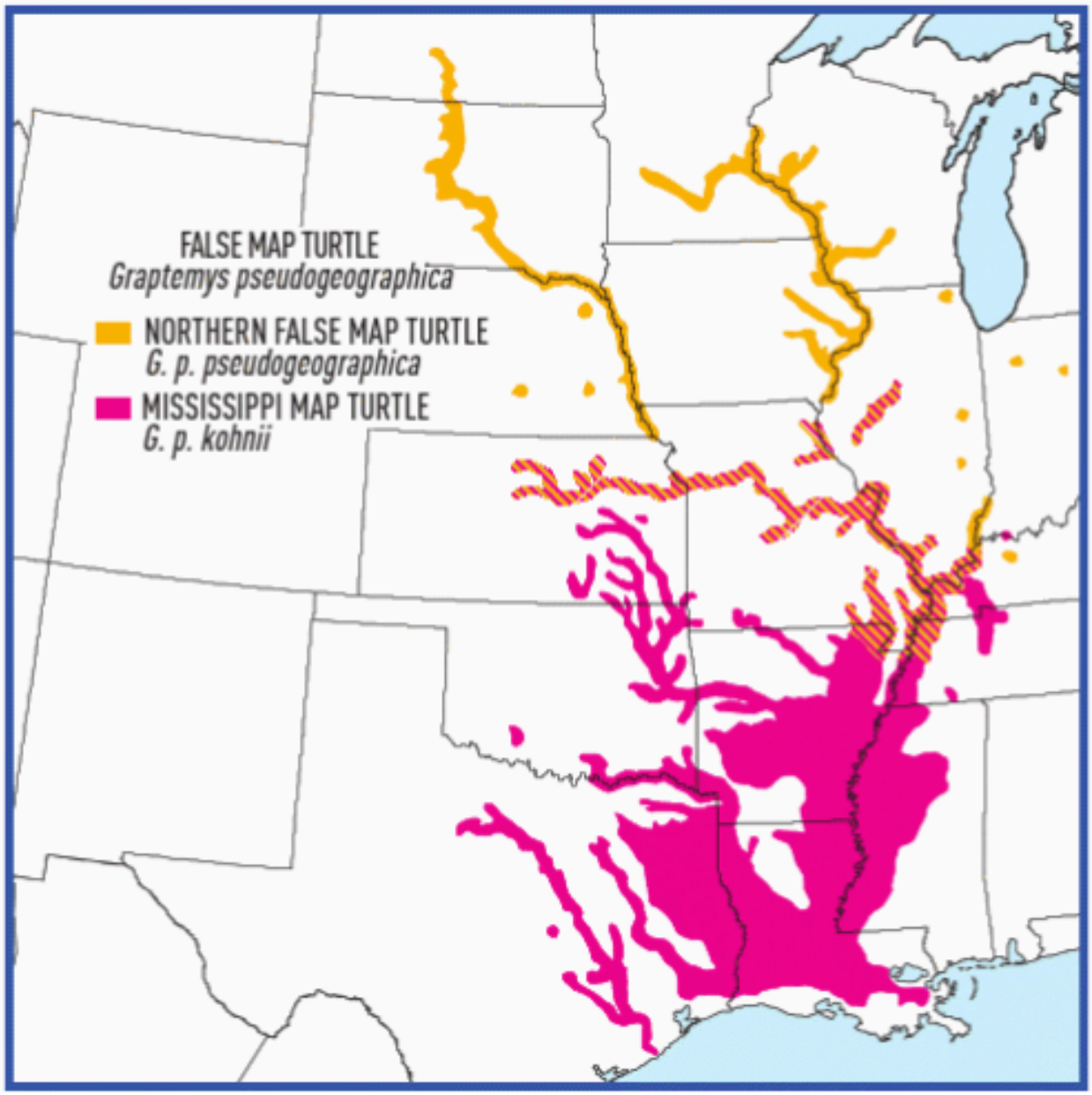


FIG. 3: False Map Turtle (*Graptemys pseudogeographica geographica; Graptemys pseudogeographica kohnii*) population distribution in the United States as of 2016. From Conant et al. 2016, p. 208.

CHAPTER I

Surveillance of *Ranavirus* Among *G. pseudogeographica* Along the Lower Missouri River, USA

INTRODUCTION

A. *Ranavirus* as a Pathogen

Among pathogens plaguing conservation efforts today, the multihost pathogen *Ranavirus* has proven debilitating to amphibian and reptilian populations alike (Daszak et al. 1999; Duffus et al. 2015). *Ranavirus* is one of five genera among the virus family *Iridovirus*, which also includes *Iridovirus*, *Chloriridovirus*, *Megalocytivirus*, and *Lymphocystivirus* (Schock et al. 2008). Iridoviruses were first discovered in 1954 in crane fly larvae (Xeros 1954), studied first for the hallmark blue iridescence it created just below the epidermis in larvae. Of the five genera, *Iridovirus* and *Chloriridovirus* both infect invertebrates including insects. Genera *Megalocytivirus*, *Lymphocystivirus*, and *Ranavirus* in contrast, infect cold-blooded vertebrates: lymphocystiviruses and megalocytiviruses can infect fish, and ranaviruses can infect fish, amphibians, and reptiles (Mao et al. 1997, Williams et al. 2005). Detection of infected populations and mass-mortality events have increased in frequency since the advent of *Iridovirus* research, particularly in the early 1990's (ie. Bradford 1991, Fellers and Droft 1993): most likely a combination of improvement of detection methods and increasing exposure to vulnerable or isolated populations (Earl and Gray 2014). However, due to the cryptic

nature of species at risk and that *Iridovirus* infection is not always symptomatic (Williams et al. 2005, Johnson et al. 2007, Price et al. 2015), geographic distribution and disease surveillance efforts are most likely underestimated.

B. *Iridoviruses*

Iridoviruses are large icosahedral viruses (ranging from 120-300 nm in diameter, Fig. 5) that are constructed in the cytoplasm of infected cells (Darlington 1966). Internally, the virus is comprised of a lipid-bound capsid and core, where the most abundant protein is the major capsid protein (MCP), an important structure in identification of iridoviruses (Williams 1996). The genome is a linear double-stranded DNA virus that is terminally redundant, thus circularly permutated (Goorha and Murti 1982) – a rarity in animal viruses. Upon infection, the virus shuts down host cell macromolecule production (including RNA transcription and translation), leading to a halt of host cellular DNA synthesis (Willis et al. 1985). Viral DNA replication occurs in the host nucleus and viral protein synthesis and assembly occurs in the host cytoplasm, preceding virion budding for viral exit (Williams et al. 2005). This method of utilizing both host nucleus and cytoplasm for replication and assembly varies from the poxvirus family, in which only the cytoplasm is used (Schramm and Locker 2005). *Ranavirus* and *Megalocytivirus* are causative agents for the most serious infections, where *Megalocytivirus* has in recent years proven deleterious in marine aquaculture species in Southeast Asia (Williams et al. 2005) and *Ranavirus* has been the causative agent for mass die-offs in amphibians and reptiles (Johnson et al. 2008, Brenes et al. 2014, Price et al. 2014).

Genus *Ranavirus* is currently separated into four or five distinct groups based on genomic analysis (Jancovich et al. 2015), but three groups have particular significance in amphibian and other ectothermic populations. The three groups of viruses of much study are frog virus 3 (FV3)-like, common midwife toad virus (CMTV)-like, and Ambystoma trigrinum virus (ATV)-like (Price et al. 2017). Viral taxonomy of *Ranavirus* shifts with increasing genomic analysis of the virus, so the systems of classification are expected to be revised over time. FV3 has undergone the most research of all iridoviruses and infects the most taxonomically diverse group of organisms and is therefore frequently used as a model for iridoviruses in general (Williams et al. 2005).

The host species able to be infected by different groups of *Ranavirus* are relatively stable, thus making further viral study and identification in infected animals simpler. Experimental infective studies in Granoff et al. (1965) and Clark et al. (1968) first introduced data suggesting that FV3-like viruses are infective to amphibians, reptiles, and bony fish, while all endotherms were not susceptible to infection of FV3-like viruses. Currently, ATV-like viruses are only known to infect tiger salamanders in western North America (Jancovich et al. 2005) and CMTV-like viruses have caused mass mortalities in six different amphibian species in Europe (Price et al. 2014). Coinciding with a diverse range of susceptible species, *Ranavirus* has been detected in organisms across the globe. This includes fish in the context of ornamental fish from Southeast Asia (Hedrick and McDowell 1995), sturgeon in the United States (Kurobe et al. 2011), sheathfish and catfish in Europe (Ahne et al. 1997); amphibians in the context of frogs from every continent except Antarctica and Africa (reviewed in Duffus et al. 2015, Price et al. 2017); and salamanders from 25 countries, including every continent except

Antarctica (reviewed in Duffus et al. 2015). *Ranavirus* has also impacts reptilian populations, where species of turtles and snakes have both been infected in Europe, Australia, Asia, and North America (Marschang et al. 1999, Chen et al. 1999, Hyatt et al. 2002, Goodman et al. 2013). Transmission of *Ranavirus* between taxonomic classes has occurred (ie. from amphibian species to reptilian species), which may give rise to increasing infections in previously uninfected species (Mao et al. 1999, Schock et al. 2008).

C. *Ranavirus* in Turtles

Because *Ranavirus* infections were more common among amphibians historically, reptilian infection with *Ranavirus* was considered a relatively sporadic event. Among chelonians, the first reported infection was in 1982 in a Hermann's tortoise (*Testudo hermanni*, Heldstab and Bestetti 1982), followed years after with an infection among a group of captive Hermann's tortoises (Muller et al. 1988). Years later, infection in captive Hermann's tortoise populations in Switzerland would confirm via PCR that the *Ranavirus* in question was in fact an FV3-like virus (Marschang et al. 1999). Since then, FV3-like *Ranavirus* infection in chelonians has been present in species including the Horsfield's tortoise (*Testudo horsfieldii*) and the Common Box Turtle (*Terrapene Carolina*, Mao et al. 1997), the Chinese Softshell Turtle (*Trionyx sinensis*, Chen et al. 1999), the Eastern Box Turtle (*Terrapene carolina carolina*, DeVoe et al. 2004, Allender et al. 2011, Kimble et al. 2017), the Painted Turtle (*Chrysemys picta*, Goodman et al. 2013), and the European Pond Turtle (*Emys orbicularis*, Blahak and Uhlenbrok 2010).

Ranavirosis, the disease caused by pathogenic viruses of the genus *Ranavirus*, is a highly virulent systemic infection (Daszak et al. 1999; Duffus et al. 2015). Generally,

Ranavirus infection in turtles is characterized externally by the presence of cutaneous abscesses, nasal or ocular discharge, oral plaque, or unenergetic behavior (Allender 2012). Internally, *Ranavirus* infection in turtles can cause systemic blood clotting and hemorrhagic necrosis of kidneys, liver, heart, spleen, and the alimentary tract, ultimately leading to death (Johnson et al. 2007). When characterized among Chinese Softshell Turtles, the FV3-like viral disease was also called the "red neck disease," due to external swelling and hemorrhaging of the neck and internal hemorrhaging of the liver (Chen et al. 1999).

Covert infection is also possible in infected organisms, where no external symptoms would indicate *Ranavirus* infection. Usually, these cases are due to an infection of low virulence instead of being early in the life cycle of a systemic and lethal infection (Williams et al. 2005). When external symptoms are not shown, infective status can still be obtained via electron microscopy of tissue (Tonka and Weiser 2000), qPCR of tissue centered around detection of major capsid protein (Forson and Storfer 2006), or end-point dilution in cell cultures for insects (Constantino et al. 2001).

Though ranaviruses have been detected across the United States, little is known about their geographic and host distribution in the Midwestern United States (Duffus et al. 2015). To our knowledge, no turtles in the region have been screened for ranaviruses, though recent efforts have detected ranaviruses in amphibians along the Missouri River in Nebraska (Davis and Kerby 2016) and in South Dakota (Davis 2018) as well as in sturgeon in the Missouri River at the Gavin's Point National Fish Hatchery in Yankton, South Dakota (Kurobe et al. 2011). Despite the presence of ranaviruses in the region, nothing is known about whether they occur locally in reptile species. Given the detection

of ranaviruses in amphibians from Nebraska and South Dakota and the ability for pathogen transmission to occur among vertebrate classes, there is concern over the potential transmission of ranaviruses to False Map Turtles. Here, we investigated the prevalence and infection load of ranaviruses in False Map Turtles from the lower Missouri River between South Dakota and Nebraska.

MATERIALS AND METHODS

A. Study Population

In this study, False Map Turtles were collected at different sites along the 59-mile stretch of the Missouri National Recreation River (MNRR) between Yankton and Elk Point, South Dakota, USA from 2015-2017 (Fig. 5). Sampling was done with the joint intent of testing for prevalence of *Ranavirus* (Butterfield et al. 2019) and collecting cloacal bacterial samples for microbiome composition analysis. Samples were taken at three distinct sections of the MNRR: the James River, Goat Island within the Missouri River, and the Vermillion River, from west to east respectively (Fig. 5).

During collection of False Map Turtles along the MNRR between 2015 and 2017, turtles were primarily collected using partially submerged hoop traps baited with sardines, but individuals were also opportunistically collected by hand. Traps were left submerged near basking surfaces (e.g., fallen trees) for 24–48 h and captured individuals were weighed, measured, and given a unique identifying notch on their marginal scutes (following Ernst et al. 1974).

B. Sample Collection

Before turtles were released, *Ranavirus* surveillance samples were obtained by either: 1) collecting a blood sample from the caudal vein using a sterile insulin syringe (EXELINT International Co., Redondo Beach, California, USA), or 2) collecting a small skin tissue sample (ca. 5 mm^2) from the webbing on the hind foot. Tissue collecting equipment (e.g., scissors, forceps) was sterilized with a 10% bleach solution between individuals and sites to prevent cross contamination and gloves were worn throughout.

All tissue samples were stored in individual tubes containing 95% ethanol and kept at -20°C until processing.

We also collected three individual False Map Turtles as voucher specimens during this study. We euthanized these individuals via an overdose of sodium pentobarbital injected through the caudal vein and collected a muscle tissue sample from the right hind limb.

C. Quantitative PCR

All tissue samples were stored in individual tubes containing 95% ethanol and kept at -20°C until processing. DNA was extracted from tissue samples using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) and following kit protocols. Extracted samples were then analyzed for *Ranavirus* infection via quantitative PCR (qPCR) following methods outlined in Forson and Storfer (2006). Numerous other studies have used this method successfully and have verified that this method provides reliable detection down to a single viral copy (Whitfield et al. 2012; Davis and Kerby 2016). Each qPCR plate included a negative control (water) and a 1/10 serial dilution series ($1e^2$-$1e^5$) of gBlocks (IDT, Coralville, Iowa, USA) containing a target sequence of a sequence of DNA known to be shared between *Ranavirus* strains, the major capsid protein. This series of dilutions was done in order to create a standard curve to quantify sample infection loads. Each sample that was analyzed was run in triplicate and C_t (cycle threshold level) values were used to determine absence/presence of *Ranavirus*; a sample was considered positive if at least two wells amplified with a $C_t < 45$. All analyses were

run on an ABI 7300 Real-time PCR System using Real-time PCR System Sequence Detection Software v1.2.3 (Applied Biosystems, Foster City, California, USA).

RESULTS AND DISCUSSION

A. Results

A total of 123 False Map Turtle genetic tissue samples were collected from 10 sites along the MNRR (Fig. 2; Table 1) including blood, skeletal muscle, and skin webbing. All samples were negative for *Ranavirus* presence (Table 1).

B. Discussion

While the negative results are promising for the conservation of the False Map Turtle in the region, transmission of ranaviruses from infected hosts still may occur. With *Ranavirus* known from sites <1.5 km away (Davis and Kerby 2016), the lack of infected False Map Turtles in this study may also suggest that ranavirosis progresses quickly, with high mortality of infected individuals. To test the vulnerability of False Map Turtles to the pathogen and ranavirosis, an experimental infection study may be a future direction to pursue. For this reason, additional research and continued surveillance for *Ranavirus* in the region, in both the False Map Turtle and other likely host species, is imperative to maintaining population health profiles and informing future conservation action for the surrounding aquatic and terrestrial ecosystems.

Lack of positive *Ranavirus* samples may have been influenced by a number of factors, including water temperature. Water temperatures are known to affect the persistence of *Ranavirus* in infected animals but do not fully explain lack of *Ranavirus* detection. In 2017, the water temperature at sampled sites where 93 of the total 123 False Map Turtles sampled was 26.1 ± 0.3°C (mean ± 1 SE). Allender (2012) found that *Ranavirus* in semi-aquatic turtles has a higher and faster mortality at lower temperatures (22°C) when compared to higher temperatures (28°C). Therefore, given that *Ranavirus*

has been historically present in the region, elevated water temperatures may reduce the likelihood of infection in False Map Turtles, though further studies should investigate this possibility.

Also potentially contributing to the negative test results, *Ranavirus* may not be as persistent within erythrocytes, muscle, or skin tissue (Johnson et al. 2007, Allender et al. 2013). While *Ranavirus* is a systemic infection with viral copies detectable in various tissues including the liver, kidney, spleen, esophagus, stomach, and both small and large intestine, infection status in muscle and epithelial skin cells was not confirmed (Johnson et al. 2007). Moreover, due to the virulence of the disease (experimental intramuscular infection caused death or euthanasia between 8 and 23 days, Johnson et al. 2007), inoculation of more superficial tissues including skin and skeletal muscle may happen after much more time had passed after infection. For this reason, it is possible our turtles sampled were infected with *Ranavirus* that would be detectable in other tissue types, but due to the time of sampling in relation to the disease onset, viral copies may not yet be detectable in the muscle or skin.

Similarly, a symptom of ranavirosis in chelonians is lethargy, continuous basking, and anorexia (Johnson et al. 2007). Another potential cause for negative results was sampling error due to animal behavior. First, infected turtles would most likely be basking continuously, instead of being in the water where the submerged hoop traps were located. Second, infected turtles would not be enticed to enter sampling traps by bait if they were experiencing anorexic symptoms. Finally, general lethargy would make infected animals less likely to enter traps in the first place, particularly when traps were set against the current of the river. Should infected animals exhibit classical behavioral

symptoms of *Ranavirus* infection, their behavior would be as such to reduce their likelihood of being trapped. For this reason, our data could potentially be skewed towards healthy turtles due to the behavioral components of *Ranavirus* infection.

Ranavirus is a deadly pathogen with a wide host range and a poorly-understood transmission method. While much study is required on transmission methods, infectivity, and reservoir species, measures such as increased surveillance sets the preliminary framework for greater connections to be made between *Ranavirus* infection and biotic and abiotic factors. Negative results for *Ranavirus* infection in a state-threatened species is overall a positive sign for conservation efforts, but much further study is required to ensure that these findings were not due to confounding variables or nuances of ranavirosis. Despite the lack of explanation as to the lack of *Ranavirus* in the population, continued surveillance of the region in all susceptible species is integral to maintain health and stability of the ecosystem.

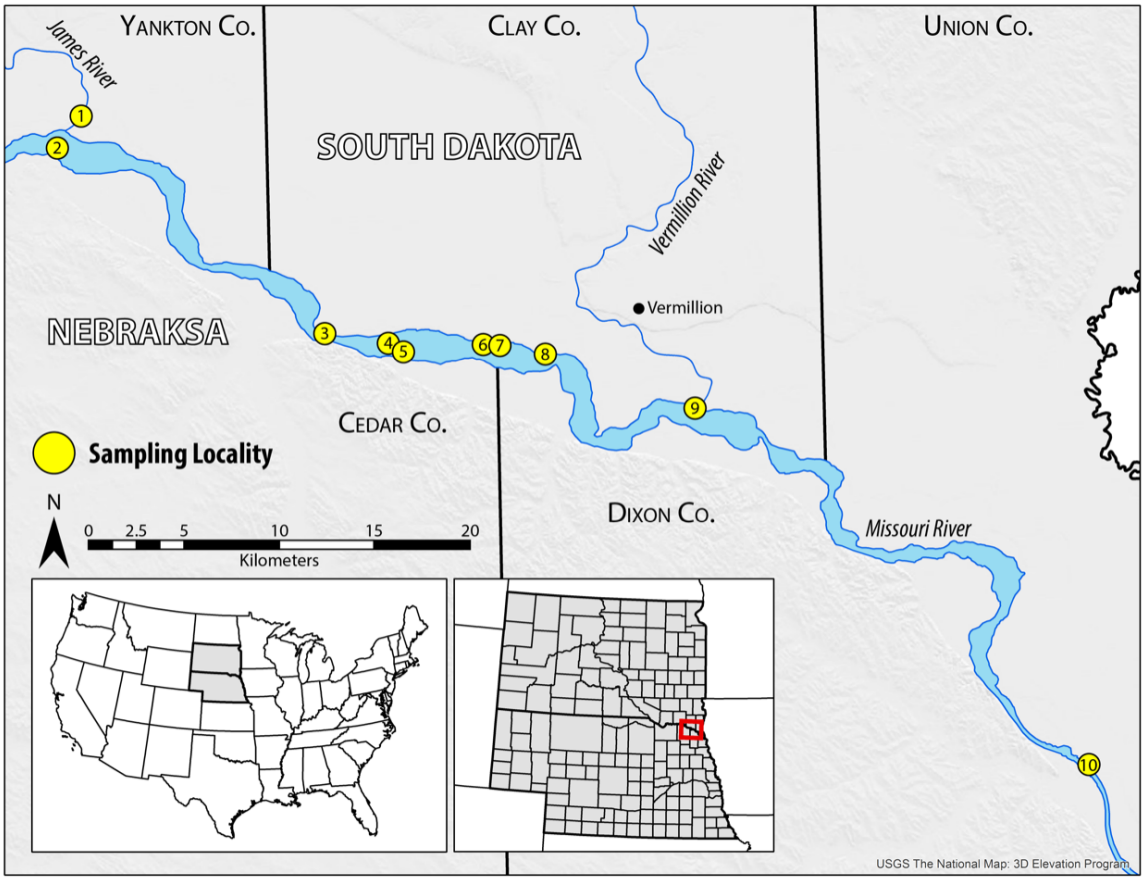


FIG. 4: Locations where False Map Turtles (*Graptemys pseudogeographica*) were sampled for *Ranavirus* along the lower Missouri River between South Dakota and Nebraska, USA. Sampling localities correspond to site numbers in Table 1.

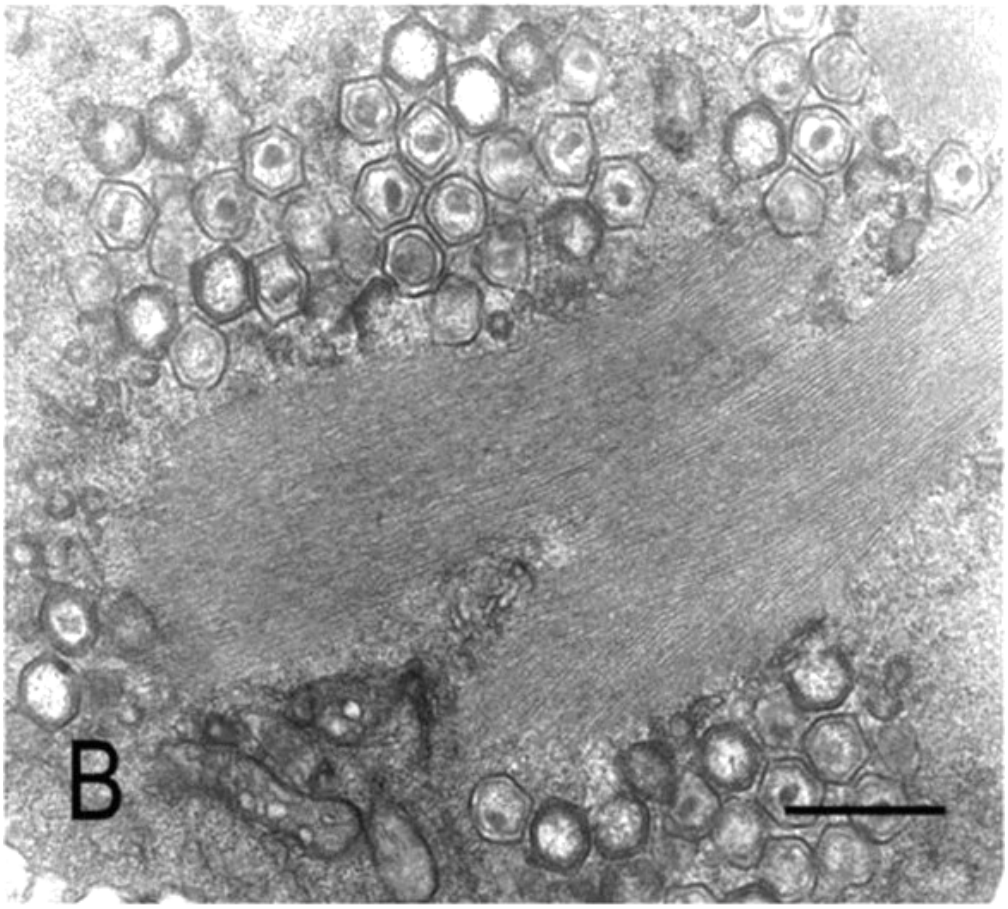


FIG. 5: Electron micrograph showing the epidermis of the pectoral fin of juvenile pallid sturgeon with iridovirus from Missouri River. Hexagonal virions show double envelope and icosahedral shape (bar=500 nm). From Kurobe et al. 2011, Fig. 2, (B).

TABLE 1. Sampling localities, sampling dates, tissue types (blood, muscle, skin), sample sizes, and *Ranavirus* (RV) prevalence of False Map Turtles (*Graptemys pseudogeographica*) sampled from the lower Missouri River between South Dakota and Nebraska, USA. Site numbers correspond to localities shown in Figure 5.

Site	Locality	Latitude	Longitude	Date	Tissue Type	Sample Size	RV Prevalence
1	James River	42.87806°N	97.27972°W	17 July 2017	Blood	7	0/7 (0%)
2	James River, at confluence with Missouri River	42.86199°N	97.29495°W	3 May 2017	Muscle	1	0/1 (0%)
3	Missouri River, Myron Grove Game Production Area	42.77300°N	97.12580°W	31 May 2017	Blood	12	0/12 (0%)
4	Missouri River, Goat Island	42.76777°N	97.08527°W	25 July 2017	Blood	11	0/11 (0%)
5	Missouri River. Goat Island	42.76364°N	97.07773°W	19 July 2017	Blood	21	0/21 (0%)
6	Missouri River, above Clay County Park	42.76557°N	97.01894°W	29 August 2017	Blood	14	0/14 (0%)
7	Missouri River, above Clay County Park	42.76541°N	97.01795°W	10 September 2015	Muscle	1	0/1 (0%)
8	Missouri River, North Alabama Bend	42.76090°N	96.98494°W	10 September 2015	Skin	27	0/27 (0%)
9	Vermillion River, at confluence with Missouri River	42.73351°N	96.88969°W	28 June 2017	Blood	28	0/28 (0%)
10	Missouri River, Rosenbaum Water Access Area	42.56042°N	96.64425°W	4 June 2016	Muscle	1	0/1 (0%)
					TOTAL	123	0/123 (0%)

CHAPTER II

Characterization of the Cloacal Bacterial Microbiome of *G. pseudogeographica*

INTRODUCTION

A. Microbiomes

Within and on the tissues of most animals on earth, host organismal cells occupy the same space as a greater number of foreign bodies: bacterial cells, viruses, fungal and archaeic cells (Qin et al 2010). These microbes (collectively microbiota) together create highly diverse and complex communities (microbiomes) on the tissues of most vertebrate organisms. Largely understudied historically in scientific literature, these communities have quietly caused the evolution of organisms, and themselves, for millennia. The ability to share genomic information between both individual microbes and entire communities for greater survival has allowed for the creation of novel and diverse microbial communities all over the biosphere. Additionally, when microbial communities inhabit surfaces on or within vertebrate hosts, genetic and physiological interaction between the microbiota and the vertebrate host occur, playing a pivotal role in the survival and evolution of vertebrate species for billions of years (Woese 2002). The role of microbiota within the development and adult physiology of the organism they inhabit is vast and varying, ranging from development, structure and function of the gut; immune response, energy metabolism, and undoubtedly other undiscovered impacts

(Backhed et al. 2005, Kelly et al. 2005, Rohde et al. 2007). Moreover, the term, "hologenome" has been coined as a representation of the genetic information of both the host and its corresponding microbial community as a unit of selection in evolution (Zilber-Rosenberg and Rosenberg 2008) due to the highly symbiotic nature of the relationship between a host and the microbial communities it supports. Microbiota of all kinds have allowed the survival of vertebrate life on earth.

Most of the research into the interaction and evolution between a microbiome and its host has been within the gut of animals, particularly in mammals (Savage 1977, Drasar 1974), though the importance of microbiomes within non-model species has become apparent and the subject of much study recently. In humans, members of the domain Bacteria predominate the microbiome, though members of Archaea and Eukaryota are also present (Eckberg et al. 2003). For this reason, the study of microbiome effect on host physiology and development is largely contained to the bacterial microbiome.

The microbiomes of organisms are structured largely during the early portion of an organism's life and may be passed down through generations (Bright and Bulgheresi 2010). The mechanism as to how microbiota are passed down varies among taxa. While many mammals develop core microbiomes through close parental interaction, reptiles infrequently have much postnatal contact and may ingest fecal material from parents to seed the gut microbiota (Troyer 1984). Microbiotas will stay relatively stable over the course of a lifetime, fluxuating in accordance with factors such as diet, environment, or infection status (Lu et al. 2014). Aquatic animals in particular have significant interaction between their assorted microbiomes and factors of the external aquatic environment, especially in the case of water salinity, pH, and temperature (Lozupone and

Knight 2007). Due to the frequent ingestion of water, the microbiome on the skin and the various diverse microbiomes along the gastrointestinal tract are impacted by the aquatic environment they inhabit (Hentschel et al. 2012). In a large proportion of animals including turtles, the gastrointestinal tract ends in a cloaca instead of a rectum, providing a direct connection between the surrounding environment as well as urogenital tract and the gastrointestinal tract. The structure of a cloaca provides an interesting and short bridge between abiotic environmental and internal bodily factors that influence the microbiome of the adjoining gut. While the microbiome of an animals is altered largely based on survival needs and homeostatic conditions of the animal based on internal indicators, having a direct external connection from the environment to the bacterial community means that the environment has the ability to profoundly alter the community structure of the gut microbiome, creating a community that is likely to be much more diverse than it would be otherwise. This influence complicates the overall characterization of a base microbiome a particular species may hold due to phylogenetic history, for microbiome comparison between species or across phyla. It does, however, allow comparisons to be made between organisms in different environments, thereby characterizing the impact that certain abiotic factors may have on species health. For this reason, and that samples of the microbiome from the cloaca can be collected without requiring the organism in question to be euthanized, the bacterial microbiome from the cloaca is the subject of study for this thesis.

B. Cloacal Microbiomes

The study of cloacal microbiomes has received much attention as a way to study the distal section of the gastrointestinal tract as a ecosystem with high efficacy, particularly in nonmammals. An analysis done by Vo and Jedlicka (2014) comparing metagenomic sequencing results from fecal samples and cloacal swab samples in nonmammalian animals revealed that cloacal swabs performed better than fecal samples in displaying a clearer picture of microbial community complexity when there was little large-matter fecal debris (ie. arthropod exoskeletal remains, bones). The cloacal microbiome has been studied extensively in birds, with many species being characterized and specific studies into research areas such as horizontal transfer of microbiota from parent to offspring (Lucas and Heeb 2005) as well as between sexual partners (Westneat and Rambo 2000). The cloacal microbiomes of young, growing turkeys (*Meleagris gallopavo*) were also characterized with respect to time by Wilkinson et al. (2017) as well as in model marsupial species *Macropus eugenii,* the tammar wallaby (Chhour et al. 2008) and *Phascolarctos cinereus*, the koala (Alfano et al. 2015).

In nonbird reptiles, the cloacal microbiome is a rapidly growing field of study. The community structure of oral and cloacal microbiomes was characterized among wild crocodiles (*Crocodylus acutus* and *Crocodylus moreletii*, Charruau et al. 2012) as well as three kinds of British snakes (*Nutrix natrix, Vipera berus,* and *Anguis fragilis*, Cooper et al. 1985), crocodile lizards (*Shinisaurus crocodilurus*, Jiang et al. 2017), and the Burmese python (*Python bivittatus*, Costello et al. 2010). In turtles, cloacal bacterial microbiome research is an understudied but growing field. Green Turtles (*Chelonia mydas*) have been studied most extensively in this regard: the cloacal bacterial microbiome community

structure has been characterized and compared across types of habitat (Price et al. 2017), the microbiomes have been compared pre- and post-hospitalization (Asahan et al. 2018), and the role of diet on microbial community structure has been analyzed (Campos et al. 2018). Additionally, the Loggerhead Sea Turtle (*Carretta carretta L.*) cloacal bacterial microbiome has been characterized (Abdelrhman et al. 2016). Within the False Map Turtle, the cloacal bacterial microbiome has been briefly characterized and the impact on it of exposure to glyphosate contaminants has been explored (Madison et al. 2018). This study is the first of its kind in analyzing the role of geographic location in differences in microbial community structure. It also significantly adds to the body of knowledge regarding cloacal bacterial microbiomes in not only the False Map Turtle, but also for turtles and reptiles in general.

Here, we investigate the cloacal bacterial microbiome of the False Map Turtle (*Graptemys pseudogeographica*) and factors that alter the microbial community structure based on location of populations within the Missouri National Recreation River (MNRR) of the lower Missouri River (SD, USA). Because of the variety in diet and feeding styles that emphasize the consumption of large quantities of water and detritus, the gut bacterial microbiome has a high level of interaction with the external environment. We are hypothesizing that varying geographic locations of the river will have significantly different water quality characteristics from one another. For this reason, we are theorizing that there will be significant difference between community structure of the cloacal bacterial microbiome from False Map Turtles inhabiting different locations within the lower Missouri River.

MATERIALS AND METHODS

A. Study Population and Sample Collection

In this study, False Map Turtles were collected at different sites along the 59-mile stretch of the Missouri National Recreation River (MNRR) between Yankton and Elk Point, South Dakota, USA from 2015-2017 (Fig. 5). Sampling was done with the joint intent of testing for prevalence of *Ranavirus* (Butterfield et al. 2019) and collecting cloacal bacterial samples for microbiome composition analysis. Samples were taken at three distinct sections of the MNRR: the James River, Goat Island within the Missouri River, and the Vermillion River, from west to east respectively (Fig. 5).

During collection of False Map Turtles along the MNRR between 2015 and 2017, turtles were primarily collected using partially submerged hoop traps baited with sardines, but individuals were also opportunistically collected by hand. Traps were left submerged near basking surfaces (e.g., fallen trees) for 24–48 h and captured individuals were weighed, measured, and given a unique identifying notch on their marginal scutes (following Ernst et al. 1974).

B. Sample Collection

Cloacal microbiome community samples were also collected prior to turtle release. To collect the sample, a sterile cotton swab (#MWE113, Medical Wire & Equipment, Corsham, Wiltshire, UK) was inserted into the cloaca, rotated in three full circles, and gently removed. The act of inserting the cotton swab into the cloaca occasionally triggered a release of the urinary bladder, causing urine to also coat the swab. Turtles that urinated were noted in data collection and were included for analysis to compare

with data from other turtles from the same location that had not urinated. Samples were stored individually in sterile microcentrifuge tubes and held on ice in the dark while in the field until transported to a -20°C freezer, where they stayed until analysis. Water sample data was taken at each site using a YSI meter for quantitative habitat comparison (Table 4).

We also collected three individual False Map Turtles as voucher specimens during this study. We euthanized these individuals via an overdose of sodium pentobarbital injected through the caudal vein and collected a muscle tissue sample from the right hind limb.

C. DNA Extraction and Purification

DNA extractions from all collected samples from Summer 2017 were completed in Winter 2018 using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Protocol included an overnight tissue digestion and proteinase K digestion at 56°C. After extraction, DNA clean-up and concentration was completed using R-9 Genomic DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA) by using standard provided protocol.

D. Library Preparation and High-Throughput Sequencing

21 turtle samples were chosen from the 78 extracted samples for continued analysis. Samples were chosen based on turtle sex, location, and whether they had urinated upon sampling. An equal balance of male and female turtle samples was attempted, despite previous data suggesting a sex-ratio bias towards females in False Map

Turtles in the lower Missouri River (Lindemann 2013). Turtles that urinated were preferentially chosen against, though in populations where most available turtles had urinated, such as the James River, we added equal numbers of turtles that had urinated and had not to have a point of comparison for its effect.

16S rRNA sequencing was completed at the Westcore facility at Black Hills State University (Spearfish, SD). Extracted DNA from each sample was quantified using the Qubit dsDNA HS Assay Kit (quantitation range: 0.2-100 ng) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Using a modified dual-indexing protocol developed by Illumina (Illumina 16S Metagenomic Sequencing Protocol [15044223 Rev. B]), up to 15 ng of DNA from each sample was used to produce a library for high-throughput sequencing. During the first round of amplification, primers targeted the V4 region of the 16S rRNA gene and each sample was run in duplicate using 2x KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA). Illumina overhang adapters were added to the 515F and 806R primers (V4_515F: 5'–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **GTG YCA GCM GCC GCG GTA A**–3' and V4_806R: 5'–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GG ACT ACH VGG GTW TCT AAT**–3'; 515F and 806R base primers in bold). System cycling protocol was followed: initial denaturation at 95°C for 3 min, followed by 25 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min on a Veriti Thermal Cycler (Thermo Fisher Scientific). All reactions were purified prior to indexing using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). For indexing and subsequent purifications, a bead solution/PCR product ratio of 0.8 (20 μL of bead solution with 25 μL of PCR product).

A secondary amplification was completed using 2x KAPA HiFi HotStart Ready Mix and a combination of two unique Nextera XT Index primers (N7xx and S5xx) on a Veriti Thermal Cycler (Thermo Fisher Scientific). The secondary amplification protocol was followed: initial denaturation at 95°C for 3 min, followed by 8 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The libraries were purified with Agencourt AMPure XP beads and quantified using Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer, normalized, and pulled together. The final library was gel re-purified using the Wizard™ SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI). Paired-end sequencing was performed on a MiSeq instrument using the MiSeq Reagent Kit v3 600 cycles (Illumina Inc., San Diego, CA).

E. Sequence Data and Statistical Analysis

Initial processing and analysis of high-throughput sequencing data was completed by using the Mothur bioinformatics software tool (version 1.39.5; Schloss et al., 2009). Briefly, a standard MiSeq SOP was followed starting with generating contigs from paired-end reads, clean-up steps (including screening, filtering, and chimera removal), alignment to the Silva database (1.32), and generation of OTUs for statistical analysis (Kozich et al., 2013; corresponding webpage accessed 29 January 2018). Community abundance, alpha diversity, unconstrained ordination, and constrained ordination were all calculated and visualized with the R statistical language (version 3.5.1; R Core Team, 2018). R packages used included Phyloseq, ggplot2, Vegan, dplyr, Scales, Grid, and Reshape2.

RESULTS

Microbial community composition varied between locations as well as individuals (Fig. 6). Unconstrained non-metric multidimensional scaling was used to visualize ordinal distances of community beta-diversity using the Bray-Curtis distance in two-dimensional Euclidean space with a square root transformation and Wisconsin double-standardization (Fig. 8, stress of fit = 0.115).

There were significant differences between microbial community composition and trapping location (PERMANOVA: pseudo-F = 2.099, $R^2 = 0.23068$, p = 0.001). A permutation test for homogeneity of groups was also conducted and yielded significant results (F = 41.936, number of permutations = 999, p = 0.001) although PERMANOVAs are robust to this assumption (Anderson 2017). A second PERMANOVA was completed excluding Goat Island observations as only a single data point was obtained from Goat Island. This was done in an attempt to obtain a more direct comparison between the two trapping sites where enough data was available to be able to draw conclusions with more certainty. There were significant differences between the microbial communities based on location (PERMANOVA: pseudo-F = 3.0108, $R^2 = 0.17699$, p = 0.001). A second permutation test for homogeneity of groups was conducted and did not yield significant results (F = 4.9691, number of permutations = 999, p = 0.051), which increases the confidence in which we can state that there are differences in microbial communities between trapping sites.

Significant differences in microbial community structure were found between trapping sites (Fig. 6). Overall, Proteobacteria dominated the microbial community across all locations, followed by Bacteroidetes in percent composition. Microbial

community composition variation occurred between individuals as well as geographic location, so some variability is due to individual age, diet, health, and interaction with the external environment. Phylum Proteobacteria was the most abundant phyla in all samples and was present across all sampling locations (averages: Vermillion River = 60.801%, James River = 52.275%, Goat Island = 43.818%). Actinobacteria, present in all samples, were relatively stable in abundance across samples and locations, yet not statistically different between locations (averages: Vermillion River: 4.8303%, James River: 5.5328%, Goat Island: 6.3582%). Phylum Verricomicrobia was only found in the Vermillion River and was identified in six samples. While the abundance of Verricomicrobia was low (average = 2.6068%), its exclusivity to presence in samples strictly in Vermillion River is intriguing.

There was no significant difference in microbial community when compared across samples from the same trapping site but differed in whether the turtle had urinated during sample collection (Fig. 7; ANOVA, Table 2). All samples were from the James River trapping site and were taken from the same day. Not all phyla of bacteria were present in all samples, but the phyla present and their proportions therein were not significantly different from one another.

Water quality variables included water temperature, conductivity, pH, barometric pressure, and dissolved oxygen under two scales (Table 4). All water quality variables were all assessed individually against sampling location (Fig. 9) and all were statistically significantly different across locations (ANOVA, Table 3). Only the Vermillion River and James River were compared, as Goat Island's singular data point would merely act as an outlier. Significant differences in water quality are encouraging, suggesting possible

explanations for differences in microbial community structure between locations. That beings said, other variables need to be taken into consideration as possible causes for microbial community diversity including habitat variation, agricultural runoff, individual diet, predation, and immune stress.

DISCUSSION

The patterns of community compositions of the cloacal bacterial microbiome in the False Map Turtles at each of the trapping sites were unique to one another based on a PERMANOVA completed comparing microbial composition and water quality data between locations. Sample data was compared once against collected samples from all trapping sites, and then a second time where samples from Goat Island were excluded. Comparing samples without Goat Island decreased how robust the PERMANOVA had to be, eliminating the need to accommodate a trapping site with n = 1 sample, thereby increasing the power of the test and strengthening the conclusion that the microbial communities between the James River and Vermillion River differ from one another.

While microbiomes differ between individuals, to have significant differences in community structure when compared to a neighboring population less than 30 miles away may lend itself to further explaining the impact of local habitat on microbial fauna within aquatic species, particularly in a riverine habitat. While a similar study had been done regarding the Green Turtle (*Chelonia mydas*) regarding variation in cloacal microbiome based on habitat, therefore feeding differences (neritic, ie. herbivorous versus pelagic, ie. omnivorous) and had found significant differences between the populations based on this factor, this study is the first to examine the impact of geographic location on such a small scale and to find significant differences. While we can state that there are significant differences between the James River and Vermillion River in terms of microbial communities harbored there, the conclusions drawn regarding Goat Island's stark differences from the other trapping sites are speculatory, due to insufficient data to draw powerful conclusions. There were significant differences in proportions of

Patescibacteria (two-sample t test: t-value = 3.0444, df = 14, p-value = 0.02267, Table 6; averages: Vermillion River = 2.5562%, James River = 4.4142%, Goat Island = 0%). Additionally, some phyla of bacteria were only found in a certain location: Verrucomicrobia, for example, was only found in the Vermillion River, but was found in six of the 13 samples. Proteobacteria dominated the bacterial communities regardless of location (averages: Vermillion River = 60.801%**,** James River = 52.275%, Goat Island = 43.818%): the second-most common phyla in terms of proportion, Bacteroidetes, comprising less than half of the amount of the present Proteobacteria (averages: Vermillion River = 18.7870%, James River = 18.6409%, Goat Island = 25.0822%). While Goat Island's proportion of Bacteroidetes is much higher than the other two trapping sites, it only consisted of a singular data point. For this reason, we can only speculate as to the cause of its irregularity in abundance.

We also found there were no significant differences in community composition and structure across urination patterns. Individuals were sampled from the same location, the James River, on the same day. Besides demographic differences attributable to the individual turtles, the only significant difference between the turtles that were sampled was that some turtles urinated during the sample collection process and some did not. Urination during sample collection has historically been a cause for concern when taking cloacal bacterial samples: as the cloaca acts as an end to the urinary tract as well as the gastrointestinal tract, there is question as to whether the presence of urine during sample collection would in any way contaminate or alter the bacterial community on the sample, thus decreasing the efficacy of swab sampling for the cloacal microbiome. Our findings show there was no significant difference between bacterial phyla present and their

proportions therein between turtles that did urinate during sample collection and those that did not. Though further laboratory study should be completed confirming this, our data suggests that urination during sample collection should not be considered a contaminant and should not impact the sample's ability to represent the individual's cloacal bacterial community.

This study aimed to examine the potential impact of water quality differences on bacterial microbiome composition. Previous work has found that water quality factors are highly impactful to determining community structure and predicting presence of specific bacterial phyla. Water salinity was determined to be the most divisive in terms of differences in microbial communities found within (Lozupone and Knight et al. 2006), which cannot be used to account for the differences in microbial communities between trapping sites as found in this study, as all samples were taken from turtles in a freshwater riverine system. pH and water temperature were also found to have an effect on the microbiomes found within these environments (Lozupone and Knight et al. 2006). Analysis of water quality data collected at each trapping site revealed significant differences in both water temperature and water pH between trapping sites (ANOVA, Table 3), thus acting as potential explanations for the significant differences in microbiome composition between samples of separate trapping sites. A small geographic region such as that in the MNRR lends itself to the idea that all turtles collected from different locations are of the same population and that differences in microbial diversity is not as simple as water quality data differences. Water quality characteristics may also be impacted by external environmental factors including atmospheric inputs, human interaction, or climate conditions (Bricker and Jones 1995). Factors impacting river

water quality may also remain relatively constant throughout the year, including wastewater discharge (Singh et al. 2004) or seasonal variations, including interflow, groundwater runoff, precipitation, or surface runoff (Vega et al. 2008). For this reason, the potential relationships described here need continued surveillance of water quality data to increase confidence in the conclusions being drawn.

Water quality overall along the MNRR was average when compared to the state of South Dakota's minimum surface water quality standards. State criterion for water conductivity is a daily maximum of 7,000 micromhos/cm (mh/cm, Criteria for fish and wildlife propagation, recreation, and stock watering waters); water sampled in the James River was measured at 15.8 mh/cm and water sampled in the Vermillion River was measured to 19.23-19.37 mh/cm. The significant increase in water conductivity in the Vermillion River suggests an increase in dissolved solids in the Vermillion River in comparison to the James River. Higher water conductivity denotes a higher proportion of solid particles in the water, thus electrical conductance to a higher degree than water with lower conductivity. The Vermillion River is downstream to the James River, so a potential cause of increased dissolved solids includes an accumulation of riverine material in the span of the river between the two confluences. River discharge speed may also impact dissolved solid concentration: faster flowing water would dilute dissolved solids further than a more stagnant river (Shrestha and Kazama 2007). pH at both locations also fell within water quality standards as administered by the state: the state criterion for water pH is between 6.0-9.5. Water sampled from the James River had a pH of 8.19 and water sampled from the Vermillion River had a pH range of 8.48-8.51. Dissolved oxygen minimums for warmwater permanent fish life is set by the state of

South Dakota at greater than 5 mg/L (Criteria for warmwater permanent fish life propagation waters), which both the Vermillion and James rivers surpass handily. Water from the James River had dissolved oxygen measuring 70.7 mg/L and water from the Vermillion River had dissolved oxygen ranging from 188.7-218.2 mg/L. The observed pH values are within the upper portion of the acceptable range and dissolved oxygen is well above minimums necessary for warmwater fish propagation, which contradicts the idea of high levels of organic dissolved solids in the water. Organic solids reduce oxygen in the river and, when the solids are hydrolyzed, reduce the pH of the water to create a more acidic environment (Shrestha and Kazama 2007). If the Vermillion River, which had a higher conductivity than the James River, also had lower dissolved oxygen levels and a lower pH, further conclusions may be drawn regarding organic solids in the water and their effect. However, this is not the case, as the Vermillion River has a higher pH and higher dissolved oxygen levels than the James River despite a greater conductivity.

Characterization on whole of the False Map Turtle cloacal bacterial microbiome exhibited present phyla that would be expected of an aquatic reptile. Phylum Proteobacteria was the most abundant phyla of bacteria across all samples, a pattern that is in agreement with studies done on other aquatic reptiles including the Green Turtle (*Chelonia mydas*, Price et al. 2017), the Loggerhead Sea Turtle (*Carretta carretta Loggerhead*, Abdelrhman et al. 2016), and the American alligator (*Alligator mississippiensis*, Keenan et al. 2013). Phylum Bacteroidetes was also identified in all samples collected, again coinciding with results found in the Green Turtle and Loggerhead Sea Turtle. Interestingly, phylum Firmicutes were only identified in four samples out of 20 across two of the three sampling locations, despite being a well-known

and abundant member of reptilian fauna (Abdelrhman et al. 2016, Keenan et al. 2013, Costello et al. 2010) and human gut microbiome (Ley et al. 2008). Many other phyla were found in community composition analysis in very low abundance, but were found across all trapping sites, including Actinobacteria, Chloroflexi, and Deinococcus-Thermus. These phyla, as well as those found in higher abundances including Proteobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia, were in part to be expected to be members of the microbial communities within our samples. These comprise some key phyla commonly found in microbial community samples from natural habitats (Costello and Schmidt 2006, Fierer et al. 2007, Bergmann et al. 2011, and Santhanam et al. 2017); as maintaining animals in captivity has been shown in multiple species to cause differences in microbiome composition when compared to samples from their wild-caught counterpart (Kohl et al. 2014, Loudon et al. 2014, Madison et al. 2018).

High abundance of phylum Proteobacteria across all samples is partially expected, as Proteobacteria dominate intestinal and cloacal microbiomes of previously-studied species (Keenan et al. 2013, Abdelrhman et al. 2016, Price et al. 2017) as well as within environmental microbial communities (Costello and Schmidt 2006, Fierer et al. 2007, Tian et al. 2009). Its high abundances in reptilian microbiomes is intriguing, as Proteobacteria as a phylum includes many pathogenic genera, including *Escherichia, Helicobacter, Salmonella, Vibrio,* and *Yersinia.* High abundances in *G. pseudogeographica* are not as concerning on a pathogenic level due to the similarity in relative abundance of Proteobacteria in other reptiles. Phyla Proteobacteria also contains many legume-symbiotic nitrogen-fixing species (Chen et al. 2003); agricultural farms in the regions surrounding our riverine trapping sites may give some rationale into the

consistently dominant relative abundance of Proteobacteria in *G. pseudogeographica* microbial communities within the region.

Phylum Bacteroidetes is a common and prominent member of reptilian intestinal microbial communities as well as in human populations in the Western hemisphere (Qin et al. 2010, Human Microbiome Project Consortium 2012). In mammals, members of Bacteroidetes are associated with digestion of polysaccharides, providing the intestine with absorbable short-chain fatty acids, aiding in energy metabolism and has been related to obesity in mammals (Ley et al. 2005). Bacteroidetes are found in varying environments, including intestinal, epidermal, marine, or soil habitats (Johnson et al. 2017) and on whole as a phyla function in breakdown of polysaccharides (Backhed et al. 2005). Genes within Bacteroidetes are largely unaffected by genetic transfer from the host, leaving internal environmental conditions to regulate abundances within a community (Davenport et al. 2015, Goodrich et al. 2016). Despite environmental factors impacting abundances, in *G. pseudogeographica*, we cannot confirm that water quality is the exclusive cause for the variations of abundances of Bacteroidetes in communities of different trapping sites, in part due to a lack of significant differences in abundances as well as other environmental factors that impact bacterial community composition.

Bacteria in phylum Firmicute are largely associated with energy storage in animal gut microbiomes and for this reason, the ratio of Bacteroidetes, associated with energy metabolism, and Firmicutes is the subject of much study in humans in relation to obesity (Mariat et al. 2009). The phyla includes notable bacterial taxa such as Clostridia and Bacilli. Clostridia has been noted to have cellulolytic function within the gut bacterial microbiome (Zhu et al. 2011) and species within both genera of Clostridia and Bacilli are

associated with fermentation (Pryor 2008, Endo et al. 2010). While Firmicutes are commonplace in reptilian microfauna, the diminished representation of the phyla among samples may be caused by a multitude of factors, including the detritivore diet of the False Map Turtle or overall resource availability. A potential direction of research is a comparison of Firmicute abundances throughout different seasons of the year, to investigate whether the less-active winter months would stimulate a higher abundance of the energy storage-related phyla.

Verrucomicrobia, the phyla of bacteria only found in the Vermillion River, is of particular interest. The facultative or obligate anaerobic phylum (Chin et al. 2001) is typically found in soils yet is very difficult to cultivate in a lab, leading to its underrepresentation in public databases (Bergmann et al. 2011). While the specific cause for Verrucombicrobia's presence exclusively in the Vermillion River is unknown, there is a significant increase in water conductivity in the Vermillion River when compared to the James River (averages: Vermillion River = 1926.69 S/m, James River = 1580 S/m; Tables 3 and 4). For this reason, a possible speculation for the presence of Verrucomicrobia in the Vermillion River is a heightened presence of soil in the water as indicated by a higher water conductivity than the James River, which may seed Verrucomicrobia in the environmental microbial community.

We have therefore shown that geographic location and water quality factors may have an impact on the cloacal microbiome composition of wild-caught *G. pseudogeographica* and that urination during cloacal sampling does not have a significant impact on cloacal microbiome composition, therefore increasing the efficacy of cloacal swabbing for microbiome sampling. The exact methods that water quality factors impact

microbiome composition remain unclear, but showing an association between the two variables acts as a first step for further study into this relationship. Other environmental impacts that would affect community composition include individual diet, available habitat resources, agricultural runoff, and comorbid infections. In addition, further controlled study of the impact of urination during sampling on the efficacy of microbial community sampling is required to be sufficiently confident of the relationship between urination and sample effectiveness. This study is also one of the first of its kind to characterize the cloacal bacterial microbiome of *G. pseudogeographica,* adding to bodies of knowledge regarding the species, reptiles, and aquatic vertebrates. Data presented therein could be used for comparative analyses of vertebral cloacal microbiomes. We suggest that further study should be done into the impact that specific water quality factors have on microbial community composition on wild-caught turtles, taking into consideration the differences noted in community structure between captive and wild turtles as noted previously. As we learn more about the impact the microbiome has on the health of organisms and the habitats and ecosystems they are a part of, research concerning the microbiome and both its effects and what it is impacted by, is of critical importance for the conservation of endangered species such as *G. pseudogeographica* and the health of ecosystems across the biosphere.

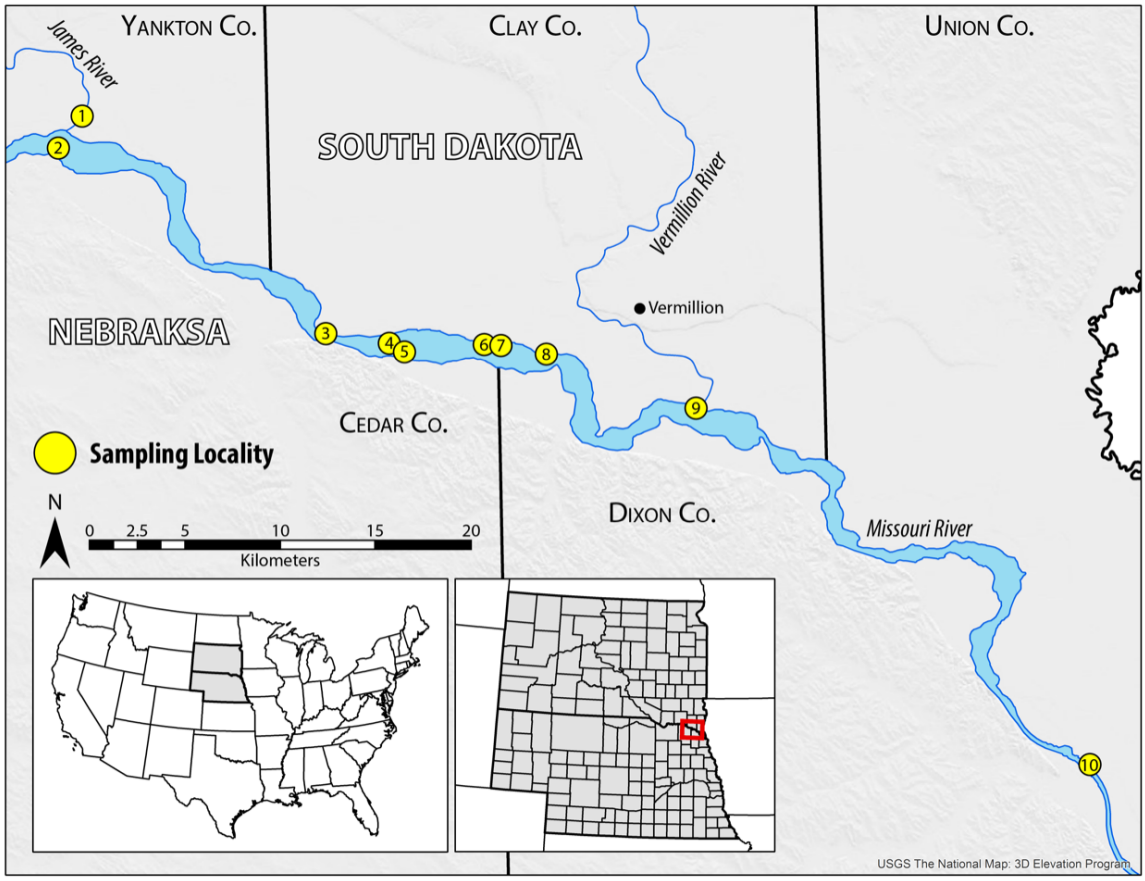


FIG. 5: Locations where False Map Turtles (Graptemys pseudogeographica) were sampled for Ranavirus along the lower Missouri River between South Dakota and Nebraska, USA. Sampling localities correspond to site numbers in Table 1.

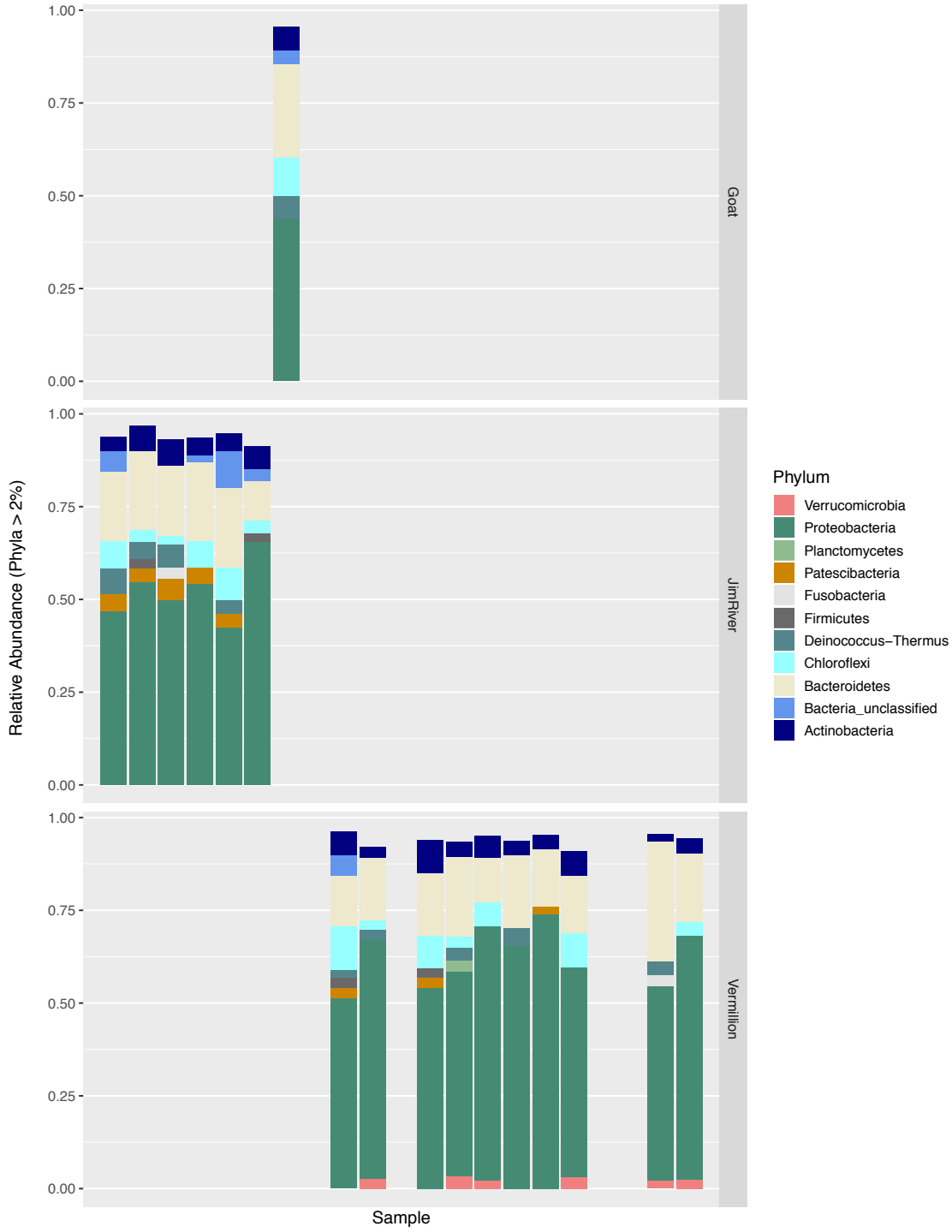


FIG. 6: Gut microbiome composition shown with each bar representing one sample. Samples are separated into trapping location – including Goat Island ("Goat"), James River ("JimRiver"), and Vermillion River ("Vermillion"). Each color is representative of a corresponding phyla (or in the case of "Bacteria_unclassified", unidentified members of the Bacteria). Bars do not fully reach 1.00 as low representation groups (<2% of total abundance) were excluded from the analysis for ease of interpretation.

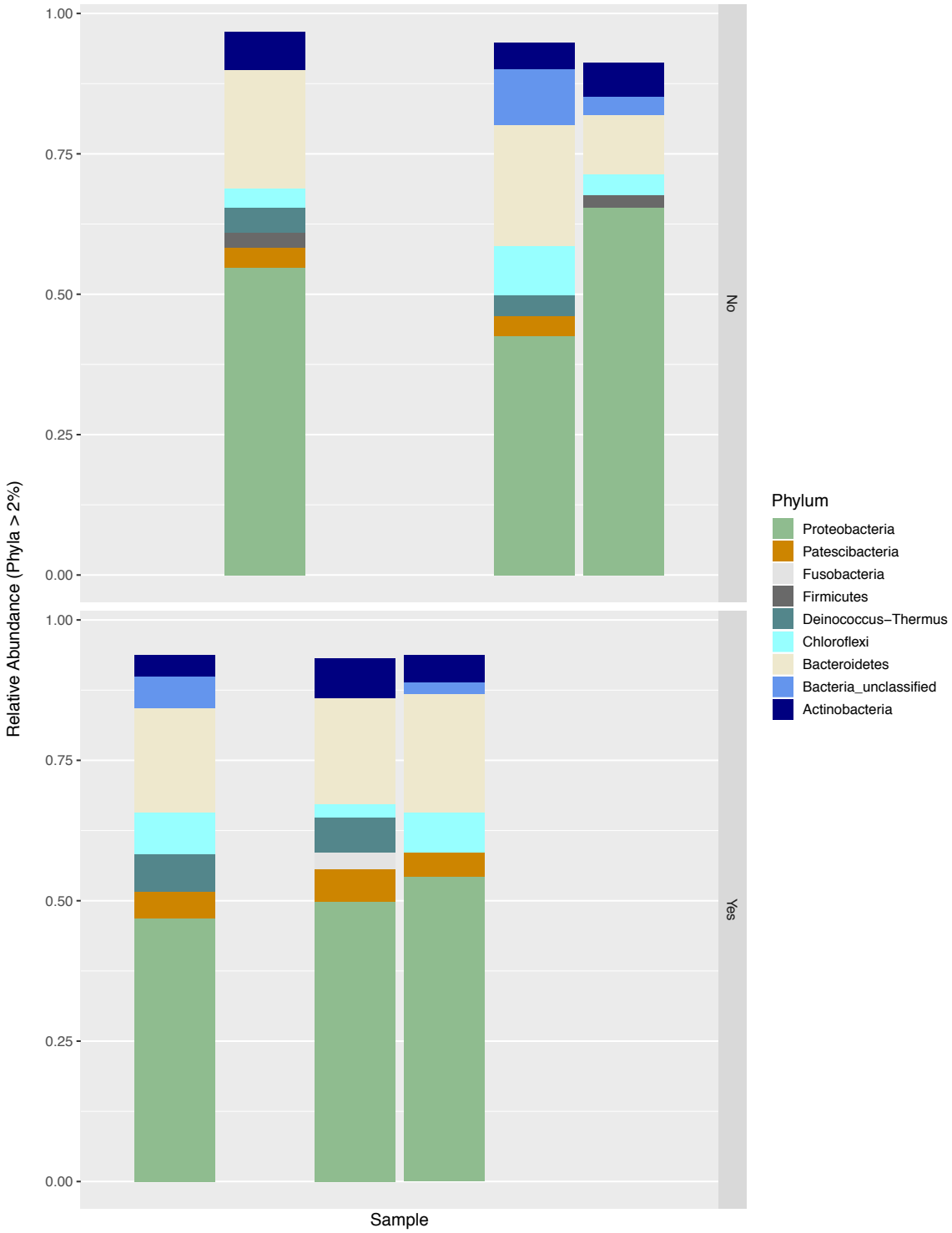


FIG.7*:* Gut microbiome composition shown with each bar representing one sample. Samples are all from the James River and are separated into urination status – whether the turtle urinated while the cloacal sample was being taken ("Yes" or "No"). Each color is representative of a corresponding phyla (or in the case of "Bacteria_unclassified", unidentified members of the Bacteria). Bars do not fully reach 1.00 as low representation groups (<2% of total abundance) were excluded from the analysis for ease of interpretation.

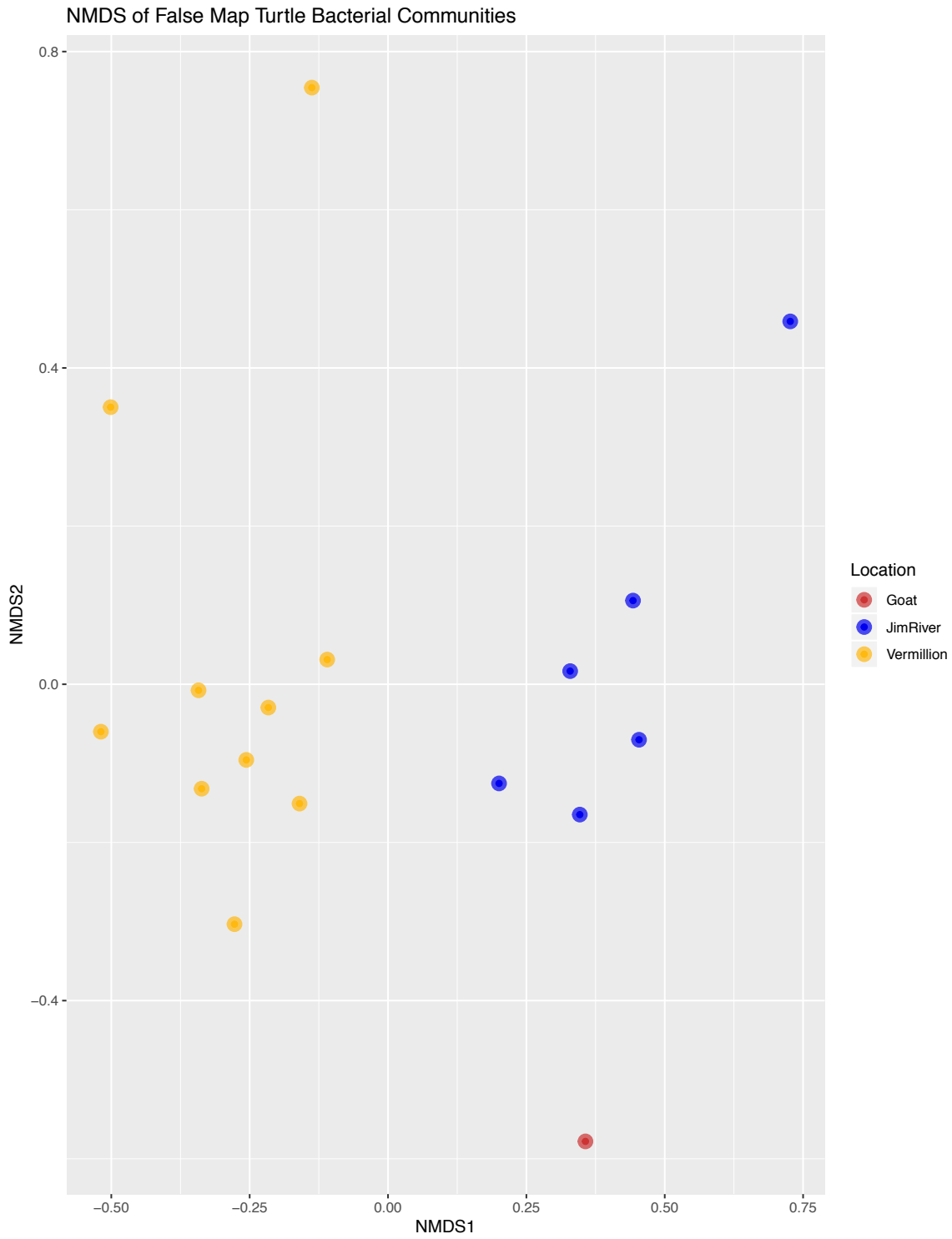


FIG. 8: Non-metric multidimensional scaling (NMDS) calculated with the Bray-Curtis distance metric using a square root transformation and Wisconsin double-standardization. Location is represented by color. Stress of fit for the ordination is reported at 0.115. Axis titles represent the two dimensions to which the data have been ordinated.

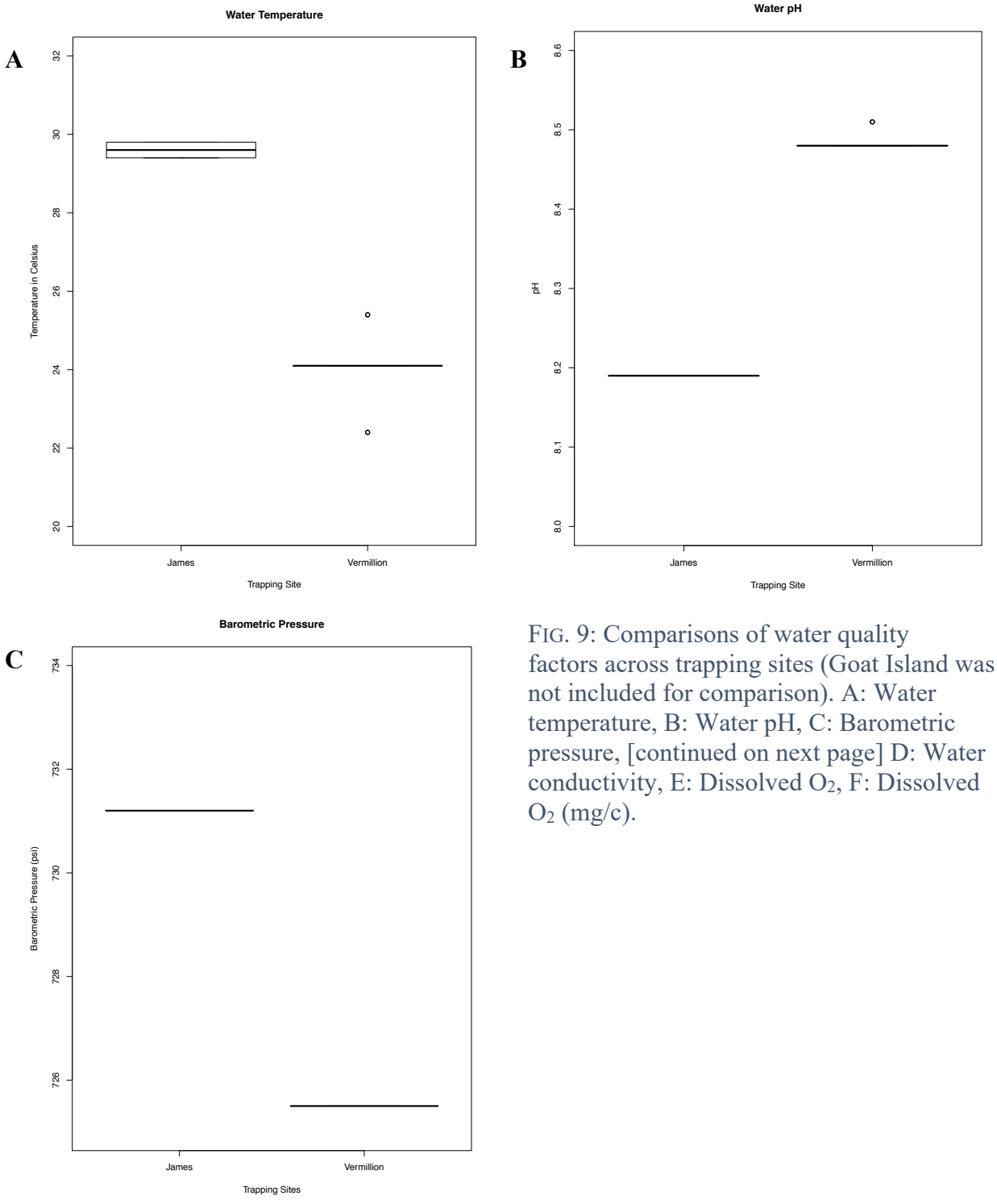
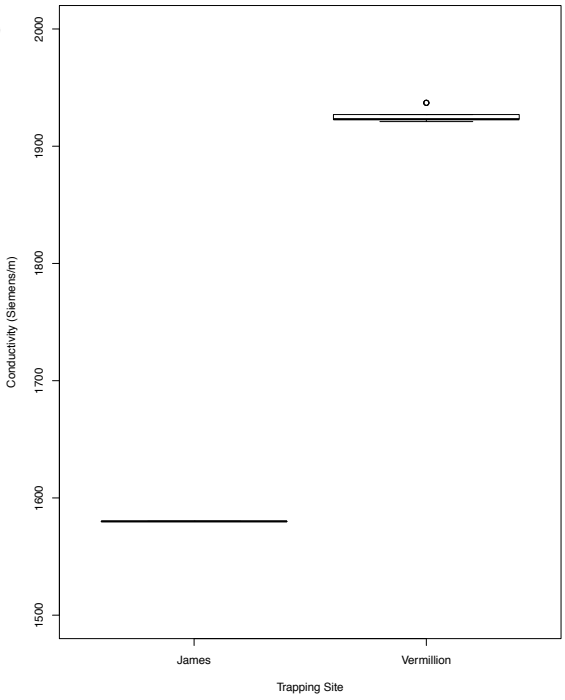
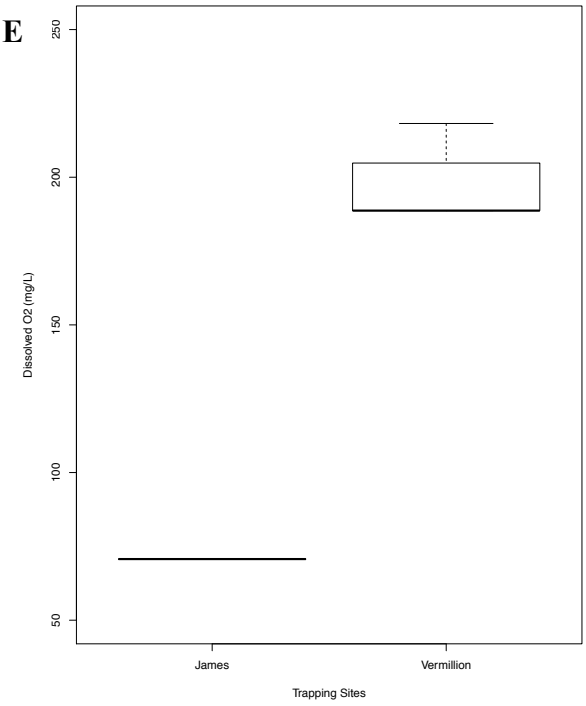


FIG. 9: Comparisons of water quality factors across trapping sites (Goat Island was not included for comparison). A: Water temperature, B: Water pH, C: Barometric pressure, [continued on next page] D: Water conductivity, E: Dissolved O_2, F: Dissolved O_2 (mg/c).

D


Water Conductivity
Conductivity (Siemens/m)
1500
1600
1700
1800
1900
2000
James
Vermillion
Trapping Site

E


Dissolved O2
Dissolved O2 (mg/L)
50
100
150
200
250
James
Vermillion
Trapping Sites

F

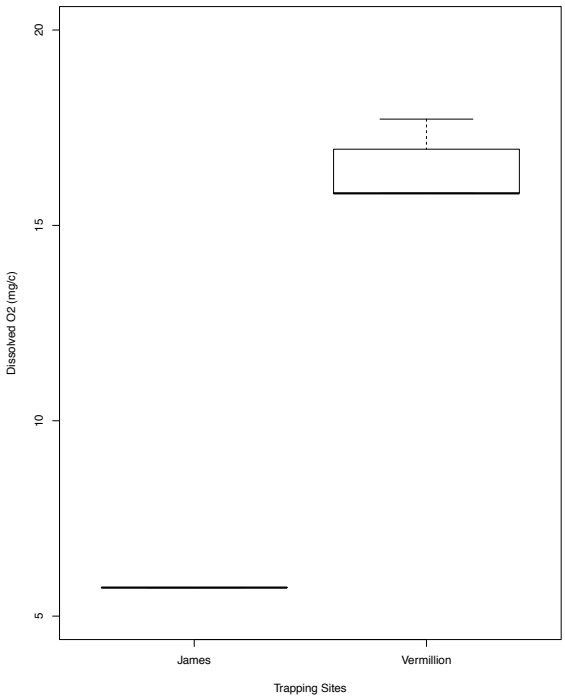

Dissolved O2 (mg/c)
Dissolved O2 (mg/c)
5
10
15
20
James
Vermillion
Trapping Sites

TABLE 1: Sampling localities, sampling dates, tissue types (blood, muscle, skin), sample sizes, and Ranavirus (RV) prevalence of False Map Turtles (Graptemys pseudogeographica) sampled from the lower Missouri River between South Dakota and Nebraska, USA. Site numbers correspond to localities shown in Figure 1.

Site	Locality	Latitude	Longitude	Date	Tissue Type	Sample Size	RV Prevalence
1	James River	42.87806°N	97.27972°W	17 July 2017	Blood	7	0/7 (0%)
2	James River, at confluence with Missouri River	42.86199°N	97.29495°W	3 May 2017	Muscle	1	0/1 (0%)
3	Missouri River, Myron Grove Game Production Area	42.77300°N	97.12580°W	31 May 2017	Blood	12	0/12 (0%)
4	Missouri River, Goat Island	42.76777°N	97.08527°W	25 July 2017	Blood	11	0/11 (0%)
5	Missouri River. Goat Island	42.76364°N	97.07773°W	19 July 2017	Blood	21	0/21 (0%)
6	Missouri River, above Clay County Park	42.76557°N	97.01894°W	29 August 2017	Blood	14	0/14 (0%)
7	Missouri River, above Clay County Park	42.76541°N	97.01795°W	10 September 2015	Muscle	1	0/1 (0%)
8	Missouri River, North Alabama Bend	42.76090°N	96.98494°W	10 September 2015	Skin	27	0/27 (0%)
9	Vermillion River, at confluence with Missouri River	42.73351°N	96.88969°W	28 June 2017	Blood	28	0/28 (0%)
10	Missouri River, Rosenbaum Water Access Area	42.56042°N	96.64425°W	4 June 2016	Muscle	1	0/1 (0%)
					TOTAL	123	0/123 (0%)

TABLE 2: Two-sample T-tests investigating the effects of urination during sampling on relative prevalence of specific phyla of bacteria. All samples are from the James River. Significance: p = 0.05

Phyla	**t-value**	**df**	**p-value**
Actinobacteria	0.49899	4	0.644
Bacteroidetes	-0.47853	4	0.6573
Chloroflexi	-0.17339	4	0.8708
Deinococcus	-0.60297	4	0.579
Firmicutes	1.984	4	0.1183
Fusobacteria	-1	4	0.3739
Patescibacteria	-2.0255	4	0.1128
Proteobacteria	0.56155	4	0.6044

TABLE 3: Analysis of Variance table investigating the effects of different trapping locations (two trapping sites, Vermillion River and James River) on various water quality factors.

Water Quality Factor	**Sums Sq**	**Df**	**F-value**	**p-value**	**R^2**
Temperature	128.39	1	157.4	5.09E-10	0.9025057
pH	0.3619	1	2962	2.00E-16	0.9942943
Conductivity	493434	1	18776	2.00E-16	0.9990954
Barometric Pressure	133.4	1	1.46E+27	2.00E-16	1
Dissolved O_2	67811	1	591.5	1.20E-14	0.972064
Dissolved O_2 (mg/c)	477.9	1	978.2	2.00E-16	0.9829188

TABLE 4: Water quality factors associated with each turtle sample from all three trapping sites. Water quality samples were taken on a YSI meter.

Sample ID	Date	Location	Temp, °C	pH	Conductivity, S/m	Barometric, psi	Dissolved O_2, mg/L	Dissolved O_2, mg/c	Sex	Carapace Length. mm	Urination
6000	7/18/17	James River	29.4	8.19	1580	731.2	70.7	5.37	F	189	Yes
6001	7/18/17	James River	29.4	8.19	1580	731.2	70.7	5.37	M	128	No
6002	7/18/17	James River	29.4	8.19	1580	731.2	70.7	5.37	F	166	Yes
6004	7/18/17	James River	29.8	8.19	1580	731.2	70.7	5.37	F	187	Yes
6005	7/18/17	James River	29.8	8.19	1580	731.2	70.7	5.37	M	146	No
495	7/18/17	James River	29.8	8.19	1580	731.2	70.7	5.37	F	190	No
6033	7/27/17	Goat Island	26						M	121	No
5032	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	75.82	M	134	No
6400	6/30/17	Vermillion River	25.4	8.48	1937	725.5	218.2	17.72	M	142	No
6700	6/30/17	Vermillion River	22.4	8.51	1921	725.5	204.8	16.95	M	124	No
5027	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	M	133	No
5031	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	M	134	No
5015	6/30/17	Vermillion River	25.4	8.48	1937	725.5	218.2	17.72	F	206	No
5017	6/30/17	Vermillion River	25.4	8.48	1937	725.5	218.2	17.72	F	219	No
1224	6/30/17	Vermillion River	22.4	8.51	1927	725.5	204.8	16.95	F	232	No
5020	6/30/17	Vermillion River	22.4	8.51	1927	725.5	204.8	16.95	F	192	No
7702	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	F	227	No
5025	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	F	217	No
5026	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	F	240	No
5029	6/30/17	Vermillion River	24.1	8.48	1923	725.5	188.7	15.82	F	223	No

TABLE 5: Two-sample T-tests investigating the effects of trapping site location (James River and Vermillion River) on relative prevalence of specific phyla of bacteria. Phyla without tests did not have a large enough sample size for adequate analysis (cutoff: n = 3). Significance: p = 0.05

Phyla	**t-value**	**df**	**p-value**
Actinobacteria	0.74015	14	0.4714
Bacteroidetes	-0.055998	14	0.9561
Chloroflexi	-0.56391	11	0.5841
Deinococcus	2.2928	7	0.05558
Patescibacteria	3.0444	6	0.02267
Proteobacteria	-2.1098	14	0.05335
Firmicutes	NA	NA	NA
Fusobacteria	NA	NA	NA
Verrucomicrobia	NA	NA	NA

LITERATURE CITED

ABDELRHMAN, K.F.A., G. BACCI, C. MANCUSI, A. MENGONI, F. SERENA, ET AL. 2016. A first insight into the gut microbiota of the sea turtle Caretta caretta. Front Microbiol 7:1060.

AHNE, W., M. BREMONT, R. P. HEDRICK, A. D. HYATT, AND R. J. WHITTINGTON. 1997. Iridoviruses associated with epizootic haematopietic necrosis (EHN) in aquaculture. World J. Microbiol. Biotechnol. 13:367-373.

ALFANO, N., A. COURTIOL, H. VIELGRADER, P. TIMMS, A.L. ROCA, ET AL. Variation in koala microbiomes within and between individuals: effect of body region and captivity status. Scientific Reports 5:10189.

ALLENDER, M. C., M. ABD-ELDAIM, J. SCHUMACHER, D. MCRUER, L. S. CHRISTIAN, ET AL. 2011. PCR prevalence of ranavirus in free-ranging eastern box turtles (*Terrapene carolina carolina*) at rehabilitation centers in three southeastern US states. J. Wildl. Dis. 47:759–764.

ALLENDER, M. C. 2012. Characterizing the epidemiology of ranavirus in North American chelonians: diagnosis, surveillance, pathogenesis, and treatment. Ph.D. Dissertation, University of Illinois at Urbana-Champaign. viii + 211 pp.

ALLENDER, M.C., AND M.A. MITCHELL. 2013. Hematological response to experimental infections for Frog Virus 3-like virus in Red Eared Sliders (Trachemys scripta elegans). J Herp Med and Surg 23(1):25-31.

ANDERSON, M. J. 2017. Permutational Multivariate Analysis of Variance (PERMANOVA). In Wiley StatsRef: Statistics Reference Online (eds N. Balakrishnan, T. Colton, B. Everitt, W. Piegorsch, F. Ruggeri and J. L. Teugels). doi:10.1002/9781118445112.stat07841

ASAHAN, M., T.B. WALTZEK, R. HUERLIMANN, AND E. ARIEL. 2018. Comparative analysis of gut bacterial communities of green turtles (Chelonia mydas) pre-hospitalization and post-rehabilitation by high-throughput sequencing of bacterial 16S rRNA gene. Microbiol Res 207:91–99.

ASHTON, D.E., AND E.M. DOWD. 2008. Fragile legacy: Rare animals of South Dakota. Third edition. South Dakota Department of Game, Fish and Parks, Pierre, South Dakota.

BACKHED F., R.E. LEY, J.L. SONNENBURG, D.A. PETERSON, AND J.I. GORDON. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.

BERGMANN, G.T., S.T. BATES, K.G. EILERS, C.L. LAUBER, J.G. CAPORASO, ET AL. 2011. The under-recognized dominance of Verrucomicrobia in soil bacterial communities. Soil Biology and Biochemistry 43:1450–1455.

BLAHAK, S., AND UHLENBROK, C. 2010. Ranavirus infections in European terrestrial tortoises in Germany. Proceedings of the 1st International Conference on Reptile and Amphibian Medicine: Munich, Germany. Edited by: Öfner, S., Weinzierl, F. Munich: Verl. Dr. Hat. 17–23.

BRADFORD, D.F. 1991. Mass mortality and extincition in a high-elevation population of *Rana muscosa*. J. Herpetol. **25**:174-177.

BRENES, R., M. J. GRAY, T. B. WALTZEK, R. P. WILKES, AND D. L. MILLER. 2014. Transmission of ranavirus between ectothermic vertebrate hosts. PLoS ONE 9:e92476.

BRICKER, O.P. AND B.F. JONES. 1995. Main factors affecting the composition of natural waters. In: Salbu, B., Steinnes, E. (Eds.), Trace Elements in Natural Waters. CRC Press, Boca Raton, FL, pp. 1-5.

BRIGHT, M. AND S. BULGHERESI. 2010. A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8(3): 218-230. doi: 10.1038/nrmicro2262

CAMPOS, P., M. GUIVERNAU, F.X. PRENAFETA-BOLDU, AND L. CARDONA. 2018. Fast acquisition of a polysaccharide fermenting gut microbiome by juvenile green turtles Chelonia mydas after settlement in coastal habitats. Microbiome 6:69-79.

CHARRUAU, P., J. PÉREZ-FLORES, J.G. PÉREZ-JUÁREZ, J.R. CEDEÑO-VÁZQUEZ, AND R. ROSAS-CARMONA. 2012. Oral and cloacal microflora of wild crocodiles Crocodylus acutus and C. moreletii in the Mexican Caribbean. Dis Aquat Org 98:27-39.

CHEN, Z.X., J.C. ZHENG, AND Y.L. JIANG. 1999. A new iridovirus isolated from soft-shelled turtle. Virus Res 63:147-151.

CHEN, W-M., L. MOULIN, C. BONTEMPS, P. VANDAMME, G. BENA, ET AL. 2003. Legume symbiotic nitrogen fixation by β-proteobacteria is widespread in nature. J Bacteriol 185(24): 7266-7272

CHHOUR, K., L.A. HINDS, E.M. DEANE, AND N.A. JACQUES. 2008. The microbiome of the cloacal openings of the urogenital and anal tracts of the tammar wallaby, Macropus eugenii. Microbiology 154:1535-1543. doi: 10.1099/mic.0.2007/014803-0

CHIN, K.J., W. LIESACK, AND P.H. JANSSEN. 2001. Opitutus terrae gen. nov., sp. nov., to accommodate novel strains of the division "Verrucomicrobia" isolated from rice paddy soil. International Journal of Systematic and Evolutionary Microbiology 51, 1965e1968.

CLARK, H.F., J.C. BRENNAN, R.F. ZEIGEL, AND D.T. KARZON. 1968. Isolation and characterization of viruses from the kidneys of Rana pipiens with renal adenocarcinoma before and after passage in the red eft (Triturus viridescens). J. Virol. 2, 629–640.

CONANT, R., J.T. COLLINS, AND R. POWELL. 2016. Peterson field guide to reptiles and amphibians of eastern and central North America, fourth edition. Houghton Mifflin Harcourt Publishing Company, Boston.

CONSTANTINO, M., P. CHRISTIAN, C. F. MARINA, AND T. WILLIAMS. 2001. A comparison of techniques for detecting *Invertebrate iridescent virus 6.* J. Virol. Meth. 98:109-118.

COOPER, J.E., J.R. NEEDHAM, AND K. LAWRENCE. 1985. Studies of the cloacal flora of three species of free-living British reptile. J of Zoology 207:521-525.

COSTELLO, E.K., J.I. GORDON, S.M. SECOR, AND R. KNIGHT. 2010. Postprandial remodeling of the gut microbiota in Burmese pythons. ISME 4:1375-1385.

COSTELLO, E.K. AND S.K. SCHMIDT. 2006. Microbial diversity in alpine tundra wet meadow soil: novel Chloroflexi from a cold, water-saturated environment. Environmental Microbiology 8:1471–1486.

Criteria for fish and wildlife propagation, recreation, and stock watering waters, SDCL § 74:51:01:52 1999.

Criteria for warmwater permanent fish life propagation waters, SDCL § 74:51:01:47 2009.

DAVENPORT, E.R., D.A. CUSANOVICH, K. MICHELINI, L.B. BARREIRO, C. OBER, ET AL. 2015. Genome-wise association studies of the human gut microbiota. PLoS One. 10(11): e0140301.

DARLINGTON, R.W., A. GRANOFF, AND D. C. BREEZE. 1966. Viruses and renal carcinoma of *Rana pipiens.* II. Ultrastructural studies and sequential development of virus isolated from normal and tumor tissue. *Virology* 29, pp. 148-156.

DASZAK, P., L. BERGER, A. A. CUNNINGHAM, A. D. HYATT, D. E., AND R. SPEARE. 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Dis. 5:735–48.

DAVIS, D.R. 2018. Effects of agricultural tile drainage on wetland habitats and species. Ph.D. Dissertation, University of South Dakota. xii + 204 pp.

DAVIS, D.R., AND J. L. KERBY. 2016. First detection of ranavirus in amphibians from Nebraska, USA. Herpetol. Rev. 47:46–50.

DeVoe, R., K. Geissler, K., S. Elmore, D. Rotstein, G. Lewbart, et al. 2004. Ranavirus-associated morbidity and mortality in a group of captive eastern box turtles (Terrapene carolina carolina). J. Zoo Wildl. Med. 35:524-543.

Drasar, B. S. 1974. The Normal Microbial Flora of Man. ed. F. A. Skinner, J. G. Carr, pp. 187-96. London: Academic.

Duffus, A. L. J., T. B. Waltzek, A. C. Stohr, M. C. Allender, M. Gotesman, et al. 2015. Distribution and host range of ranaviruses. In Gray, M. and V. G. Chinchar, (eds.), Ranaviruses: Lethal pathogens of ectothermic vertebrates, pp. 9–57. Springer International Publishing, Cham.

Earl, J.E. and M.J. Gray. 2014. Introduction of ranavirus to isolated wood frog populations could cause local extinction. Ecohealth. doi:10.1007/s10393-014-0950-y

Eckberg, P.B., P.W. Lepp, and D.A. Relman. 2003. Archaea and their potential role in human disease. Infect Immun. 71(2):591-596.

Endo, A., Y. Endo-Futagama, and L.M.T. Dicks. 2010. Diversity of Lactobacillus and Bifidobacterium in feces of herbivores, omnivores and carnivores. Anaerobe. 2010(16):590–6.

Ernst, C. H., M. F. Hershey, and R. W. Barbour. 1974. A new coding system for hard-shelled turtles. Trans. Kentucky Acad. Sci. 35:27–28.

Fierer, N., M.A. Bradford, and R.B. Jackson. 2007. Toward an ecological classification of soil bacteria. Ecology 88: 1354–1364.

Fellers, G.M. and C.A. Drost. 1993. Disappearance of the cascades frog *Rana cascadae* at the southern end of its range, California, USA. Biol. Conserv. **65**:177-181.

Forson, D., and A. Stofer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecol. Appl. 16:2325–2332.

Gibbons, J.W., D.E. Scott, T.J. Ryan, K.A. Buhlmann, T.D. Tuberville, et al. 2000. The global decline of reptiles, déjà vu amphibians: reptile species are declining on a global scale. BioScience, 50(8): 653-666.

Goodman, R.M., D.L. Miller, and Y.T. Ararso. 2013. Prevalence of ranavirus in Virginia turtles as detected by tail-clip sampling versus oral-cloacal swabbing. Northeast. Nat. 20:325–332.

GOODRICH, J.K., E.R. DAVENPORT, M. BEAUMONT, M.A. JACKSON, R. KNIGHT, ET AL. 2016. Genetic determinants of the gut microbiome in UK twins. Cell Host Microbe. 19:731-743.

GRANOFF, A., P.E. CAME, AND K.A.J. RAFFERTY. 1965. The isolation and properties of viruses from Rana pipiens: their possible relationship to the renal adenocarcinoma of the leopard frog. Ann. N. Y. Acad. Sci. 126, 237–255. http://dx.doi.org/10.1111/j.1749- 6632.1965.tb14278.x.

HEDRICK, R.P., T.S. MCDOWELL. 1995. Properties of iridoviruses from ornamental fish. Vet Rsrch 26 (5-6), pp.423-427.

HELDSTAB, A. AND G. BESTETTI. 1982. Spontaneous viral hepatitis in a spur-tailed Mediterranean land tortoise (Testudo hermanni). J. Zoo Anim. Med. 13:113-120.

HENTSCHEL, U., J. PIEL, S.M. DEGNAN, AND M.W. TAYLOR. 2012. Genomic insights into the marine sponge microbiome. Nature Rev Microbiol 10:641-654. doi: :10.1038/nrmicro2839

HUMAN MICROBIOME PROJECT CONSORTIUM. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486(7402):207-14.

HYATT, A.D., M. WILLIAMSON, B.E.H. COUPAR, D. MIDDLETON, S.G. HENGSTBERGER, ET AL. 2002. First identification of a ranavirus from green pythons (*Chondropython viridis*). J. Wildlife Dis. 38(2)239-252.

JANCOVICH, J.K., E.W. DAVIDSON, N. PARAMEWARAN, J. MAO, V. G. CHINCHAR ET AL. 2005 Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Mol. Ecol. 14: 213–224.

JANCOVICH, J.K., N.K. STECKLER, AND T.B. WALTZEK. 2015. Ranavirus taxonomy and phylogeny. In: Gray, M.J., Chinchar, V.G. (Eds.), Ranaviruses. Springer International Publishing, 59–70. http://dx.doi.org/10.1007/978-3-319-13755-1_3.

JIANG, H-Y., J-E. MA, J. LI, X-J. ZHANG, L-M. LI, ET AL. 2017. Diets alter the gut microbiome of crocodile lizards. Front Microbiol 8:2073.

JOHNSON, A.J., A.P., PESSIER, J.F.X. WELLEHAN, R. BROWN, AND E.R. JACOBSON. 2005. Identification of a novel tortoise herpesvirus from a California desert tortoise (Gopherus agassizii). Vet. Microbiol. 111:107-116.

JOHNSON, A.J., A.P. PESSIER, AND E.R. JACOBSON. 2007. Experimental transmission and induction of ranaviral disease in Western Ornate Box Turtles (*Terrapene ornata ornata*) and Red-Eared Sliders (*Trachemys scripta elegans*). Vet. Pathol. 44:285–297.

JOHNSON, A.J., A.P. PESSIER, J.F.X. WELLEHAN, A. CHILDRESS, T.M. NORTON, ET AL. 2008. Ranavirus infection of free-ranging and captive box turtles and tortoises in the United States. J. Wildl. Dis. 44:851–863.

KELLY, D., S. CONWAY, AND R. AMINOV. 2005. Commensal gut bacteria: mechanisms of immune modulation. Trends in Immunol. 26(6): 326-333.

KIMBLE, S., J.A.J. JOHNSON, R.N. WILLIAMS, AND J.T. HOVERMAN. 2017. A severe ranavirus outbreak in captive, wild-caught box turtles. EcoHealth 14:810–815.

KLEMENS, M.W. (Ed.) 2000. Conservation of river turtles. In Turtle Conservation, pp. 127-155. Smithsonian Institute Press, Washington and London.

KOHL, K.D., M.M. SKOPEC, AND M.D. DEARING. 2014. Captivity results in disparate loss of gut microbial diversity in closely related hosts. Conservation Physiology 2:10. 1093/conphys/cou009.

KOZICH, J.J., S.L. WESTCOTT, N.T. BAXTER, S.K. HIGHLANDER, AND P.D. SCHLOSS. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology 79: 5112–5120.

KUROBE, T., E. MACCONNELL, C. HUDSON, T. S. MCDOWELL, F. O. MARDONES, ET AL. 2011. Iridovirus infections among Missouri River sturgeon: Initial characterization, transmission, and evidence for establishment of a carrier state. J. Aquat. Anim. Health. 23:9–18.

LEY, R.E., F. BACKHED, P. TURNBAUGH, C.A. LOZUPONE, R.D. KNIGHT, ET AL. 2005. Obesity alters gut microbial ecology. Proc Natnl Acad Sci USA 102(31):11070-5.

LEY, R.E., C.A. LOZUPONE, M. HAMADY, R. KNIGHT, AND J.I. GORDON. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6, 776–788. doi: 10.1038/nrmicro1978

LOUDON, A.H., D.C. WOODHAMS, L.W. PARFREY, H. ARCHER, R. KNIGHT, ET AL. 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (Plethodon cinereus). The ISME Journal 8:830–840.

LU, K., R.P. ABO, K.A. SCHLIEPER, M.E. GRAFFAM, S. LEVINE, ET AL. 2014. Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated metagenomics and metabolomics analysis. Environ Health Perspect 122:284-291.

LUCAS, F.S. AND P. HEEB. 2005. Environmental factors shape cloacal bacterial assemblages in great tit Parus major and blue tit P. caeruleus nestlings. Journal of Avian Biology 36: 510–516. doi: 10.1111/j.0908-8857.2005.03479.x

MADISON, J.D., S. AUSTIN, D.R. DAVIS, AND J.L. KERBY. 2018. Bacterial microbiota response in Graptemys pseudogeographica to captivity and roundup exposure. Copeia 106(4):580-588.

MAO, J., R.P. HEDRICK, AND V.G. CHINCHAR. 1997. Molecular characterization, sequence analysis and taxonomic position of newly isolated fish iridoviruses. Virol. 229:212-220.

MARIAT, D., O. FIRMESSE, F. LEVENEZ, V.D. GUIMARAES, H. SOKOL, ET AL. 2009. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiology 9:123.

MARSCHANG, R.E., P. BECHER, H. POSTHAUS ET AL. 1999. Isolation and characterization of an iridovirus from Hermann's tortoises (Testudo hermanni). Arch Virol 144:1909–1922

MOLL, D. 1976. Food and feeding strategies of the Ouchita map turtle (*Graptemys pseudogeographica ouachitensis*). American Midland Naturalist 96:478-482.

MULLER, M., N. ZANGGER, AND T. DENZLER. 1988. Iridovirus-epidemie bei der griechischen Landschildkrote (Testudo hermanni hermanni), p.271-274. Verhandl Ber 30. Int Symp Erkr Zoo-und Wildtiere, Sofia.

NUMBERS OF THREATENED SPECIES BY MAJOR GROUPS OF ORGANISMS (1996–2018). (2018). [Table illustration of summary of threatened species by taxa]. *IUCN Red List.* Retrieved from:https://nc.iucnredlist.org/redlist/content/attachment_files/2018_2_RL_Stats_Table_1_new_errata.pdf

ORRIGI, F.C., C.H. ROMERO, D.C. BLOOM, P.A. KLEIN, J.M. GASKIN, ET AL. 2004. Experimental transmission of a herpesvirus in Greek tortoises (Testudo graeca). Vet. Pathol. 41:50-61.

PRICE, S.J., T.W.J. GARNER, R.A. NICHOLS, F. BALLOUX, C. AYRES, ET AL. 2014. Collapse of amphibian communities due to an introduced *Ranavirus*. Curr. Biol. 24:2586-2591.

PRICE, J.T., F.V. PALADINO, M.M. LAMONT, B.E. WITHERINGTON, S.T. BATES, ET AL. 2017. Characterization of the juvenile green turtle (Chelonia mydas) microbiome through an ontogenetic shift from pelagic to neritic habitats. PLoS ONE 12(5): e0177642.

PRYOR, G.S. 2008. Anaerobic bacteria isolated from the gastrointestinal tracts of bullfrog tadpoles (Rana catesbeiana). Herpetol Conserv Biol. 3:176–81.

QUESNELLE, P.E., L. FAHRIG, AND K.E. LINDSAY. 2013. Effects of habitat loss, habitat configuration and matrix composition on declining wetland species. Biol Conserv 160:200-208.

QIN J., R. LI, J. RAES, M. ARUMUGAM, K.S. BURGDORF, ET AL. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59–65. doi: 10.1038/nature08821

RHODIN, A.G.J., C.B. STANFORD, P.P. VAN DIJK, C. EISEMBERG, L. LUISELLI ET AL. 2018. Global conservation status of turtles and tortoises (order Testudines). Chelonian Conserv and Biol 17(2):135-161.

ROHDE, C.M., D.F. WELLS, L.C. ROBOSKY, M.L. MANNING, C.B. CLIFFORD, ET AL. 2007. Metabonomic evaluation of Schaedler altered microflora rats. Chem. Res. Toxicol. 20:1388-1390.

SANTHANAM, R., Y. OH, R. KUMAR, A. WEINHOLD, V.T. LUU, ET AL. 2017. Specificity of root microbiomes in native-grown Nicotiana attenuata and plant responses to UVB increase Deinococcus colonization. Molecular Ecology 26:2543–2562.

SAVAGE, D.C. 1977. Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 31:107-33.

SCHLOSS, P.D., S.L. WESTCOTT, T. RYABIN, J.R. HALL, M. HARTMANN, ET AL. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and com- paring microbial communities. Applied and Environmental Microbiology 75:7537–7541.

SCHOCK, D.M., T.M. BOLLINGER, V.G. CHINCHAR, J.K. JANCOVICH, J.P. COLLINS. 2008. Experimental evidence that amphibian ranaviruses are multi-host pathogens. Copeia 1:133-143.

SCHRAMM, B. AND J.K. LOCKER. 2005. Cytoplasmic organization of poxvirus DNA replication. Traffic. **6**:839-846.

SHRESTHMA, S. AND F. KAZAMA. 2007. Assessment of surface water quality using multivariate statistical techniques: A case study of the Fuji river basin, Japan. Environ Model and Software 22:464-475.

SINGH, K.P., A. MALIK, D. MOHAN, AND S. SINHA. 2004. Multivariate statistical techniques for the evaluation of spatial and temporal variations in water quality of Gomti River (India): a case study. Water Research 38:3980-3992.

TIAN, F., Y. YU, B. CHEN, H. LI, Y-F. YAO, ET AL. 2009. Bacterial, archaeal and eukaryotic diversity in Arctic sediment as revealed by 16S rRNA and 18S rRNA gene clone libraries analysis. Polar Biology 32(1):93-103.

TIMKEN, R. L. 1968. *Graptemys pseudogeographica* in the upper Missouri River of the northcentral United States. J. Herpetol. 1:76–82.

TONKA, T., AND J. WEISER. 2000. Iridovirus infection in mayfly larvae. J. Invertebr. Pathol. 76:229-231.

TROYER, K. 1984. Behavioral acquisition of the hindgut fermentation system by hatchling Iguana iguana. Behav Ecol Sociobiol 14: 189–193.

VEGA, M., R. PARDO, E. BARRADO, AND L. DEBAN. 1998. Assessment of seasonal and polluting effects on the quality of river water by exploratory data analysis. Water Research 32:3581-3592.

VOGT, R.C. 1981. Food partitioning in three sympatric species of map turtle, genus Graptemys (Testudinata, Emydidae). Amer. Midland Nat. 105(1):102-111.

WESTNEAT, D.F. AND R.B. RAMBO. 2000. Copulation exposes female red-winged blackbirds to bacteria in male semen. J. Avian Biol. 31:1–7.

WIKINSON, T.J., A.A. COWMAN, H.E. VALLIN, L.A. ONIME, L.B. OYAMA, ET AL. 2017. Characterization of the microbiome along the gastrointestinal tract of growing turkeys. Front Microbiol 8:1089. doi: 10.3389/fmicb.2017.01089

WILLIAMS, T. 1996. The iridoviruses. *Adv. Virus Res.* 46 pp. 345-412.

WILLIS, D.B., R. GOORHA, V.G. CHINCHAR. 1985. Macromolecular synthesis in cells infected by frog virus 3. Curr. Topics Microbiol. Immunol. 116:77-106.

XEROS, N. 1954. A second virus disease of the leather jacket, Tipula paludosa. Nature (London) 174 pp. 562-563.

WHITFIELD, S.M., M. DONNELLY, E. GEERDES, AND J.L. KERBY. 2012. Ranavirus infection in native amphibians at La Selva Biological Station, Costa Rica: the first report of ranavirus in Central America. Herpetol. Rev. 43(3): 425-427.

WOESE, C.R. 2002. On the evolution of cells. PNAS 99(13):8742-8747.

ZHU, L., Q. WU, J. DAI, S. ZHANG, AND F. WEIT. 2011. Evidence of cellulose metabolism by the giant panda gut microbiota. PNAS. 108:17714–9.